UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/541,071	08/14/2019	Willem Hendrik Lindhout	VONL:004USC1

CONFIRMATION NO. 4348

125771
DENTONS US LLP
P.O. Box 061080
Chicago, IL 60606

PUBLICATION NOTICE

OC000000114332450

Title: Hybrid Seed Potato Breeding

Publication No.US-2020-0029520-A1
Publication Date:01/30/2020

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Public Records Division. The Public Records Division can be reached by telephone at (571) 272-3150 or (800) 972-6382, by facsimile at (571) 273-3250, by mail addressed to the United States Patent and Trademark Office, Public Records Division, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently https://portal.uspto.gov/pair/PublicPair. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

To: patents.us@dentons.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 125771

Jan 31, 2020 03:46:13 AM

Dear PAIR Customer:

DENTONS US LLP
P.O. Box 061080
Chicago, IL 60606
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 125771 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

Disclaimer:
The list of documents shown below is provided as a courtesy and is not part of the official file wrapper. The content of the images shown in PAIR is the official record.

Application	Document	Mailroom Date	Attorney Docket No.
16541071	NTC.PUB	01/30/2020	VONL:004USC1

To view your correspondence online or update your email addresses, please visit us anytime at https://sportal.uspto.gov/secure/myportal/privatepair.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Willem Hendrik Lindhout *et al.*	Group Art Unit: 1662
Serial No.: 16/541,071	Examiner: Unknown
Filed: August 14, 2019	Atty. Dkt. No.: VONL:004USC1
For: Hybrid Seed Potato Breeding	Confirmation No.: 4348

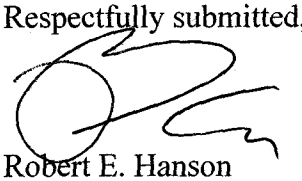
ASSOCIATE POWER OF ATTORNEY

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Commissioner:

I hereby grant, Richard Peet, Intellectual Property Manager at Solynta, Registration No. 35,792, an associate Power of Attorney to prosecute this application and transact all business in the U.S. Patent and Trademark Office connected herewith.

Respectfully submitted,



Robert E. Hanson
Reg. No. 42,628
Attorney for Applicants

Dentons US LLP
2000 McKinney Avenue, Suite 1900
Dallas, Texas 75201
(214) 259-0933

Date: February 3, 2020

Electronic Acknowledgement Receipt

EFS ID:	38479513
Application Number:	16541071
International Application Number:	
Confirmation Number:	4348
Title of Invention:	Hybrid Seed Potato Breeding
First Named Inventor/Applicant Name:	Willem Hendrik Lindhout
Customer Number:	125771
Filer:	Robert E. Hanson/LeRey Coker
Filer Authorized By:	Robert E. Hanson
Attorney Docket Number:	VONL:004USC1
Receipt Date:	03-FEB-2020
Filing Date:	14-AUG-2019
Time Stamp:	18:41:18
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	VONL004USC1_Associate_POA.pdf	31702 e4677318b34af644568edab126072069a6adccc1	no	1

Warnings:

Information:	
Total Files Size (in bytes):	31702

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Willem Hendrik Lindhout *et al.*	Group Art Unit: 1662
Serial No.: 16/541,071	Examiner: Russell T Boggs
Filed: August 14, 2019	Atty. Dkt. No.: VONL:004USC1
For: Hybrid Seed Potato Breeding	Confirmation No.: 4348

REVOCATION OF ASSOCIATE POWER OF ATTORNEY

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Commissioner:

I hereby revoke the associate Power of Attorney filed on February 3, 2020 for, Richard Peet, Intellectual Property Manager at Solynta, Registration No. 35,792. Mr. Peet no longer has the authority to prosecute this application and transact any business in the U.S. Patent and Trademark Office connected herewith.

Respectfully submitted,

/Robert E. Hanson/

Robert E. Hanson
Reg. No. 42,628
Attorney for Applicants

Dentons US LLP
2000 McKinney Avenue, Suite 1900
Dallas, Texas 75201
(214) 259-0931

Date: March 17, 2020

Electronic Acknowledgement Receipt

EFS ID:	38887266
Application Number:	16541071
International Application Number:	
Confirmation Number:	4348
Title of Invention:	Hybrid Seed Potato Breeding
First Named Inventor/Applicant Name:	Willem Hendrik Lindhout
Customer Number:	125771
Filer:	Robert E. Hanson/LeRey Coker
Filer Authorized By:	Robert E. Hanson
Attorney Docket Number:	VONL:004USC1
Receipt Date:	17-MAR-2020
Filing Date:	14-AUG-2019
Time Stamp:	14:32:32
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	VONL004USC1_Revocation_Associate_POA.pdf	122685 736f2a4b62601e7de75b21a9a9ede15101fcda1a	no	1

Warnings:

Information:	
Total Files Size (in bytes):	122685

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/541,071	08/14/2019	Willem Hendrik Lindhout	VONL:004USC1	4348

125771 7590 03/27/2020

DENTONS US LLP
P.O. Box 061080
Chicago, IL 60606

EXAMINER
BOGGS, RUSSELL T

ART UNIT	PAPER NUMBER
1663	

NOTIFICATION DATE	DELIVERY MODE
03/27/2020	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

patents.us@dentons.com

Applicant-Initiated Interview Summary	**Application No.** 16/541,071	**Applicant(s)** Lindhout et al.	
	Examiner RUSSELL T BOGGS	**Art Unit** 1663	**AIA (FITF) Status** No

All participants (applicant, applicants representative, PTO personnel):

(1) RUSSELL T. BOGGS.

(2) ROB HANSON.

(3) SHEILA WALKER.

(4) _____.

Date of Interview: 18 March 2020.

Type: ☑ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☐ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☑112 ☐102 ☑103 ☐Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: 35 and 41.

Identification of prior art discussed: Maris.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

The parties discussed how to proceed extensively including the facts in the instant application as well as the allowed scope of the claims in the parent application. One potentially promising approach was one or more declarations and/or letters in support. No agreement was reached..

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/RUSSELL T BOGGS/ Examiner, Art Unit 1663	

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiners responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicants correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,-
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
(The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicants record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiners version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, Interview Record OK on the paper recording the substance of the interview along with the date and the examiners initials.

To: patents.us@dentons.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 125771

Mar 27, 2020 04:47:44 AM

Dear PAIR Customer:

DENTONS US LLP
P.O. Box 061080
Chicago, IL 60606
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 125771 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

Disclaimer:
The list of documents shown below is provided as a courtesy and is not part of the official file wrapper. The content of the images shown in PAIR is the official record.

Application	Document	Mailroom Date	Attorney Docket No.
16541071	INTV.SUM.APP	03/27/2020	VONL:004USC1

To view your correspondence online or update your email addresses, please visit us anytime at https://sportal.uspto.gov/secure/myportal/privatepair.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Willem Hendrik Lindhout *et al.*	Group Art Unit: 1663 Examiner: Russell T. Boggs
Serial No.: 16/541,071	Atty. Dkt. No.: VONL:004USC1
Filed: August 14, 2019	
For: Hybrid Seed Potato Breeding	Confirmation No.: 4348

RESPONSE TO NON-FINAL OFFICE ACTION DATED DECEMBER 5, 2019

Mail Stop Amendment
Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

This paper is submitted in response to the non-final Office Action dated December 5, 2019, for which the date for response is May 5, 2020, in view of the enclosed Petition for Extension of Time and fees. No additional fees are believed due in connection with the filing of the instant paper. However, should any such fees become due under 37 C.F.R. §§ 1.16 to 1.21 for any reason relating to the instant response, the Commissioner is authorized to deduct said fees from Dentons US LLP's Deposit Account No. 19-3140/VONL:004USC1.

Amendments to the Specification begin on page 2.

Amendments to the Claims begin on page 11.

Telephonic Interview Summary begins on page 15.

Remarks begin on page 16.

Aardevo Exhibit 1010
Page 0510

SPECIFICATION AMENDMENT

(1) Please amend the paragraph in the specification at page 9, lines 3-9 as follows:

Proper (commercial) sources of tetraploid cultivars are provided through the European Cultivated Potato Database (ECPD) available on the world wide web at: ~~http://~~www.europotato.org/menu.php and/or the Potato Pedigree Database of the expertise group Plant Breeding (a cooperation between the Laboratory of Plant breeding of Wageningen UR, the Netherlands) and the business unit Biodiversity and breeding of Plant Research International (PRI), Wageningen, the Netherlands available on the world wide web at: ~~http://~~www.plantbreeding.wur.nl/potatopedigree/.

(2) Please amend the paragraph in the specification at page 51, lines 2-5 as follows:

This resulted in 279 and 453 unique BLAST hits representing the occurrence of only one copy in the potato genome sequence of *Solanum tuberosum* Group Phureja DM1-3 516R44 (CIP801092) as known hitherto (Potato Genome Sequencing Consortium, www.potatoge[[m]]nome.net).

(3) Please amend the paragraph in the specification at page 51, lines 18-23 as follows:

Though twenty-five sequences did not meet the criteria described above, they were kept as these were [[were]]known to be highly informative in the GoldenGate SNP assays. These SNPs even allowed to discriminate between the five genotype classes that occur in tetraploids (Voorrips et al, EAPR-EUCARPIA congress "Potato Breeding after completion of the DNA Sequence of the Potato Genome", 27-30 June 2010, Wageningen, the Netherlands, e-publication available on the world wide web at: ~~http://~~edepot.wur.n1/143559, pg 42).

(4) Please amend the paragraph in the specification at page 52, lines 2-7 as follows:

Aardevo Exhibit 1010
Page 0511

The 100 SNP sequences selected as described above were used in a KASPar SNP genotyping system (KBioscience Ltd. Hoddesdon, UK; available on the world wide web at: ~~http://~~www.kbioscience.co.uk/lab%20services/SNP%20Genotyping/genotyping_chemistry.html) . The markers were tested on a set of 20 diploid potato genotypes (see Figure 4) including the three diploid parents used in the present study and further on the donor of the Sli gene, clone 07-1004-1 (see also Example 2 herein above).

(5) Please amend the paragraph in the specification at page 52, lines 19-23 as follows:

All markers showed a high frequency of occurrence in the diploid potato germplasm used in this experiment (Table 6~~Appendix III~~). The heterozygote frequency in the germplasm by using[[suing]] these 100 markers ranged from 26 % to 77%, while the Sli gene donor was 100% homozygous as is in accordance with expectations (Phumichai et al. 2005 Genome 48: 977-984).

(6) Please amend the paragraph in the specification at page 33, lines 11-22 as follows:

Plants according to the present invention include the line with breeders reference AGVD1, representative seeds of said line having been deposited on 23 October 2009 with the NCIMB, Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB219YA, Scotland under accession number NCIMB 41663 and breeders reference AGVD1, which plant represents an F2 obtained by crossing a plant of parent IVP07-1001/4 (see Example 1), which is an essentially homozygous, diploid, fertile and self-compatible but non-vigorous potato line with a plant of parent D1, which is an elite diploid breeding clone that is essentially non-homozygous, and is not self-compatible, but which has the desirable agronomic trait of yellow flesh color and exhibits good cooking quality in that the flesh is not greying after cooking, and selfing the resulting Fl offspring plants. It is estimated that AGVD1 is about 50% homozygous, meaning that about 50% of the loci for which the parents differ are homozygous.

Aardevo Exhibit 1010
Page 0512

(7) Please amend the paragraph in the specification at page 33, lines 23-31 as follows:

Plants according to the present invention also include the line with breeders reference AGVD2, representative seeds of said line having been deposited on 23 October 2009 with the NCIMB, Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB219YA, Scotland under accession number NCIMB 41664 and under breeders reference AGVD2, which line represents an (F1 x Fl) line (or pseudo F2) obtained by crossing a plant of parent IVP07-1001/4 as indicated above with a plant of parent D1 as indicated above, and mutually crossing individual Fl offspring plants, that share the IVP07-1001/4 parent but have different individuals of the D1 parent. It is estimated that AGVD2 is about 25% homozygous for the loci for which the parents differ.

(8) Please amend the paragraph in the specification bridging page 33, line 33 to page 34, line 6 as follows:

Plants according to the present invention also include the line with breeders reference AGVD3, representative seeds of said line having been deposited on 23 October 2009 with the NCIMB, Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB219YA, Scotland under accession number NCIMB 41665 and under breeders reference AGVD3, which line represents a BC1 population obtained by crossing a plant of parent IVP07-1001/4 as indicated above with a plant of parent D1 as indicated above, and backcrossing the resulting Fl offspring plants with the IVP07-1001/4 parent. It is estimated that AGVD3 is about 75% homozygous for the loci for which the parents differ.

(9) Please amend the paragraph in the specification at page 34, lines 7-14 as follows:

Plants according to the present invention also include the line with breeders reference AGVD17, representative seeds of said line having been deposited on 5 October 2010 with the

Aardevo Exhibit 1010
Page 0513

NCIMB, Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB219YA, Scotland under accession number NCIMB 41765 and under breeders reference AGVD17, which line represents an F3 population obtained by crossing a plant of parent IVP07-1001/4 as indicated above with a plant of parent D2 as indicated above, and backcrossing the resulting Fl offspring plants with the IVP07-1001/4 parent, followed by two steps of selfing. It is estimated that AGVD17 is > 80% homozygous for the loci for which the parents differ.

(10) Please amend the paragraph in the specification at page 37, lines 5-19 as follows:

The present inventors used the strategy of repeated selfing, backcrossing and crossing of potato germplasm of a single self-compatible potato line and one or more self-incompatible potato lines harbouring agronomic traits. The homozygous plants of the present invention were obtained by continuous selfing of diploid potato plants and selection for fertile offspring (besides the inherent selection for self- compatibility). This is an entirely new approach, as in the prior art methods, plants are obtained by selection for agronomical traits, such as tuber formation or general growth characteristics of the offspring plants, not by selection for fertile offspring from selfings. Moreover, and unlike the prior art methods the procedures are performed using diploid plant lines, instead [[to]]of tetraploid plants. Unexpectedly, this radically new approach proved successful for obtaining increasingly homozygous, diploid offspring plants, that were self-~~compatibel~~compatible and fertile. In addition, when (out)crossed with plants of diploid lines harbouring beneficial traits, such as resistance or white tuber color, these traits could be introduced into the essentially homozygous germplasm without loss of self-compatibility and fertility.

(11) Please amend table 5A in the specification as follows:

Table 5A. Sequences of 100 SNP markers in potato

Aardevo Exhibit 1010
Page 0514

SNPs

SNPID	SNPNum	AlleleY	AlleleX	Sequence	Sequence Identifier
PotSNP002	11940065	G	C	ACCTGGTGCAGGCCT[C/G]CGTATTGATACGATT	SEQ ID NO:1
PotSNP011	11940098	C	T	GACTGTGATTCGAAA[T/C]GTTTACCTATCTCTT	SEQ ID NO:2
PotSNP021	11940075	C	T	TCGTAGAAATGACAT[T/C]TGGGGTGCTAGAGAT	SEQ ID NO:3
PotSNP026	11940044	C	T	AGTTTCCAGCTGATG[T/C]TATTCTAGGAGACGA	SEQ ID NO:4
PotSNP032	11940037	G	A	AGAGGCTCTTGAGGC[A/G]CAGCGAAAAGAGGAA	SEQ ID NO:5
PotSNp038	11940016	C	T	TGGTGACGTTCATGT[T/C]GGACGTAATGCTTCT	SEQ ID NO:6
PotSNP043	11940036	C	A	AGTATCTCCGTCCGA[A/C]TCACAGTCCTACTCT	SEQ ID NO:7
PotSNP045	11940094	G	A	GGTGCTAAGATTCGC[A/G]TTGATGATTCACTAG	SEQ ID NO:8
PotSNP047	11940054	C	T	CCAACACTGGAAAAA[T/C]TATGGAAGCAGAAAG	SEQ ID NO:9
PotSNP052	11940097	C	T	GCTAGCATTAGAGCT[T/C]GCTAAGCGACTCAAT	SEQ ID NO:10
PotSNP055	11940021	G	A	GCCTTGGAGTAAGAG[A/G]TTTTCTGTTTTGGGC	SEQ ID NO:11
PotSNP057	11940040	C	T	GGATGTTCATCAACG[T/C]CGACTTCGACAAGCT	SEQ ID NO:12
PotSNP061	11940032	C	T	ACAACCTGCAAGAGC[T/C]TTGCATCTGTTTAGT	SEQ ID NO:13
PotSNP068	11940023	C	T	TACAATCATGATCCT[T/C]TTGCCTAATTGCCCT	SEQ ID NO:14
PotSNP072	11940005	G	A	CTGAGCTAGTAGATC[A/G]ATCAAACGGTGCTCC	SEQ ID NO:15
PotSNP073	11940076	G	T	GACTGTATGGACAGT[T/G]ATTGGGTCAGAGGAC	SEQ ID NO:16
PotSNP080	11940068	C	T	AAGATGACGTGGAGT[T/C]CGGCACTGCGCCGTT	SEQ ID NO:17
PotSNP081	11940047	C	T	GCCGCACAAGGTGCT[T/C]GCCGTCGATGTTAGC	SEQ ID NO:18
PotSNP083	11940033	C	T	AGGTAGTGTTGCAAA[T/C]ACAATTAGAGGGTTG	SEQ ID NO:19
PotSNP089	11940062	G	A	AAGTACATTTATGAG[A/G]AGATTAACAAGTGTT	SEQ ID NO:20
PotSNP099	11940092	C	T	ACTGCTGGAACTGAG[T/C]TGAACAATGCTATTA	SEQ ID NO:21
PotSNP100	11940083	C	T	GAGTGCCGGCAGCGT[T/C]ACAGATCCGACGATG	SEQ ID NO:22

Aardevo Exhibit 1010
Page 0515

PotSNP102	11940070	G	A	TATGGCAGAGAAAGA[A/G]ATGGAGTATAGAGTG	SEQ ID NO:23
PotSNP1026	11940059	G	C	TAAACTCCAACATAT[C/G]AACACAAATTAGTGC	SEQ ID NO:24
PotSNP1034	11940001	G	A	TTCTACATCCTTCGC[A/G]GGCATTTTTGAGGAG	SEQ ID NO:25
PotSNP1049	11940051	T	C	TTTGGGGAGGAACAC[C/T]TGTGCTTTAGAACTC	SEQ ID NO:26
PotSNP1057	11940022	A	C	TGTCACTAAGAAAGC[C/A]CAAGAGGCAATGCAG	SEQ ID NO:27
PotSNP106	11940010	G	T	GCAAGCTACTTGTTC[T/G]AAAGGACCTGCTACT	SEQ ID NO:28
PotSNP1072	11940027	G	T	AGCGAGTTTGGATGC[T/G]GAGCTTTTGCAGTTA	SEQ ID NO:29
PotSNP1083	11940019	C	T	TACAAAGTTCACTGC[T/C]GAGGAGGTCATGCAG	SEQ ID NO:30
PotSNP1105	11940041	A	C	GATGAGGAAAGGCAC[C/A]GGCGGAGGTGTTGGA	SEQ ID NO:31
PotSNP1115	11940071	T	C	CACCACCGTCCAAAC[C/T]GGTTTTGCCAATCGC	SEQ ID NO:32
PotSNP1122	11940060	T	C	GGTCCCTGAGCAAGG[C/T]AATCAACGAATTAGG	SEQ ID NO:33
PotSNP1139	11940004	G	A	AGTCTCTACAAACCC[A/G]TGGTTTCGATCTTGA	SEQ ID NO:34
PotSNP116	11940058	G	A	TAATGTGGTCGGCAC[A/G]CTGACGCTTGCTGAT	SEQ ID NO:35
PotSNP118	11940091	G	A	AGATGCATTCTATGC[A/G]GAACCACAAAGATAT	SEQ ID NO:36
PotSNP120	11940082	G	A	TTCGTGTTGCACTCA[A/G]CGATTCAGTTGGATG	SEQ ID NO:37
PotSNP121	11940099	C	T	AGCTTGTGCTAATAG[T/C]TGTGCTGATTTTCTC	SEQ ID NO:38
PotSNP122	11940078	G	A	TATGCAATCCCAGCC[A/G]ACATATCCGATCCAT	SEQ ID NO:39
PotSNP124	11940055	G	A	TCATACTTCACATAT[A/G]TCAGAGTGGTCTGAT	SEQ ID NO:40
PotSNP130	11940050	C	T	AGAGAAAAAAGCTGT[T/C]GTTGTTGATGACGAG	SEQ ID NO:41
PotSNP134	11940080	C	T	CGCGTATTTCCCTTA[T/C]TATAACTCGTTCTCT	SEQ ID NO:42
PotSNP138	11940052	C	T	TTACATATGCAATAG[T/C]GGTGGTGATCTTTAT	SEQ ID NO:43
PotSNP152	11940077	C	T	GAAGTTGTCTTACAT[T/C]GCTCTTGACTATGAA	SEQ ID NO:44
PotSNP162	11940074	C	T	CTATGATGCATTTAG[T/C]TCATGGAGCAAACTT	SEQ ID NO:45
PotSNP165	11940009	G	A	AGTTCCTTTTTCTCC[A/G]AGTTCTCAGACTCCG	SEQ ID NO:46

Aardevo Exhibit 1010
Page 0516

PotSNP178	11940026	G	T	GAGGAATTGATGCGT[T/G]CTGAACTGGCACAGT	SEQ ID NO:47
PotSNP182	11940095	G	A	ATACACAGGAAGGGT[A/G]AAGTGCTTCAAATTG	SEQ ID NO:48
PotSNP184	11940038	G	A	TTTGACCATTCTTTC[A/G]GTCCTAGCTAACCAC	SEQ ID NO:49
PotSNP185	11940002	C	T	AAGATAAGAAGAAAG[T/C]TAAGAGAACCAAGAT	SEQ ID NO:50
PotSNP194	11940064	C	T	GGCCCACTTTCCACA[T/C]ATTCAATTTGGTCTC	SEQ ID NO:51
PotSNP205	11940042	G	A	TCAACTCACCACTGG[A/G]TTTTACTCAAAATCA	SEQ ID NO:52
PotSNP213	11940096	G	A	TAATATGTTGTGTCT[A/G]TAATTTTTGGGTCAT	SEQ ID NO:53
PotSNP220	11940013	C	T	CATTAACCAAGGTTG[T/C]ATCGTTCGAGAGATC	SEQ ID NO:54
PotSNP229	11940056	C	T	TCCTTTGTCATTGTA[T/C]GCCATGGAAGAAGCA	SEQ ID NO:55
PotSNP392	11940003	T	A	GCTTTGTTTAAGAAC[A/T]GCCAGAAGAAGTTGC	SEQ ID NO:56
PotSNP401	11940012	G	A	GCCTACGCGGCATGA[A/G]GATGTGGATATTGTT	SEQ ID NO:57
PotSNP402	11940072	T	C	AAAGCTCAAATTAAA[C/T]GACGATGTTCAGGAA	SEQ ID NO:58
PotSNP423	11940020	A	G	GGCCAACCAATTCCA[G/A]CGTCTTCTCCCAGAC	SEQ ID NO:59
PotSNP446	11940081	C	T	AAAACCATTTGGTCT[T/C]GTTGATAATTCTGAG	SEQ ID NO:60
PotSNP458	11940069	A	G	TACATAGCTCTACAA[G/A]CTTGGAAACATGTCA	SEQ ID NO:61
PotSNP470	11940043	T	A	GACACAAAATTCATA[A/T]TGGCTGATACTTTGT	SEQ ID NO:62
PotSNP543	11940049	T	A	GCAACTTGTCGTCGG[A/T]AAACTCGACTAGATT	SEQ ID NO:63
PotSNP567	11940014	A	C	CTAGAAACAAAATCA[C/A]AATATAAGTTACATA	SEQ ID NO:64
PotSNP569	11940028	G	A	GTTGTTTGCTTTGTT[A/G]TGCTGACTTATTGTA	SEQ ID NO:65
PotSNP573	11940034	C	T	ATGCTTCTTGGTTGC[T/C]GTTTGTTTAGGCTCT	SEQ ID NO:66
PotSNP580	11940087	T	C	AAATATGTTGTTCCA[C/T]TAGAAAAAAGAGTAA	SEQ ID NO:67
PotSNP586	11940088	C	T	GTGAATACTGGTATC[T/C]TTCCAATTGAAGTCA	SEQ ID NO:68
PotSNP587	11940067	C	T	AATATTCTTGTAGTA[T/C]ATATTGTCTAAAATC	SEQ ID NO:69
PotSNP607	11940084	T	C	CAACGATATCGAGAA[C/T]TATTGTTCTAAGGGA	SEQ ID NO:70
PotSNP626	11940045	G	A	GCTGAAAGTAAAGAC[A/G]GTGTTCAACATCTTG	SEQ ID NO:71

Aardevo Exhibit 1010
Page 0517

PotSNP645	11940007	T	G	CATGCAATTGTAATA[G/T]TATCAATGTTTGTTG	SEQ ID NO:72
PotSNP652	11940024	T	C	TGCTCAAGCTGCCAA[C/T]GCTAATCCGCTTTAC	SEQ ID NO:73
PotSNP700	11940061	G	A	CAAGATATATGCAGC[A/G]GAGTCCCTTCGAGCA	SEQ ID NO:74
PotSNP702	11940039	A	T	TTATGGTTATGGTGG[T/A]GGAGGTTGGATTAAT	SEQ ID NO:75
PotSNP712	11940057	C	G	GGGAGGGTGAAAATA[G/C]TCGAAAACGGGCAAC	SEQ ID NO:76
PotSNP713	11940073	G	A	GATTGTTATCCTTCC[A/G]CCACCTCCAACAGAT	SEQ ID NO:77
PotSNP731	11940035	A	G	CAACTTCCAGTCGTG[G/A]GAGTGGGGTTAACGG	SEQ ID NO:78
PotSNP753	11940046	G	A	ATGGTGTCTCCAAGG[A/G]ACTATTACAGTCACT	SEQ ID NO:79
PotSNP759	11940090	C	T	AACATCGATAGGACT[T/C]TGAAGGCCGCGGATG	SEQ ID NO:80
PotSNP766	11940089	T	C	CTGCCACTGCGGCTG[C/T]AGGTTCTGCAGCTGC	SEQ ID NO:81
PotSNP775	11940079	G	A	TGAAATGATTGGATT[A/G]TGACCCATAGTTTAG	SEQ ID NO:82
PotSNP796	11940025	C	A	ATCTCTAAGTTGGGG[A/C]AACCCATAATTTCTA	SEQ ID NO:83
PotSNP827	11940053	T	A	CGCTTGGGAAGAACC[A/T]TTCGGTCCAGTTTTG	SEQ ID NO:84
PotSNP834	11940006	T	C	CCAATTCCGACGGCC[C/T]TCGTCGTTTCACCGC	SEQ ID NO:85
PotSNP853	11940066	A	C	GGTCGACCATTGGGG[C/A]CCCGTAGAGGGTATA	SEQ ID NO:86
PotSNP866	11940048	G	T	AAGGCGAATTCCGGT[T/G]CCCGAAATGGATCTC	SEQ ID NO:87
PotSNP893	11940011	A	G	TCATTACATCAAAAT[G/A]TTTTATCAATGCCCA	SEQ ID NO:88
PotSNP908	11940100	T	A	TGATCCATTTGGTCT[A/T]GACCAGTTCTTGACA	SEQ ID NO:89
PotSNP910	11940017	T	A	TGCTGTGCTCCTTCT[A/T]TTCCTCATCTCTCAC	SEQ ID NO:90
PotSNP928	11940030	A	T	TCATTCTCACTCTCC[T/A]GCTGAACAAGCCATT	SEQ ID NO:91
PotSNP947	11940015	T	G	AAAAGACAAGAATCT[G/T]GTTCAGATTCTCCAG	SEQ ID NO:92
PotSNP948	11940063	T	A	TGGGGAAAACTTAAT[A/T]TGACAGCCAAGGCGT	SEQ ID NO:93
PotSNP960	11940031	C	T	GGTTTTGTGGACTGC[T/C]AACACTGAAAGATAC	SEQ ID NO:94
PotSNP964	11940029	A	T	TTATTTCAATGAAGC[T/A]TCTGGTGGACGTTAT	SEQ ID NO:95
PotSNP982	11940085	C	T	TCTGTGGTTTTTTCA[T/C]CACCTCCATCTTCAA	SEQ ID NO:96

Aardevo Exhibit 1010
Page 0518

PotSNP983	11940086	G	A	TAAGATTAGTTCTAA[A/G]CTTAGCGCAGAAGAC	SEQ ID NO:97
PotSNP985	11940018	A	G	GAGCTCTATAAGTGC[G/A]CCTGTTACTCCACCT	SEQ ID NO:98
PotSNP987	11940008	C	A	GGGCTATGGTTGGTT[A/C]GGAAAAAAGGTGCAA	SEQ ID NO:99
PotSNP996	11940093	C	T	GGATATTGGGTTCGT[T/C]CCATGATTCGTGGCA	SEQ ID NO:100

Aardevo Exhibit 1010
Page 0519

CLAIM AMENDMENT

Please amend the claims as follows:

1-34. (Cancelled)

35. (Currently Amended) A ~~*Solanum* species~~ plant of a potato line, wherein said potato~~plant~~ line is diploid, fertile, self-compatible, produces at least 200 grams fresh weight of tubers per plant, and at least 50% of the genomic loci in said potato[[plant]] line are homozygous, wherein said self-compatibility in said potato line is conferred by the S-locus inhibitor (*Sli*) gene from *Solanum chacoense*, and wherein a copy of said gene is present in potato lines AGVD1, AGVD2, AGVD3, and AGVD17, representative seeds of said lines having been deposited with the NCIMB under NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, and NCIMB accession number 41765, respectively.

36. (Cancelled)

37. (Currently Amended) The ~~*Solanum* species~~ plant [[line]]of claim 35, wherein said plant[[line]] further comprises at least one ~~additional~~ agronomically desirable trait[[s]] selected from the group consisting of: insect tolerance, nematode tolerance, disease resistance, herbicide tolerance, cold tolerance, drought tolerance, flooding tolerance, tolerance to dry and wet rot, salinity tolerance, yellow flesh color, and cold-sweetening~~sweeting~~ resistance.

38. (Currently Amended) The ~~*Solanum* species plant~~potato line of claim 35, wherein said ~~plant~~potato line has a fresh weight of the foliage and shoots of at least 500 grams per plant at maturity.

39. (Cancelled)

40. (Currently Amended) A seed or tuber of the plant of claim 35[[39]].

41. (Currently Amended) A method of producing a ~~*Solanum* species~~ plant of a potato line, wherein said potato~~plant~~ line is diploid, fertile, self-compatible, produces at least 200 grams fresh weight of tubers per plant, and at least 50% of the genomic loci in said plant line are homozygous, comprising the steps of:

(a) providing a first potato plant, wherein said first potato plant is the plant~~diploid, fertile, self-compatible and essentially homozygous~~according to claim 35;

Aardevo Exhibit 1010
Page 0520

(b) providing a second potato plant, wherein said second potato plant is a diploid ~~*Solanum* species~~ plant, and wherein said second potato plant comprises~~having~~ any level of homozygosity;

(c) cross-pollinating said first potato plant and said second potato plant to produce~~provide a~~ seed and collecting said seed to thereby provide a hybrid offspring seed;

(d) growing a multiplicity of said hybrid offspring seed in a population of hybrid offspring potato plants and selecting from said population a self-compatible potato plant;

(e) inbreeding said self-compatible potato plant by selfing ~~or backcrossing~~ said self-compatible potato plant to a potato plant of said line of said first potato plant, for between 1 to 8 selfings ~~or backcrosses~~ to thereby increase the degree of homozygosity in each successive generation; and

(f) selecting within each generation of inbreeding for a fertile offspring plant to provide a diploid, fertile, self-compatible ~~*Solanum* species~~potato plant[[line]], wherein at least 50% of the genomic loci in said plant [[line]]are homozygous.

42. (Cancelled)

43. (Currently Amended) The method of claim 41[[42]], ~~wherein step (b) comprises the provision of a second potato plant,~~ wherein said second potato plant is a donor for an ~~additional~~ agronomically desirable trait~~, wherein said trait is~~ selected from the group consisting of: insect tolerance, nematode resistance, disease resistance, herbicide tolerance, cold tolerance, drought tolerance, flooding tolerance, tolerance to dry and wet rot, salinity tolerance, yellow flesh color, and cold-sweetening resistance.

44. (Currently Amended) ~~The *Solanum* species~~A potato plant [[line]]produced by the method of claim 41.

45. (Currently Amended) A method of producing a uniform hybrid potato~~*Solanum* species~~ seed comprising:

(a) providing a first ~~*Solanum* species~~potato plant, wherein said first potato plant is a plant of a first potato line, wherein said first potato line is~~that is a first~~ diploid, fertile, self-compatible and ~~essentially~~wherein at least 50% of the genomic loci in said first

Aardevo Exhibit 1010
Page 0521

potato line are homozygous ~~*Solanum* potato line~~, and wherein said self-compatibility in said first potato line is conferred by the S-locus inhibitor (*Sli*) gene from *Solanum chacoense*, wherein a copy of said gene is present in potato lines AGVD1, AGVD2, AGVD3, and AGVD17, representative seeds of said lines having been deposited with the NCIMB under NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, and NCIMB accession number 41765, respectively;

(b) providing a second ~~*Solanum* species~~potato plant, wherein said second potato plant is a plant of a second potato line, wherein said second potato line is ~~that is a~~ diploid ~~or tetraploid,~~ fertile, self-compatible and ~~essentially~~wherein at least 50% of the genomic loci in said second potato line are homozygous ~~*Solanum* potato plant~~, wherein said self-compatibility in said second potato line is conferred by the S-locus inhibitor (*Sli*) gene from *Solanum chacoense*, wherein a copy of said gene is present in potato lines AGVD1, AGVD2, AGVD3, and AGVD17, representative seeds of said lines having been deposited with the NCIMB under NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, and NCIMB accession number 41765, respectively, and wherein said ~~first and~~ second potato plant contains at least 20% contrasting homozygous loci as determined by molecular marker analysis when compared to said first potato plant;

(c) cross-pollinating said first and second potato plants and allowing one of said potato~~parent~~ plants to produce berries with seeds; and

(d) collecting said seeds from said berries to provide a hybrid ~~*Solanum* species~~potato seed.

46. (Cancelled)

47. (Currently Amended) The method according to claim 45, wherein at least one of said first and second ~~*Solanum* species~~potato plants comprises at least one agronomically desirable trait selected from the group consisting: of insect tolerance, nematode resistance, disease resistance, herbicide tolerance, cold tolerance, drought tolerance, flooding tolerance, tolerance to dry and wet rot, salinity tolerance, yellow flesh color, and cold-sweetening resistance.

Aardevo Exhibit 1010
Page 0522

48. (Currently Amended) [[The]]A uniform hybrid ~~*Solanum* species~~potato seed produced according to the method of claim 45.

Aardevo Exhibit 1010
Page 0523

TELEPHONIC INTERVIEW SUMMARY

On March 18, 2020, Applicants' undersigned representatives, Robert E. Hanson, Reg. No. 42,628, and Sheila Walker, Reg. No. 71,087, spoke telephonically with Examiner Boggs regarding the outstanding rejections in the present Office Action. The Examiner indicated that amendments to claim 35 to replace recitation of "*Solanum* species plant line" with language found in the specification, such as "a plant of a potato line," and to incorporate the *Sli* gene language of claim 36 while also referencing the deposit as a source of the *Sli* gene would help overcome a number of issues. The possibility of amending claim 41 to recite similar limitations to those proposed for claim 35 along with referencing the plant of claim 35 itself, given that the product claims were not subject to the art rejection, was also discussed.

The Examiner also noted that his primary concerns remain with the supporting letters filed in the parent case, which he has interpreted as evidence demonstrating that success in obtaining the claimed plants is dependent on the genetic background of the starting germplasm, and the Janksy review article from 2016 that cites the inventor's work with the subject matter being claimed, which he has interpreted as showing that the claimed methods can be used for certain germplasm and do not solve the larger problem of creating diverse vigorous diploid self-compatible inbred potato lines. The Examiner indicated that a 132 Declaration that provides evidence to the contrary would be helpful in advancing prosecution.

Applicants thank Examiner Boggs for the time taken to discuss the case.

Aardevo Exhibit 1010
Page 0524

REMARKS

A. Status of the Claims

Claims 35-48 are pending in the application. Claims 35-48 are rejected. Claims 35, 37, 38, 40, 41, 43-45, and 47-48 are amended and claims 36, 39, 42, 46 are cancelled herein. Support for the amendments can be found, at least, in the Specification and claims as filed.

B. Specification Objection

The Action objects to the specification for containing informalities. Applicants note that the Specification is amended herein to address the issues raised in the Action. The objection is thus believed to be moot and withdrawal thereof respectfully requested.

C. Sequence Rules

The Action objects to the Specification as missing sequence identifiers for the linker sequences in Table 5A. Applicants refer to the Specification Amendment presented herewith, which adds sequence identifiers to Table 5A. Support for the amendment can be found in the sequence listing as filed. As the Specification fully complies with 37 CFR 1.821-1.825, withdrawal of the objection is respectfully requested.

D. Claim Objections

The Action objects to claims 37 and 39 for containing informalities. Applicants note that claim 37 is amended herein and claim 39 is cancelled herein. The objection is thus believed to be moot and withdrawal thereof respectfully requested.

E. Rejection Under 35 U.S.C. § 112, Second Paragraph

The Action rejects claims 35 and 41-42 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly clam the subject matter which the inventor regards as the invention. Applicants respectfully traverse but note that claims 35

Aardevo Exhibit 1010
Page 0525

and 41 are amended herein in the interest of compact prosecution. The rejection is thus believed to be moot and withdrawal thereof is respectfully requested.

F. Rejection of Claims Under 35 U.S.C. §112, First Paragraph - Written Description

The Action rejects claims 35-48 under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement. The Action asserts that the Specification provides written description for deposited lines AGVD1, AGVD2, AGVD3 and AGVD17 but fails to describe working examples of the broad genus of any other *Solanum* lines, which if combined with the *Sli* gene, that fall within the scope of the claims. The Action further asserts that one of skill in the art would not believe Applicants to be in possession of the genus as broadly claimed, especially in view of the discussion of the art by Applicants and the letter dated 11 December 2012 of Dr. Jonathan D G Jones. Applicants respectfully traverse.

Submitted herewith is the Declaration of Dr. Willem Hendrik Lindhout under 37 C.F.R. § 1.132. At paragraphs 10-12 of the Declaration, Dr. Lindhout provides the breeding histories for potato lines D1, D2, and D3, which are the diploid donor lines used to produce deposited lines AGVD1, AGVD2, AGVD3 and AGVD17. At paragraphs 13-15 of the Declaration, Dr. Lindhout discusses the wide genetic diversity of potato lines D1, D2, and D3. At paragraphs 16-17 of the Declaration, Dr. Lindhout describes a study performed to determine the genetic variation between lines D1, D2, and D3, as well as the genetic variation between these lines and a random set of 22 commercial tetraploid *Solanum tuberosum* varieties and explains that the results show that D1, D2, and D3 represent a random sample of *Solanum tuberosum*.

In paragraphs 21-23 of the Declaration, Dr. Lindhout discusses the letter of 11 December 2012 of Dr. Jones cited in the Action. At paragraph 22 of the Declaration, Dr. Lindhout provides an excerpt from a response letter from Dr. Jones that further explains the statements he made in

Aardevo Exhibit 1010
Page 0526

the 11 December 2012 letter. In the response letter, which is provided in the Declaration as **Exhibit A**, Dr. Jones states:

> "I herewith inform you [...] that I never made a statement to that effect, and that my words are misinterpreted. What I stated is that we attempted to test the *Sli* gene in *S. phureja*, but were not successful, and that we found that, after only a few rounds of selfing with the original material from Hosaka, inbreeding depression was already so extensive that we terminated the program, because we were unable to propagate the material. We drew no specific conclusions at the time, other than that we considered that it might have been due to the poor germplasm we worked with. However, I was well aware at the time of my letter that this was not the case, as I was convinced from personal communications, and your scientific publication in Potato Research (2011) Vol. 54:301–312, that we should have persisted inbreeding and selecting plants with seed carrying berries.
>
> I considered that your method was widely applicable to potato, and you had certainly shown that it was widely applicable to *S. tuberosum*."

Furthermore, at paragraph 34 of the Declaration, Dr. Lindhout explains that the present inventors were able to develop the experimental test-hybrids into a potato hybrid breeding system and provides publications demonstrating its use. A person of ordinary skill in the field of plant biotechnology would be able to generate similar results using the teaching in the application with only routine experimentation. The results further demonstrate that one of skill in the art as of the filing date of the patent application would have been able to, using routine experimentation, use any diploid potato donor lines to generate a plant of a potato line that is diploid, fertile, self-compatible, produces at least 200 grams fresh weight of tubers per plant, and at least 50% of the genomic loci in said plant line are homozygous, wherein said self-compatibility in said line is conferred by the S-locus inhibitor (*Sli*) gene from *Solanum chacoense*, and wherein a copy of said gene is present in potato lines AGVD1, AGVD2,

AGVD3, and AGVD17. Thus, the invention is not limited to a potato lines of a specific genotype, such as deposited lines AGVD1, AGVD2, AGVD3 and AGVD17, and the lines described in the specification are commensurate with the full scope of the claimed invention. Applicants were therefore in possession of the invention as claimed. In view of the foregoing, the rejection is believed to be moot and withdrawal thereof is respectfully requested.

G. Rejection of Claims Under 35 U.S.C. §112, First Paragraph - Enablement

The Action rejects claims 35-48 under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement. Specifically, the Action asserts that the Specification describes four deposited lines however the claimed genus is much broader. The Action further asserts that extensive guidance would be required to enable the invention as broadly claimed, given that success in practicing the claimed invention appears to be unpredictable and dependent on the genotype of the potato lines used, however such guidance is not provided in the application. Applicants respectfully traverse.

Submitted herewith is the Declaration of Dr. Willem Hendrik Lindhout under 37 C.F.R. § 1.132. At paragraphs 13-15 of the Declaration, Dr. Lindhout discusses the wide genetic diversity of potato lines D1, D2, and D3, which are the diploid donor lines used to produce deposited lines AGVD1, AGVD2, AGVD3 and AGVD17. At paragraphs 16-17 of the Declaration, Dr. Lindhout describes a study performed to determine the genetic variation between lines D1, D2, and D3, as well as the genetic variation between these lines and a random set of 22 commercial tetraploid *Solanum tuberosum* varieties and explains that the results show that D1, D2, and D3 represent a random sample of *Solanum tuberosum*.

With regard to the assertion made in the Action that success in practicing the claimed invention appears to be unpredictable and dependent on the genotype of the potato lines used,

Aardevo Exhibit 1010
Page 0528

Dr. Lindhout addresses this in paragraphs 21-23 of the Declaration. At paragraph 22 of the Declaration, Dr. Lindhout provides an excerpt from a response letter from Dr. Jonathan D G Jones that further explains statements made in the original letter of 11 December 2012 of Dr. Jones cited in the Action. In the response letter, which is provided in the Declaration as **Exhibit A**, Dr. Jones states:

> "I herewith inform you [...] that I never made a statement to that effect, and that my words are misinterpreted. What I stated is that we attempted to test the *Sli* gene in *S. phureja*, but were not successful, and that we found that, after only a few rounds of selfing with the original material from Hosaka, inbreeding depression was already so extensive that we terminated the program, because we were unable to propagate the material. We drew no specific conclusions at the time, other than that we considered that it might have been due to the poor germplasm we worked with. However, I was well aware at the time of my letter that this was not the case, as I was convinced from personal communications, and your scientific publication in Potato Research (2011) Vol. 54:301–312, that we should have persisted inbreeding and selecting plants with seed carrying berries.
>
> I considered that your method was widely applicable to potato, and you had certainly shown that it was widely applicable to *S. tuberosum*."

Dr. Lindhout states in paragraph 23 of the Declaration that the claimed methods can be successfully used in species other than *Solanum tuberosum*.

Applicants further note that the entire scope of the claimed subject matter is fully enabled because all that is required to satisfy the enablement requirement is that the specification teaches one reasonably skilled in the art how to make and use the claimed invention without undue experimentation. In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). Some experimentation and routine screening will not preclude enablement, particularly when a

Aardevo Exhibit 1010
Page 0529

reasonable amount of guidance with respect to the direction in which the experimentation should proceed is provided. *In re Wands*, 858 F.2d at 737.

Thus, the invention is not limited to a potato lines of a specific genotype, such as deposited lines AGVD1, AGVD2, AGVD3 and AGVD17. Thus, one reasonably skilled in the art viewing the application as filed and coupled with information known in the art, is enabled to make and use the full scope of the claims. As such, the rejection is believed to be moot and withdrawal thereof is respectfully requested.

H. Rejection of Claims Under 35 U.S.C. §112, Fourth Paragraph

The Action rejects claim 37 under 35 U.S.C. §112, fourth paragraph, as being of improper dependent form. Applicants respectfully traverse but note that claim 37 is amended herein in the interest of compact prosecution. The rejection is thus believed to be moot and withdrawal thereof is respectfully requested.

I. Rejection Under 35 U.S.C. § 103

The Action rejects claims 41-48 under 35 U.S.C. § 103 as being unpatentable over Phumichai *et al.* (*Genome* 48:977-84, 2005), in view of Maris (*Euphytica* 46:15-33, 1990) in further view of Watanabe *et al.* (*Breed Sci* 46:329-36, 1996). Applicants respectfully traverse but note that claim 41 is amended herein to reference claim 35 and claim 45 is amended herein to recite the same limitations as claim 35, which is not subject to the rejection and consistent with the Telephonic Interview Summary above. The rejection is thus believed to be moot and withdrawal thereof is respectfully requested.

J. Double Patenting

The Action provisionally rejects claims 35-48 on the ground of nonstatutory double patenting as being unpatentable over claims 1, 3-6, 16, 18-21, 29-30, 32, 35, 37-39, and 42-43 of

Aardevo Exhibit 1010
Page 0530

copending Application Ser. No. 13/504,156. Applicants respectfully traverse but note that a Terminal Disclaimer will be submitted upon indication of otherwise allowable subject matter in order to expedite prosecution.

K. Request for Information Under 37 C.F.R. § 1.105

Applicants provide the following information and remarks regarding the requests made in the Request for Information under 37 C.F.R. § 1.105 communication to the extent such information is known and readily available pursuant to 37 C.F.R. § 1.105:

Items a)-e)

All information on designations/denominations of the original parental lines is provided in Table 6 on page 57 of the Specification as filed. In principle, all these parental lines are publicly availability and the breeders are indicated in Table 6, although lines may be proprietary.

Seed of the Sli gene donor "H" was a gift from Prof. Dr. K. Hosaka, Food Resources Education and Research Center, Kobe University, Japan, to Dr. Ir. H.J. Van Eck, Department of Plant Sciences, Wageningen University and Research, Wageningen, Netherlands. The seeds were highly inbred (S6-S8) obtained by selfing from lines 4H127-19, 4H128-16, 4H130-7 and line 5H130-5. These seeds are progeny of the Sli-donor F1-1 as described in Phumichai *et al.* (2005) *Genome Vol.* 48:977-984, Phumichai and Hosaka (2006) *Euphytica* 149:251–258, and Phumichai *et al.* (2006) *Euphytica* 148: 227–234. The "Hosaka" seed lines 4H127-19, 4H128-16, 4H130-7 and 5H130-5 (from 2H32-3) received the IVP code "07-1001/4" (for "Instituut Voor Plantenveredeling" – Institute for Plant Breeding, Wageningen) upon receipt. IVP07-1001/4 is 100% homozygous.

The various diploid *S. tuberosum* clones used in the Examples of the Specification include clones designated D1-D19. All of these are described in Table 6 of the Specification.

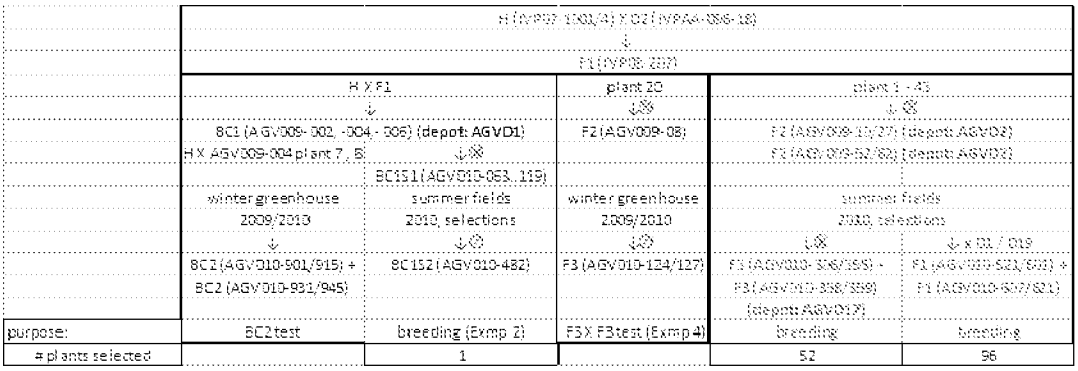
Aardevo Exhibit 1010
Page 0531

Lines D1, D2, and D3 (designations or culture collection codes IVP97-079-9, IVPAA-096-18, and SH83-92-488, respectively) were used for introgression of the *Sli*-gene from the *Sli* donor described above. D1-D3 represent dihaploid clones (2n = 2x =24) produced by haploidization of cultivated tetraploid *S. tuberosum* using conventional methods of gynogenesis, wherein haploidization occurs via the 'maternal,' or the seed parent's genome. In this process, specific *S. phureja* (2n = 2x = 24) selections, known as 'haploid inducers,' contribute the paternal gametes for pollination of the desired haploid progenitor. Lines D1-D3 were produced through gynogenesis, also referred to as "prickle pollination". The clones are available from Ronald Hutten, Laboratory for Plant Breeding, Wageningen University, as indicated in Table 6. IVP97-079-9, IVPAA-096-18, and SH83-92-488 are essentially heterozygous.

The breeding method used was not a single seed descent, nor bulk method, but was what the inventors refer to as "breeding with selection of offspring." Using this method, essentially starting with parental lines IVP07-1001/4 (as *Sli* gene donor) and IVP97-079-9, IVPAA-096-18, and SH83-92-488 (as diploid *S. tuberosum Sli*-gene recipients), plants according to claim 35 were obtained without further crossings to other diploid *S. tuberosum* (e.g. deposit AGVD17). AGVD17 is >80% homozygous, fertile with 300 gr of tubers. The plants according to claim 35, may thus comprise F3 plants, but may also comprise BC1S2 generations, F1 generations, and "pseudo F3" generations (Figure 2 of the Specification, reproduced below).

FIGURE 2

Aardevo Exhibit 1010
Page 0532



To further improve the stability of the plant lines obtained, of which may show reduction in vigour, self-compatibility and fertility upon continued selfing, the lines were crossed with a plant of another diploid *S. tuberosum* line (D1-D19). Such plants are indicated in Figure 2 above as "F1" plants.

As further explanation of this method (in red in the figure above), the middle part of Figure 2 is provided wherein the breeding program starts with a cross between diploid self-compatible *Sli*-donor IVP07-1001/4 and dihaploid *S. tuberosum* line IVPAA-096-18. The resulting F1 seed (IVP08-207) is grown into plants numbered 1–43. These plants are selfed, indicated by " ⊗ ", resulting in an F2. From these F2 populations (representative F2 seeds having been deposited under breeders reference AGVD2), fertile and self-compatible plants are selected and either selfed (left leg of right column) resulting in F3 populations (representative F3 seeds having been deposited under breeders reference AGVD17), or crossed (right leg of right column) with a plant of yet another dihaploid *S. tuberosum* line (D1-D19), resulting in an F1. Further breeding included steps of improving for agronomical traits through conventional breeding, using crossings, backcrossings and/or selfings.

Item f)

Aardevo Exhibit 1010
Page 0533

Patent applications or patents in which sibs of the instant plant are claimed include U.S. provisional patent application No. 62/879,769, line designated "Solyntus" and U.S. provisional patent application No. 62/933,151 (no specific designation claimed).

L. Conclusion

In light of the foregoing, Applicants submit that the case is in condition for allowance, and an early indication to that effect is earnestly solicited. The Examiner is invited to contact the undersigned at (214) 259-1890 with any questions, comments or suggestions relating to the referenced patent application.

DENTONS US LLP
2000 McKinney Avenue, Suite 1900
Dallas, TX 75201-1858
214-259-1890
214-259-0910 (fax)

Respectfully submitted,

/Sheila Walker/

Sheila Walker
Reg. No. 71,087
Agent for Applicant

Date: May 5, 2020

Aardevo Exhibit 1010
Page 0534

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Willem Hendrik Lindhout *et al.*	Group Art Unit: 1663
Serial No.: 16/541,071	Examiner: Russell T. Boggs
Filed: August 14, 2019	Atty. Dkt. No.: VONL:004USC1
For: Hybrid Seed Potato Breeding	Confirmation No.: 4348

DECLARATION OF WILLEM HENDRIK LINDHOUT, PH.D. UNDER 37 C.F.R. § 1.132

I, Willem Hendrik Lindhout, hereby declare as follows:

1. I am a Founder and Director of R&D at Florere B.V. and Agventure B.V., operating under the tradename Solynta, and am an inventor on the patent application identified above ("the Application").

2. I hold an MSc degree in Biology, an MSc degree in Chemistry, and a Ph.D. in Molecular Biology from the University of Leiden, in the Netherlands. Until 2006, I was an Associate Professor of Plant Breeding at Wageningen University in the Netherlands. During this time period, I published more than 100 scientific publications related to plant breeding, served as the Coordinator of Bachelors and Masters Studies in Plant Sciences at Wageningen University, and served as the National Coordinator of Tomato Genomics Research as part of the Centre for Biosystems Genomics (CBSG). Previously, I served as Director of R&D at De Ruiter Seeds. After the acquisition of De Ruiter Seeds by Monsanto, I was appointed Monsanto's Global Breeding Lead of the Vegetable Seed Division.

3. I understand that the United States Patent and Trademark Office is in the process of evaluating the patentability of U.S. patent application serial No. 16/541,071 and am familiar with the contents of the Office Action issued by the United States Patent and Trademark Office on December 5, 2019.

4. I am providing the present Declaration to provide evidence demonstrating that the dihaploid lines designated D1, D2, and D3 are genetically diverse and are representative of the entire range of genomic diversity in the *Solanum tuberosum* germplasm that is currently available and that the application therefore provides a sufficient number of working examples to support the plants as presently claimed and the wide applicability of the claimed methods across diverse *S. tuberosum* germplasm.

5. I am further providing the present Declaration to show that the Examiner is misinterpreting the letter of Dr. Jonathan D G Jones of 11 December 2012 in support of the assertion that the claimed method is not shown to work in *S. tuberosum* lines other than lines D1, D2, and D3, or the deposits made in connection with the application. In particular, I will provide further evidence to support that the success in practicing the claimed invention is not unpredictable and does not depend on the genotype of the potato lines used.

6. Finally, I am providing the present Declaration to show that the Examiner is misinterpreting the publication of Jansky et al. (2016) "Reinventing Potato as a Diploid Inbred Line-Based Crop." Crop Sci. 56:1412-22, when concluding therefrom that, approximately seven years after the filing date of the instant application, the problem of creating vigorous inbred diploid *S. tuberosum* plants apparently still existed. In particular, I will provide evidence that the Applicant was in possession of the invention as broadly as claimed at the time of filing.

Aardevo Exhibit 1010
Page 0536

Genetic diversity of lines D1, D2, and D3

7. Diploid potato lines D1, D2, and D3 (IVP97-079-9, IVPAA-096-18, and SH83-92-488, respectively, as indicated in Table 6 of the application) are heterozygous lines of a diploid potato breeding program at the Department of Plant Breeding, Wageningen University, the Netherlands. This program spans a period of over 50 years, and was initiated using a wide range of dihaploids generated from tetraploid potato cultivars in the 1960's.

8. In support of the diverse genetic background and wide applicability of the plants and methods as claimed, the complete pedigree showing the generation of diploid clones D1, D2, and D3 is provided as Figure 1 below. A majority of the information of Figure 1 is available from the public Potato Pedigree Database (https://www.plantbreeding.wur.nl/PotatoPedigree/index.html).

9. In the pedigree, the original tetraploid ($2n = 4x = 48$) *Solanum tuberosum* cultivars are shown in green, the dihaploids ($2n = 2x = 24$) generated from these tetraploids are shown in red, the diploid breeding lines of the program that are ancestors of D1, D2 and D3 are shown in orange, and diploid lines D1-D3 are shown in yellow. Red colored lines show parental mother, blue colored lines show parental father. The tetraploid cultivars (green) represent commercial cultivars, such as Chippewa, Fennema, Grata, Merrimack and Katahdin. Names of the diploid breeding lines (orange) often include a reference to their production year (e.g. DH63-72, the dihaploid from Maritta was developed in 1963).

Aardevo Exhibit 1010
Page 0537

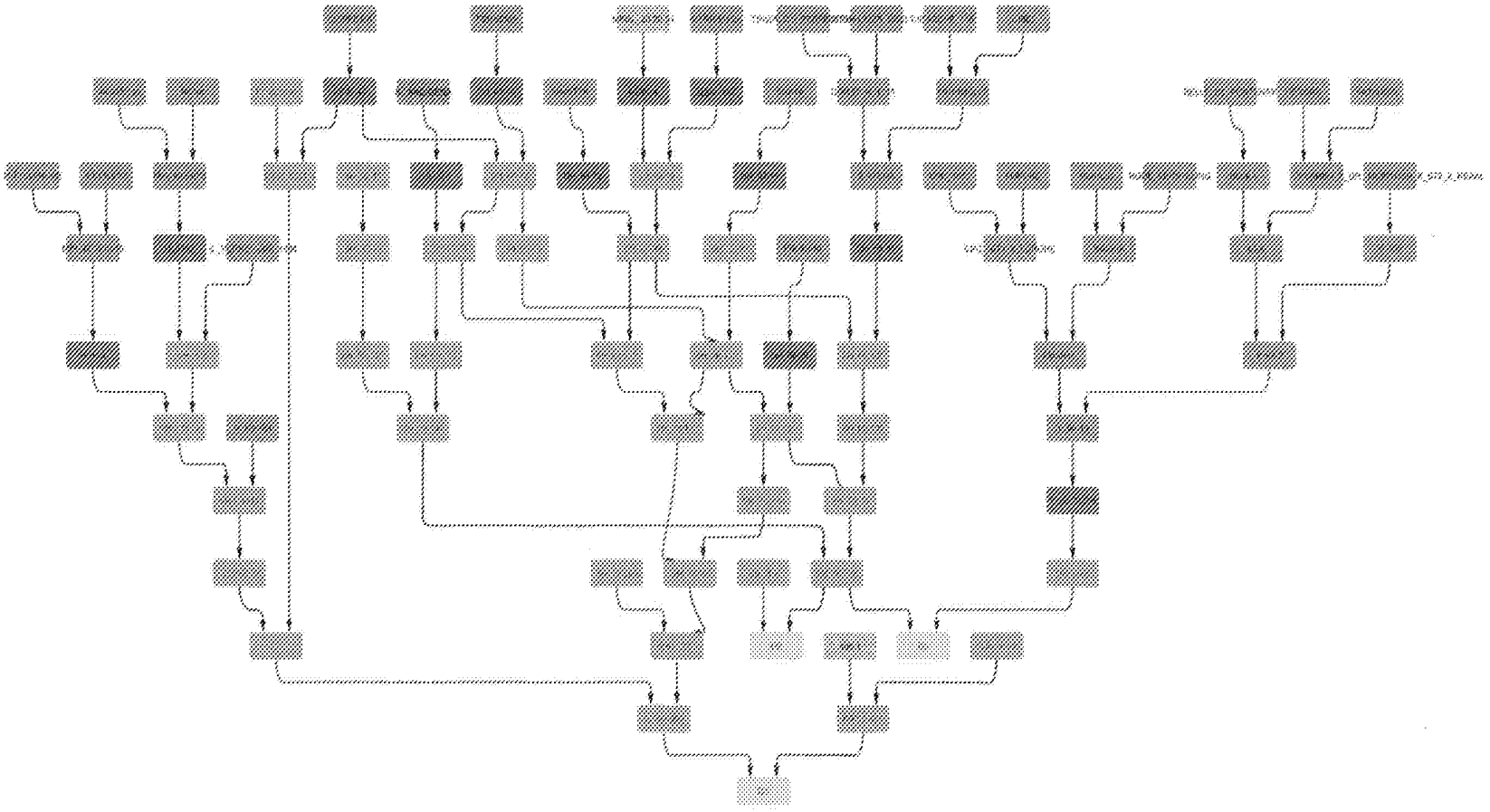


Figure 1. Pedigree of the diploid breeding lines D1, D2 and D3 (yellow) as described in the Application.

Aardevo Exhibit 1010
Page 0538

10. D1 is a diploid resulting from a cross between IVP92-053 (mother) and IVP92-027 (father). These parental lines were produced in 1992. The mother line is a descendant of dihaploids generated from the original tetraploid cultivars "Primura", "Grata", "Fennema", "Chippewa", "Maritta", "Minn-20-20-35" "Merrimack", "Mittelfrühe", "Bona", and wild relative "*S. andigena*".

11. D2 is a diploid resulting from a cross between DB-207 (mother) and SH_76-128 (father). The father line is a descendant of dihaploids generated from the original tetraploid cultivars "Chippewa", "Fennema", "Maritta", "Minn-20-20-35" "Merrimack", "Grata", "Primura", "Sirtema", and wild relative "*S. andigena*". "Sirtema" finds ancestry in cultivars "Fruhmolle", "Jubel", "Berlikumer Geeltje", and "Tinwald's Perfection".

12. D3 is a diploid resulting from a cross between DH_80-8 (mother) and SH_76-128 (father). The mother line is a descendant of a dihaploid generated from the original tetraploid cultivars "Furore", "Rode Eersteling", "Herald", "Saskia", and "Amaryl" as paternal ancestors, and "McIntosh", "Record", "Katadhin", "Belle de Fontenay", "Belka" and "Bea" as maternal ancestors.

13. D1, D2, and D3 represent such large genetic variation, that the use of each of these plants in crosses yield offspring plants with a wide variation in tuber characteristics as skin color and pattern (white to deep purple, solid color, multi-colored), flesh color and color pattern (solid, ringed, white to deep purple), tuber shape (oval spherical, flat, long), tuber "eye" configuration (depth, number, location, color), tuber starch content, fry quality, cook quality, as well as non-tuber characteristics such as flower color, etc. This is typical for dihaploids derived from tetraploid potatoes. The same was observed in an F2 population created by self-pollinating a

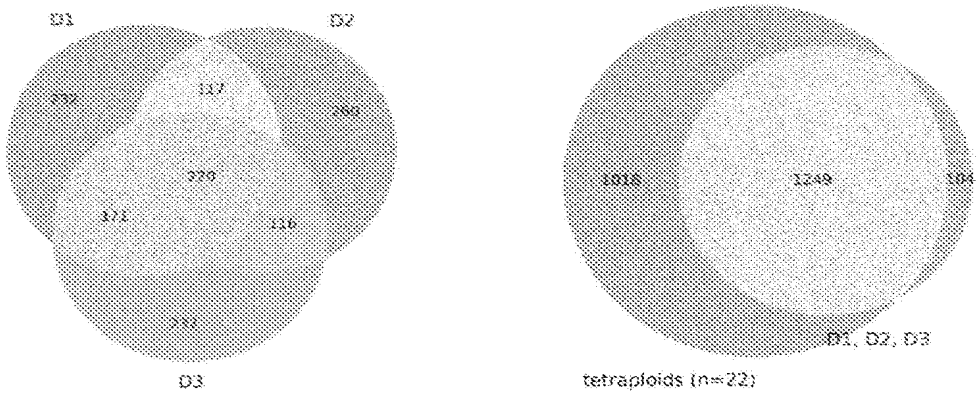
Aardevo Exhibit 1010
Page 0539

single F1 plant from a cross between two diploid potato clones (Jansky et al. 2015. Am. J. Botany 102: 1213): "*The tuber shape and color variability derived from self-pollinating a single diploid F 1 plant approaches that of the landrace collections maintained in potato genebanks*".

14. The genetic background of the plants of D1-D3 is thus extremely diverse. In fact, all *S. tuberosum* diploids, whether produced 60 years ago, today, or in the future, contain a random sample of the genes of the *S. tuberosum* tetraploid(s) from which they were derived. In that sense, D1-D3 are not unique and do not represent any specific genotype of potato lines.

15. Therefore, any *S. tuberosum* diploid is equally suitable for use as starting material in a method of the invention, that is, to serve as a recipient of the *Sli*-gene to produce a self-compatible diploid line that can be selfed in order to increase its level of homozygosity.

16. As a further substantiation of the diverse genetic background and wide applicability of the plants and methods as claimed, I provide results of a study on the genetic variation between lines D1, D2, and D3, as well as the genetic variation between these lines and a random set of 22 commercial tetraploid *Solanum tuberosum* varieties (*i.e.* Vr808, Vitabella, Spunta, Sarpo Mira, Russet Burbank, Nicola, Miss Mignonne, Lady Claire, Innovator, Colomba, Brooke, Bionica, Ivpx-041-01, Avito, Altus, Lady Anna, Barcelona, Toronto, Taisiya, Mondial, Melody, and Madrid). The genetic variation was determined by using 426 multi-allelic markers that were proportionally and randomly distributed over the twelve chromosomes. These results are summarized in Figure 2, below.



A B

Figure 2. Haplotype overlap between individual diploid lines D1-D3 (A); Haplotype overlap between the combination of the diploid lines D1-D3 and 22 random tetraploids (B).

17. It was found that lines D1, D2, and D3 represent a total of 1353 haplotypes, of which only 220 haplotypes (16%) were shared by all three (Figure 2A). By contrast, the 22 tetraploids (comprising a total of 22x4=88 genomes) represented a total of 2267 haplotypes (Figure 2B), of which no less that 1249 (55%) were also present in lines D1, D2, and D3 (together comprising only 3x2=6 genomes). Only 104 haplotypes (4%) were unique to lines D1, D2, and D3, which is a small fraction of the wide genetic variety between the 25 clones compared (Figure 2B). That D1, D2, and D3 share only a fraction of their alleles shows that they are genetically diverse. That no less than 96% of the alleles of D1, D2, and D3 is also present in the 22 commercial tetraploids shows that D1, D2, and D3 represent a random sample of *Solanum tuberosum*.

18. For these reasons, lines D1-D3 should be considered to be representative of the genetic diversity that is present in a large group of commercial tetraploid *Solanum tuberosum* varieties presently available to breeders.

Aardevo Exhibit 1010
Page 0541

19. It is characteristic of current potato breeding that the four genomes of tetraploid parents are mixed in each generation. Because all homologous chromosomes can pair with each other (quadrivalence), these four genomes are repeatedly mixed up. The three diploid lines D1, D2 and D3 are a good example thereof, and show how this mixing results in a wide genetic variation. Again, D1-D3 are not unique and do not represent any specific genotype of potato lines. Other than the Examiner appears to suggest, there is no such thing as a "genetically unique potato" or a "genetically diverse potato". Each genotype is a sample from a great raffle of possibilities and is therefore just as good as any other sample. That is also why D1, D2 and D3 have such a large overlap with tetraploid potatoes as shown in Figure 2B.

20. Thus, (i) the D1-D3 pedigree is rooted in a wide variety of tetraploid cultivars, (ii) no specific "sample" from tetraploid potato germplasm was taken and no selection occurred when choosing D1-D3, (iii) that the four genomes of tetraploid germplasm was randomly sampled is evidenced by the showing that the genetic variation within D1-D3 almost entirely overlaps with that of 22 tetraploid cultivars, and (iv) crosses with D1-D3 result in a segregation of a wide variation of tuber and non-tuber characteristics. This demonstrates that lines D1-D3 as starting material for introgression of the *Sli*-gene and the development of self-compatible vigorous diploids of the invention, represent three fully independent working examples to demonstrate the applicability within diverse *Solanum tuberosum* germplasm to one of skill in the art of plant breeding.

Aardevo Exhibit 1010
Page 0542

Practicing the claimed invention is predictable in view of the teaching in the patent application and does not depend on the genotype of the potato lines used

21. I reiterate the comments I made in paragraph 16 of my Declaration dated January 29, 2019, submitted in U.S. patent application serial No. 13/504,156, now U.S. Patent No. 10,524,436:

> *"More recent, independent research, using completely different diploid S. tuberosum clones as recipient of the Sli gene, has confirmed that it is possible to generate fertile, essential homozygous S. tuberosum potato lines when selecting for fertile offspring in self progenies (Jansky et al., 2014. J Plant Registrations, doi:10.3198/ jpr2013.05.0024crg; Jansky et al., 2016 Crop Sci 56: 1412-1422). This independent research supports my statement that the specific starting material is not essential to the success of the claimed method of producing the Solanum tuberosum plants, and further supports the effectiveness of the method."*

Thus, independent research published in 2014 shows that D1, D2 or D3 may be substituted with entirely different diploid clones, such as DM1-3 and US-W4.

22. I further understand from the Office Action that the Examiner cites the letter of Dr. Jonathan D G Jones of 11 December 2012 for the argument that the claimed method would not credibly work in *Solanum tuberosum* lines other than lines D1, D2, and D3. As I consider this to not be consistent with the conclusions of Dr. Jones, I asked Dr. Jones to further explain the paragraph referred to by the Examiner. The following is a quote from the response letter I received from Dr. Jones:

"I herewith inform you [...] that I never made a statement to that effect, and that my words are misinterpreted. What I stated is that we attempted to test the Sli gene in S. phureja, but were not successful, and that we found that, after only a few rounds of selfing with the original material from Hosaka, inbreeding depression was already so extensive that we terminated the program, because we were unable to propagate the material. We drew no specific conclusions at the time, other than that we considered that it might have been due to the poor germplasm we worked with. However, I was well aware at the time of my letter that this was not the case, as I was convinced from personal communications, and your scientific publication in Potato Research (2011) Vol. 54:301–312, that we should have persisted inbreeding and selecting plants with seed carrying berries.

I considered that your method was widely applicable to potato, and you had certainly shown that it was widely applicable to S. tuberosum."

The letter of Dr. J.D. Jones is attached as **Exhibit A**.

23. It is therefore incorrect to conclude that Dr. Jones's letter supports the Examiners assertion that the success of the method was due to a specific selection of diploid germplasm, *i.e.* the diploid *Solanum tuberosum* lines D1-D3. On the contrary, nothing suggests that this is the case. In fact, I believe that the claimed methods can be successfully used in species other than *Solanum tuberosum*. As *Solanum tuberosum* is a natural tetraploid, deleterious genes can be hidden in the tetraploid genome, and are maintained in the dihaploid genome of *Solanum tuberosum* diploid plants, making diploid *S. tuberosum* a more "difficult" potato.

Aardevo Exhibit 1010
Page 0544

24. As a showing of what Dr. Jones and others will have observed, I point to Example 2 of the Application, where the self-compatible diploid *Sli*-gene donor (H, for Hosaka – the same *Sli*-gene donor to which Dr. Jones refers) was crossed with the three self-incompatible diploid potato clones D1, D2, and D3. Some of the characteristics of the early stage breeding plants are provided in Table 2 of the Application, reproduced below.

Table 2. Some characteristics of winter grown plants of a diploid potato breeding program to introduce the *Sli* gene.

	# plants	compatibility frequency (%)	yield (gram per SC plant) average	variation
Sli donor	2	100	22*	0 - 43
F1 (HxD3)	9	89	443**	258 - 634
BC1 (Hx(HxD1)	49	81	206**	0 - 524
BC1 (Hx(HxD2)	64	72	237**	28 - 543
F2 selfed (HxD1)	23	22	252**	131 - 376
F2 selfed (HxD2)	20	25	69**	0 - 281

* from tuber

** from seedling

25. As can be seen from this Table, the two individual *Sli* donor plants (referred to in Table 6 of the application as "H" or "07-1004-1") were fully self-compatible, and crosses HxD1, HxD2 and HxD3 all resulted in the generation of self-compatible offspring plants, albeit in various degrees. Yet, self-compatible plants were found in all instances, indicating successful *Sli*-gene introgression from parent "H". Even in the F1 of cross HxD3, which yielded very few seeds, 8 out of 9 plants (89%) were found to be self-compatible.

26. As also shown in Table 2 above, selfings of initial hybrids (F2 selfed (HxD1); F2 selfed (HxD2)) resulted in an overall decrease in self-compatibility towards 22-25%. This is attributed to inbreeding depression.

27. As shown in Table 3 of the Application, which is reproduced below, of the eight self-compatible plants of the F1(HxD3) noted above, eight selfed populations were prepared from seed to generate a total of 1602 field plants. Of these, only ten plants (about 0.6%) were self-compatible, and of these ten self-compatible plants, only seven showed sufficient seed set. Fertility is thus greatly if not especially affected by inbreeding depression in the early crosses.

Table 3. Breeding scheme to introduce self-compatibility (SC) into diploid potato. In bold the most promising materials are indicated.

F	F1 (HxD)	F2: selfed (HxD)				BC1S1: selfed (HxD1xD)			
parents	plants (DxH)	Populations tested	plants in the field	self compatible plants	plants selected*	populations tested	plants in the field	self compatible plants	plants selected
	#	#	#	#	#	#	#	#	#
H = SH donor									
D1	33	15	2480	± 50	7	28	2521	< 10	1
D2	45	29	**5598**	**> 580**	**52**	24	1280	< 10	1
D3	8	8	1602	± 10	7				

* sufficient seedset

28. This means that only four in 1,000 plants were selected and 99.6% of the offspring were discarded. The relevance of this step is indicated in the application on page 37, line 27 – page 38, line 7:

"No, or at least little, attention is paid to the agronomical appearance of the plants at this stage. They may look withered and frail, with only a few flowers. Nonetheless, the selfing is repeated at least three times, but preferably four or five or even six to eight times. In this manner, a line of essentially homozygous, fertile, self-compatible, and diploid potato plants is obtained, essentially by selfing and selecting for plants that produce fertile offspring.

Next, an agronomically desirable trait is added to the essentially homozygous, fertile, self-compatible, diploid potato genotype by crossing a plant of that line with a plant of an unrelated essentially heterozygous diploid potato line, preferably a line containing an agronomically desirable trait. This essentially heterozygous line (which term merely reflects that the line is not an essentially homozygous line as used herein) functions as a donor for an agronomically desirable trait, in this case vigour."

Thus, the plants that were not discarded but used for further breeding were the frail, fertile plants, while the vigorous less fertile plants were discarded.

29. The Examiner appears to suggest that prior to the invention selection of progeny plants for subsequent crosses based on a plant's fertility was a well-understood and routine activity. However, this was not at all the case in conventional potato breeding. Selection for fertile offspring or persistent self-compatibility in the selfed progeny of inbred diploid potato has shown to require the generation of large numbers of plants and selecting those that are increasingly homozygous and fertile. From the large population, the plants that held potential of yielding fertile progeny were shown to be the seemingly useless plants which often carried only a few berries, each having only a few seeds. This meant that the vigorous and abundantly

Aardevo Exhibit 1010
Page 0547

flowering plants were not the plants to be selected as these were the plants that were the least homozygous. Given the observed reduction in self-compatibility after every subsequent inbreeding step, and the large number of plants required to identify the homozygous fertile plants after three or four steps of inbreeding (not to mention that such plants may look withered, frail, and utterly invaluable, and that a markers system needed to be developed in order to test homozygosity), there was nothing obvious to one of ordinary skill in the art to proceed with such an inbreeding program prior to the invention, let alone have any expectation of success in obtaining the plants as presently claimed.

Possession of the Invention

30. The publication of Jansky et al. (2016) "Reinventing Potato as a Diploid Inbred Line-Based Crop." Crop Sci. 56:1412-22, fully supports the findings of the present inventors. In fact, it advocates the radical change to the potato breeding industry using an inbred-line-based breeding system: "*We call on leaders of the public and private organisations to come together to explore the feasibility of this radical and exciting new strategy in potato breeding*". The publication describes at great length why classic potato breeding is not effective, and further describes that attempts at the tetraploid level were never successful: "*Efforts to self-pollinate potato at the tetraploid level were initiated in the mid-twentieth century in response to the success of the inbred-hybrid methods developed in maize. Low yield and poor fertility were observed in S5 and S6 generations, so this effort was terminated. [...]. Consequently, these efforts to self-pollinate tetraploid potato clones never actually tested the value of creating inbred lines to eliminate deleterious alleles and fix desirable ones*" (emphasis added).

31. This is entirely in line with what I have stated before: Potato experts were so discouraged by all failures that no further attempts to produce homozygous lines by inbreeding were made prior to the current invention.

32. It is important to note that more than mere scientific proof is needed to change an established crop production system that has existed for hundreds of years. The authors of Jansky et al. 2016 did not question the possibility of producing fertile and vigorous homozygous diploids and methods for their production as claimed by the present inventors. They indicated that much work still needed to be done before a diploid inbred line breeding system could replace the conventional potato breeding system. As explained on page 1414, right column, first §, such a system would require the assignment of inbreds to heterotic pools within each market class (red, white, russet, specialty) that can be crossed to combine important traits, such as time to senescence, tuber yield, flesh texture, tuber composition, disease resistance, and maintenance of tuber quality in storage.

33. This leaves untouched the fact that the present inventors have provided the pivotal ingredients for the new potato crop production system: fertile and vigorous homozygous diploids. In showing that the inventors were in possession of such plants, I simply refer to the deposits, notably line AGVD17.

34. Using the technology of the instant application, the present inventors were able to develop the experimental test-hybrids into a potato hybrid breeding system. This is exemplified by publications of de Vries et al. in 2016 (Open Agriculture. 2016; 1: 151–156), wherein hybrids tested in East-Africa showed a yield of up to 29 ton/ha, and of Lindhout et al. in 2017 (Hybrid potato breeding for improved varieties. In: Achieving sustainable cultivation of potatoes Vol. 1

Aardevo Exhibit 1010
Page 0549

Breeding, nutritional and sensory quality. Edt Burleigh & Dodds, Science publishing, Cambridge, UK), wherein the scientific principles and applied aspects of hybrid potato breeding are provided. (copies enclosed). In Lindhout et al. in 2017, it is indicated that (Page 18):

> *"Since the first crosses in 2008, the Solynta research efforts have been focused on the development of a hybrid potato breeding system. This research has now reached the stage where hybrid potato breeding will become reality. This has recently been supported by two leading potato breeding companies in EU, KWS and HZPC, who have also expressed their conviction that hybrid potato breeding will be future main breeding system (KWS, 2016). In addition, 21 leaders in the industry and potato science in the United States have expressed their opinion on ' Reinventing potato as a diploid inbred line-based crop ' with a scientific base for diploid hybrid potato breeding (Jansky et al., 2016)."*

35. Furthermore, Stockem et al. in 2020 Potato Res. DOI 10.1007/s11540-019-09444-x, describing experiments of 2016, confirmed that diploid hybrids can exhibit the same average yields as an established commercial cultivar (Abstract), despite the fact that such diploid hybrids were never selected for high yielding capacity under field conditions (Results section "Seedling Tuber Production").

36. Therefore nothing in the sentence of Jansky et al. 2016 "*Diploid inbred line breeding in potato is being tested by the private sector in Europe (Lindhout et al., 2011)*" bears any relation to a suggestion that, approximately seven years after the filing date of the instant application, the problem of creating vigorous inbred diploid *S. tuberosum* plants apparently still existed. On the

contrary, in the paper of Jansky et al. 2016, the existence of a breeding program based on diploid inbred lines is only confirmed.

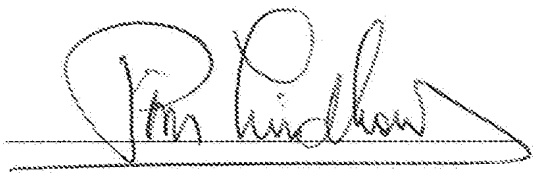
37. All the above is further supported by independent development of diploid *Sli*-gene-containing *S. phureja* and *S. tuberosum* inbreds wherein the *Sli-gene* was introgressed from *S. chacoense* M6 into diploid DM1-3 and US-W4 (Jansky et al. 2014. Journal of Plant Registrations 8:195–199), showing that D1, D2 or D3 can successfully be substituted with diploid clones DM1-3 and US-W4 (page 198, right column, second §), and by the publication of Jansky and Spooner 2018 "The Evolution of Potato Breeding", Plant Breeding Reviews, Volume 41, First Edition. Edited by Irwin Goldman. John Wiley & Sons, Inc., which states on page 201:

> *"Next, Pim Lindhout, in the Netherlands, reported on the development of diploid inbred lines with acceptable tuber shape and size (Lindhout et al., 2011). F3 plants were uniform for tuber quality and yield, so they were intercrossed to make F1 hybrid prototypes. Some F1 hybrids were uniform and exhibited good tuber quality. This proof of concept paper has set the stage for the development of diploid F1 hybrids in potato."*

Aardevo Exhibit 1010
Page 0551

38. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: May 1st, 2020



Willem Hendrik Lindhout

EXHIBIT A

THE SAINSBURY LABORATORY

John Innes Centre
Norwich Research Park
Colney · Norwich NR4 7UH · UK
T +44 (0) 1603 450400 F +44 (0) 1603 450011
admin@TSL.ac.uk www.TSL.ac.uk

16th April 2020

Dear Pim,

I write to you in response to your request for explanation of a paragraph in my letter of 11 December 2012, which letter was attached to an Inventor's declaration that you submitted on 15 August 2016, and which letter is now part of the patent prosecution proceedings in the case of your US application 16/541,071.

I understand that the US Examiner is putting weight on the paragraph wherein I state:

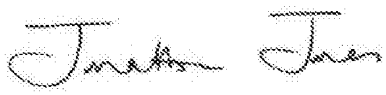
"*(I)n the mid 2000s we attempted to produce hybrid potato ourselves. No successes had ever been reported with S. tuberosum. In order to work at the diploid level, we back-crossed the S. chacoense Sli- gene into Solanum phureja. In addition, we selfed the lines that carry the Sli- gene. However, we never succeeded in developing any promising material in the form of fertile homozygotes that could be useful for hybrid breeding and that could tolerate recurrent selfing. We considered that this was due to our inability to cross the Sli- self-compatibility locus into good enough or diverse enough diploid Solanum germplasm*." (underlining added by Examiner).

In particular, I understand that the Examiner interprets my statement as a showing that your method of recurrent inbreeding to recover the vigor of the diploid potatoes only works in selected *Solanum* germplasm, i.e. that your success depended on the genotype of your diploid potato lines.

I herewith inform you, and you may share this with the Examiner, that I never made a statement to that effect, and that my words are misinterpreted. What I stated is that we attempted to test the *Sli* gene in *S. phureja*, but were not successful, and that we found that, after only a few rounds of selfing with the original material from Hosaka, inbreeding depression was already so extensive that we terminated the program, because we were unable to propagate the material. We drew no specific conclusions at the time, other than that we considered that it might have been due to the poor germplasm we worked with. However, I was well aware at the time of my letter that this was not the case, as I was convinced from personal communications, and your scientific publication in Potato Research (2011) Vol. 54:301–312, that we should have persisted inbreeding and selecting plants with seed carrying berries.

I considered that your method was widely applicable to potato, and you had certainly shown that it was widely applicable to *S. tuberosum*.

Best regards,



Prof Jonathan D G Jones FRS

The Sainsbury Laboratory is a registered charity, number 1065510, and a company limited by guarantee, registered number 3346853 in England and Wales
Registered office: JIC · Norwich Research Park · Norwich NR4 7UH · UK

PTO/AIA/22 (03-13)
Approved for use through 7/31/2016. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)

Docket Number (Optional): VONL:004USC1

Application Number: 16/541,071

Filed: August 14, 2019

For: Hybrid Seed Potato Breeding

Art Unit: 1663

Examiner: Russell T. Boggs

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above-identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	Micro Entity Fee	
☐	One month (37 CFR 1.17(a)(1))	$200	$100	$50	$
☑	Two months (37 CFR 1.17(a)(2))	$600	$300	$150	$ 300.00
☐	Three months (37 CFR 1.17(a)(3))	$1,400	$700	$350	$
☐	Four months (37 CFR 1.17(a)(4))	$2,200	$1,100	$550	$
☐	Five months (37 CFR 1.17(a)(5))	$3,000	$1,500	$750	$

☑ Applicant asserts small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29.
Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☑ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 19-3140/VONL:004USC1.

☑ Payment made via EFS-Web.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant.

☑ attorney or agent of record. Registration number 71,087.

☐ attorney or agent acting under 37 CFR 1.34. Registration number _____.

/Sheila Walker/ — Signature

May 5, 2020 — Date

Sheila Walker — Typed or printed name

(214) 259-1890 — Telephone Number

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

☐ * Total of _____ forms are submitted.

This collection of information is required by 37 CFR 1.136(a). The information is required to obtain or retain a benefit by the public, which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 6 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Mail Stop PCT, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Willem Hendrik Lindhout *et al.*	Group Art Unit: 1663
Serial No.: 16/541,071	Examiner: Russell T. Boggs
Filed: August 14, 2019	Atty. Dkt. No.: VONL:004USC1
For: HYBRID SEED POTATO BREEDING	Confirmation No.: 4348

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Commissioner:

In compliance with the duty of disclosure under 37 C.F.R. § 1.56, it is respectfully requested that this Supplemental Information Disclosure Statement be entered and the documents listed on attached Form PTO/SB/08 be considered by the Examiner and made of record. Copies of the listed documents required by 37 C.F.R. § 1.98(a)(2) are enclosed for the convenience of the Examiner.

In accordance with 37 C.F.R. §§ 1.97(g), (h), this Supplemental Information Disclosure Statement is not to be construed as a representation that a search has been made, and is not to be construed to be an admission that the information cited is, or is considered to be, material to patentability as defined in 37 C.F.R. § 1.56(b).

A fee as set forth in 37 C.F.R. § 1.17(p) in the amount of $120.00 has been authorized for payment through use of a company credit card. If the authorized amount is insufficient, the Commissioner is authorized to deduct the appropriate fee from Dentons US LLP's Deposit Account 19-3140/VONL:004USC1.

DENTONS US LLP
2000 McKinney Avenue, Suite 1900
Dallas, TX 75201-1858
214-259-1890
214-259-0910 (fax)

Respectfully submitted,

/Sheila Walker/

Sheila Walker
Reg. No. 71,087
Agent for Applicant

Date: May 5, 2020

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16541071
Filing Date	2019-08-14
First Named Inventor	Willem Hendrik Lindhout
Art Unit	1663
Examiner Name	Russell T. Boggs
Attorney Docket Number	VONL:004USC1

U.S.PATENTS

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1					

If you wish to add additional U.S. Patent citation information please click the Add button.

U.S.PATENT APPLICATION PUBLICATIONS

Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1					

If you wish to add additional U.S. Published Application citation information please click the Add button.

FOREIGN PATENT DOCUMENTS

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] ¡	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1							☐

If you wish to add additional Foreign Patent Document citation information please click the Add button

NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	16541071
	Filing Date	2019-08-14
	First Named Inventor	Willem Hendrik Lindhout
	Art Unit	1663
	Examiner Name	Russell T. Boggs
	Attorney Docket Number	VONL:004USC1

	1	USPTO: Non-Final Office Action regarding U.S. Application No. 14/112,747 (Atty. Dkt. No. VONL:006US), dated January 16, 2020.	☐
	2	DE VRIES et al., "Hybrid potato breeding for improved varieties," Open Agriculture 1:151–156, 2016.	☐
	3	JANSKY et al., "How do we address the disconnect between genetic and morphological diversity in germplasm collections?," American Journal of Botany 102(8):1213-1215, 2015.	☐
	4	JANSKY et al., "The Evolution of Potato Breeding", Plant Breeding Reviews, Vol. 41, pp. 169-214, 2018.	☐
	5	LINDHOUT et al. "Hybrid potato breeding for improved varieties," In: Achieving sustainable cultivation of potatoes Vol. 1. Breeding, nutritional and sensory quality. Edt. Burleigh & Dodds, Science Publishing, Cambridge, UK). 2017.	☐
	6	STOCKEM et al., "Contribution and Stability of Yield Components of Diploid Hybrid Potato," Potato Research, 2020. doi.org/10.1007/s11540-019-09444-x	☐

If you wish to add additional non-patent literature document citation information please click the Add button

EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16541071
Filing Date	2019-08-14
First Named Inventor	Willem Hendrik Lindhout
Art Unit	1663
Examiner Name	Russell T. Boggs
Attorney Docket Number	VONL:004USC1

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☐ See attached certification statement.

☒ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Sheila Walker/	Date (YYYY-MM-DD)	2020-05-05
Name/Print	Sheila Walker	Registration Number	71,087

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/327073074

Hybrid potato breeding for improved varieties

Chapter · August 2018

DOI: 10.19103/AS.2018.0046.04

CITATIONS
8

READS
1,183

6 authors, including:



Michiel E. de Vries
Solynta
15 PUBLICATIONS **215** CITATIONS
SEE PROFILE



Ying Su
Solynta
1 PUBLICATION **8** CITATIONS
SEE PROFILE



Adriaan W van Heusden
Wageningen University & Research
127 PUBLICATIONS **2,968** CITATIONS
SEE PROFILE

Some of the authors of this publication are also working on these related projects:



Ornamental Research View project



Irrigated Rice in Africa View project

All content following this page was uploaded by Michiel E. de Vries on 09 September 2018.

The user has requested enhancement of the downloaded file.

Aardevo Exhibit 1010
Page 0561

Hybrid potato breeding for improved varieties

Pim Lindhout, Michiel de Vries, Menno ter Maat, Su Ying, Marcela Viquez-Zamora and Sjaak van Heusden, Solynta, the Netherlands

1 Introduction
2 The scientific basis for hybrid potato breeding
3 The state of the art of hybrid potato breeding
4 Production of and commercialization of hybrid seed cultivars
5 Inbred lines for genetic research
6 Cropping systems based on true seeds
7 Case studies
8 Conclusion
9 Where to look for further information
10 Acknowledgements
11 References

1 Introduction

The cultivated potato, *Solanum tuberosum*, can be reproduced generatively through seeds and vegetatively through tubers. This may have evolutionary advantages: seeds may provide better survival under extreme conditions, such as frost or drought, and can remain viable in the soil for years. When conditions are mild, tubers survive in a dormant state for a couple of months. When conditions become favourable again, their fast and strong sprouting provides a clear competitive advantage over other plants in the same ecological niche.

In traditional potato breeding, each breeding cycle starts with a cross between two genotypes, usually tetraploid varieties, followed by many years of selection and multiplication (see Chapters 2 and 3). The advantage of this approach is uniformity: the tubers are clones and thus genetically identical. The disadvantage is the low genetic gain in each lengthy breeding cycle, as the genetic composition of the two parental genotypes is just reshuffled, including alleles which negatively affect plant growth and development. As a result, potato yield has not significantly been improved over the past century (Douches

http://dx.doi.org/10.19103/AS.2016.0016.04
© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

AQ: Please check if 'is less than a factor 10 per season' is OK as is.

et al., 1996; Vos et al., 2015). In addition, the reproduction of seed tubers is less than a factor 10 per season. It takes many years to build up sufficient quantities of seed tubers for commercial production, and the risk of contamination by pathogens increases with each multiplication step.

True potato seed (TPS) has been promoted as an alternative for seed tubers because TPS is easy to store and devoid of most soil-borne pathogens. In South Asia, East Africa and the Andes, TPS is used mainly by subsistent farmers (Almekinders et al., 1996). TPS is produced by crossing parent plants that have been selected to produce a hybrid variety. The parents are propagated vegetatively, similar to seed tuber propagation. As the parents of a TPS variety are heterozygous, all seeds of a TPS cultivar are genetically different. This results in a highly variable crop that is not acceptable in most markets, such as the high value markets of Europe and North America.

Since the success of hybrid breeding in corn in the 1930s, breeders have adapted a hybrid breeding system for many crops (Fig. 1; Crow, 1998; Troyer, 2006; Hua et al., 2003). Typically, hybrid cultivars produce higher yields and show high crop uniformity (Rijk et al., 2013). In addition, the breeding system is fast and efficient and new traits can rapidly be introduced by marker-assisted introgression.

These advantages are also expected for potato: hybrid potato varieties will be higher yielding, will need less crop protection chemicals due to disease resistance and will have better quality for processors and consumers (FAO et al., 2015). A hybrid breeding system for potato offers two additional advantages: fast multiplication of hybrid seeds and easier logistics, as clean true seeds can easily be produced, transported and stored (Duvick et al., 2005).

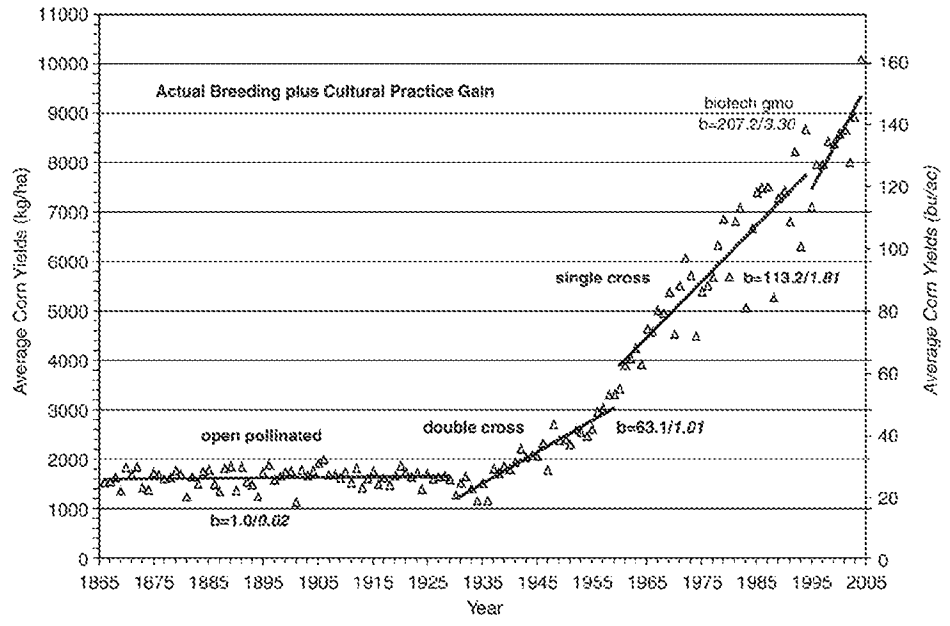


Figure 1 Fivefold increase in corn yields since the introduction of hybrids (Troyer, 2006).

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0563

However, self-incompatibility and inbreeding depression have for long hindered progress in hybrid potato breeding (De Jong and Rowe, 1971; Charlesworth and Willis, 2009; Jansky et al., 2016). These two limitations have recently been overcome by introducing a self-compatibility restorer gene and by large-scale and consistent breeding (Lindhout et al., 2011a).

This chapter describes the scientific principles and applied aspects of hybrid potato breeding. The successful introduction of the principle of hybrid potato breeding was described in 2011 (Lindhout et al., 2011a). Since then, we have focused on further developing the potato hybrid breeding system, especially genetic studies to establish a genetics-driven hybrid breeding system. In a recent paper, Jansky et al. (2016) have confirmed the possibilities of such approach.

This is the first publication on the state of the art of a hybrid potato breeding programme. The authors, all working at Solynta, want to emphasize that scientific papers on this topic are not available yet, and hence we have to rely entirely on the results of the Solynta breeding and research programme. Still, in presenting these results, we hope to contribute to a better understanding of the principles and applied aspects of hybrid potato breeding.

2 The scientific basis for hybrid potato breeding

2.1 The principle of hybrid breeding

The basic idea of hybrid breeding is to combine the genes of two parent genotypes, both of which may harbour suboptimal alleles, resulting in weaker performance. If parents have different suboptimal alleles, hybrid offspring can show increased vigour and yield, designated 'heterosis', as the suboptimal alleles in one parent may be compensated by the favourable genes from the other parent (Birchler et al., 2010; Gopal, 2014; Fig. 6). If the parents are completely homozygous, the resulting hybrid offspring will be partially heterozygous and genetically uniform. By testing many hybrid offspring under relevant cultivation conditions, the best combining parents are identified. These are maintained and propagated in separate groups as 'heterotic pools' for further breeding (Brown and Cagliari, 2011).

Thus, hybrid breeding has two distinct processes: development of homozygous parent lines and production and testing of experimental hybrids.

2.2 Diploids are more efficient than tetraploids for hybrid breeding

Homozygous diploids are faster to generate than homozygous tetraploids. For instance, seven generations of selfing are required to obtain 50% homozygous loci starting from a tetragenic tetraploid heterozygote (carrying four different alleles). The same homozygosity level is reached starting from a heterozygous diploid by only one generation of selfing (Haldane, 1930; Fig. 1). For this reason, hybrid potato breeding is more efficient at the diploid level.

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0564

2.3 Generation of homozygous diploid potato genotypes via haploidization and via inbreeding

Haploid genotypes can be generated from an egg cell (gynogenesis) or from pollen, often via anther culture (androgenesis). In potato, some haploids have been generated by anther cultures. The resulting haploids were converted into diploids by chromosome doubling. However, the resulting homozygous diploids were very weak and sterile (van Breukelen et al., 1977; Uijtewaal et al., 1987b), hampering their usage in breeding.

Haploidization has been more successful in crossable species like *S. chacoense* (Cappadocia, 1990; Phumichae et al., 2005; Phumichae and Hosaka, 2006) and *S. phureja* (Chani et al., 2000).

A reason for the failure to produce vigorous doubled haploids may be the transition to complete homozygosity in one step. Inbreeding depression may be so severe that homozygous plants are too weak to survive. Repeated selfing, on the other hand, might lead to a more gradual improvement of homozygosity. However, in potato, inbreeding is seriously limited by self-incompatibility, which prevents self-fertilization. Still some rare examples of homozygous diploid plants have been generated by inbreeding but again the homozygous diploid plants always showed a strong inbreeding depression, which limited their usage in breeding (De Jong and Rowe, 1971; Charlesworth and Willis, 2009).

2.4 Large genetic variation in potato causes inbreeding depression

The tetraploid and outcrossing nature of commercial potato is likely responsible for the large genetic variation. In a study on the allelic composition of 800 genes in 83 potato cultivars, an average frequency of 3,2 alleles per locus within a genotype was identified (Uitdewilligen et al., 2013). Among the 83 cultivars, often more than ten alleles per locus

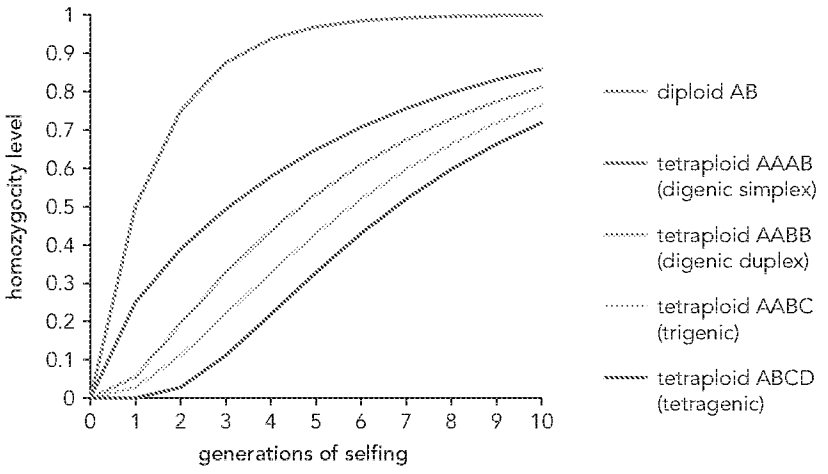


Figure 2 The theoretical increase in homozygosity in diploids and tetraploids through inbreeding, adjusted from Haldane et al. (1930).

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0565

were observed. In addition, the frequency of single nucleotide polymorphisms (SNPs) in potato is 1 in 15–30 base pairs (PGCS, 2011; Visser et al., 2014). This means that the genetic distance between the two sets of chromosomes within one diploid potato genotype is four times larger than the genetic distance between the genomes of man and chimpanzee (CSAC, 2005).

The high frequency of allelic variation has the inevitable consequence that 'weak alleles' that have a negative effect on plant fitness are maintained. Such alleles remain hidden in the large buffer of four genomes, but reveal themselves upon inbreeding when the chance for homozygosity increases. This is even more manifested at the diploid level where the homozygosity level more rapidly increases upon inbreeding (Fig. 2).

This large genetic variation is also helpful for breeding as it forms a genetic reservoir of useful genes. It is a challenge to identify alleles that contribute most to plant performance. As potato has 39000 genes, the identification and usage of the 'best alleles' per locus, including interactions between them (epistasis), will gradually take place over decades of research and breeding (PGSC, 2011). Corn may serve as a good example, whereby, after a century of dedicated breeding by numerous breeders worldwide, a genetic gain of over 1% per year is still achieved (Troyer, 2006).

2.5 Crossable diploid species and tetraploids increase genetic reservoir for diploid breeding

The potato germplasm available for breeding comprises many species, including diploid species (Jansky and Peloquin, 2006). These have been used as source to introduce resistance genes into cultivated germplasm. Breeders often use diploid potato to rapidly combine favourable traits that can be introduced into the tetraploid germplasm by direct crossings, bridge crossings or via chromosome doubling (De Mainea, 1982; Chauvin et al., 2003). The diploid breeding programme at Wageningen University (Hutten, 1994) has generated donor lines that harbour the most important traits for potato breeding (Table 1). Additional diploid germplasm is available from potato gene banks and public research institutes such as University of Wisconsin-Madison, United States; Potato Germplasm Enhancement Laboratory, Japan; Gene Bank at Gatersleben, Germany and International Potato Centre, Peru.

Another source of diploid germplasm is tetraploids that can be prickle pollinated to generate diploid offspring, designated dihaploids (Uijtewaal et al., 1987a). A collection of dihaploids obtained from one tetraploid harbours the full set of genes from the tetraploid and can be exploited in a diploid potato breeding programme.

In conclusion, the large genetic variation in potato and in its wild relatives, combined with the technology to switch between ploidy levels, provides a tremendous wealth of germplasm available for diploid hybrid breeding.

2.6 Diploid potato may perform equal to tetraploids

Most important food crops such as rice, corn and soybean are diploid. Sugar beet cultivars were initially tetraploid, then triploid and since 2000, all new cultivars are diploid. In potato, it has long been assumed that tetraploids outperform diploids (Rowe, 1967; Hutten et al., 1994). Occasional observations have contradicted this assumption: Progeny of diploid potato USW4 with *S. chacoense* M6 produced large tubers and high yield (Lipman and

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0566

Table 1 The *Sli*-gene donor, designated DS and 16 diploid potato germplasm, designated D1–D16, used for hybrid breeding at Solynta. The trait abbreviations are according to Hutten (1994)

Abbreviation	Short description
DS	*Sli*-gene donor
D1	Early (maturity), long, *Y*, Qcook
D2	Early, *Y*, Qcook
D3	*R3*, *H1*, *Gpa2*, *RXadg*, *Y* (yellow flesh)
D4	Grp1, early, long (shape), *Ro1* (*H1*?)
D5	Early, long, *Y*, Qfry
D6	Long, *Y*, Qfry, *H1*, Qcook
D7	Early, long, *Y*, *H1*, Qcook, *Zep* (orange flesh)
D8	Early, *y* (white flesh)
D9	Qstarch, *Y*
D10	Wild species hybrid: *phyt avl*
D11	Wild species hybrid: *phyt rch*
D12	Round (shape), Qcook, Qfry
D13	Early, round, *Zep*, *Y*, Spectacled, Qcook, blue anthocyans
D14	Wild species hybrid: *phyt tar*
D15	Wild species BC1: early, *phyt vnt1*, round, *Y*, *H1*
D16	Early, round, *y* (white flesh)

Zamir, 2007; Jansky et al., 2014). Uijtewaal et al. (1987b) observed that heterozygous diploid potato outperformed all homozygous di- and tetraploid derivatives. These results from potato and from other crops support the expectation that diploid potato will eventually replace tetraploid potato for commercial usage.

3 The state of the art of hybrid potato breeding

In 2008, Solynta started its research by making crosses between diploid potato germplasm, obtained from a pre-breeding programme from Wageningen University (Rutten, 1994), and a homozygous accession of the wild species *S. chacoense*, carrying the dominant self-compatibility controller gene *Sli* (Hosaka and Hanneman, 1998a,b; Phumichai et al., 2005; Lindhout et al., 2011a). The F_1 plants were extremely vigorous and about half of them produced many berries upon self-pollination. This was considered a major breakthrough as these were, to our knowledge, the first vigorous, self-compatible diploid potato plants ever obtained. These F_1 plants were highly heterozygous. The first generation after selfing (designated F_2) should harbour at least 50% homozygous loci. As many of these loci might

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0567

harbour 'weak alleles', it was expected that F_2 plants would be too weak to survive. We also made BC_1 populations by backcrossing to the *S. chacoense* parent.

As expected, the F_2 and BC_1 showed weak growth and many died in the field. However, a number of plants survived and 10% of the surviving F_2 plants even proved self-compatible (Lindhout et al., 2011a). We generated the second inbred generation, designated F_3 and made crosses between self-compatible individual F_3 plants. These inbred plants were tested with SNP markers to confirm their genetic identity as real inbreds. These results indicated that breeding hybrid potato was now feasible (Lindhout et al., 2011b).

The weak plant performance, the poor tuber quality and the low yield of the inbreds was not only due to inbreeding depression, but was also caused by the wild *S. chacoense*, a species that hardly produces tubers. So, by this approach, we not only started a hybrid potato breeding system, but also the process of domestication of a new 'diploid potato' based on an interspecific cross of diploid *S. tuberosum* and the wild species *S. chacoense*.

We hypothesized that developing vigorous inbred lines is challenging as for each of the 39 000 loci the most favourable alleles should be identified and combined. Unfavourable genes with large effects on plant performance are identified easily and hence selecting increased plant performance is initially easy and fast. Undesired characters from the *S. chacoense* parent such as abundant stolons, small leaves and twisted, small and low-yielding tubers were removed in a few breeding generations.

Sli is a dominant gene (Phumichai et al., 2005; Phumichai and Hosaka, 2006). However, the successful expression of this gene requires a vigorous plant that is fertile and supports self-pollination. Often these criteria are not met. Therefore, the frequency of self-compatible plants is usually lower than expected based on a monogenic trait. Inbreeding depression is exhibited as weaker plant growth upon higher generations of inbreeding (Fig. 3). As a consequence, the self-compatibility level tends to decrease upon further selfings. The first inbred lines obtained by Solynta, containing over 95% homozygous

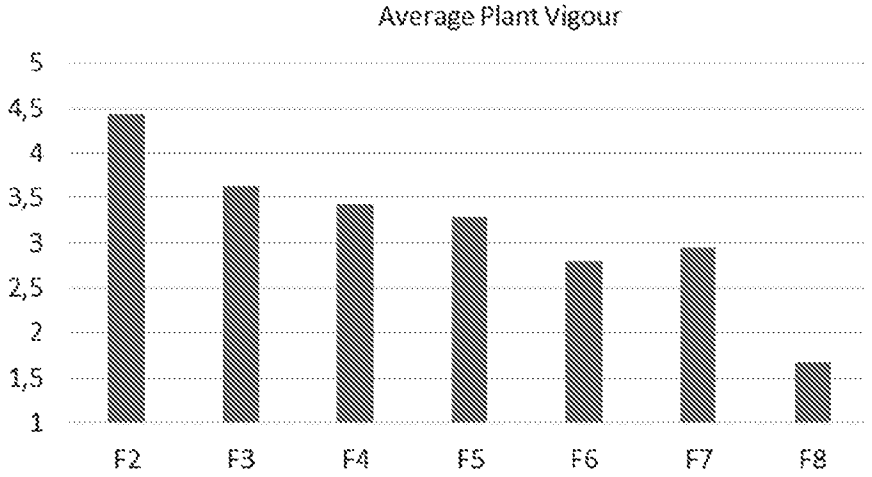


Figure 3 Inbreeding in the diploid Solynta germplasm in winter 2014–15. The Fx indicates x-1 generation of selfing after the last cross was made. Data are averaged over the complete trial consisting of over 5000 plants. The scale of plant vigour ranges from 1 = very weak via 3 = average to 5 = very strong.

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0568

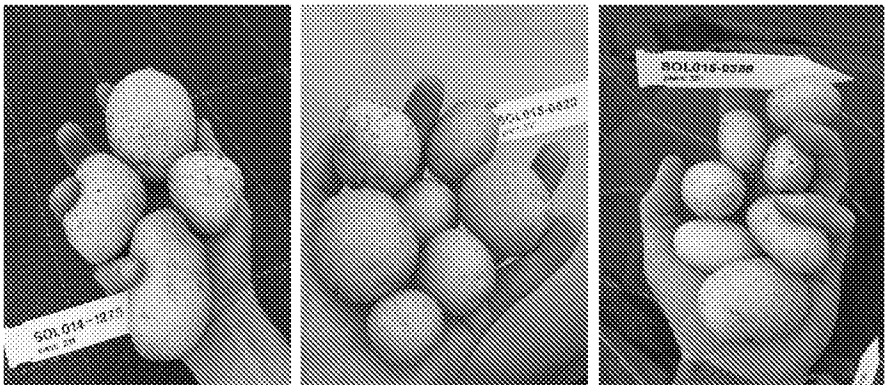


Figure 4 Examples of Solynta diploid germplasm. Plants were raised from seedlings and grown in the greenhouse in the summer season of 2014 (left panel) and 2015 (middle and right panel). They were among the best genotypes in a breeding programme comprising over 15000 plants.

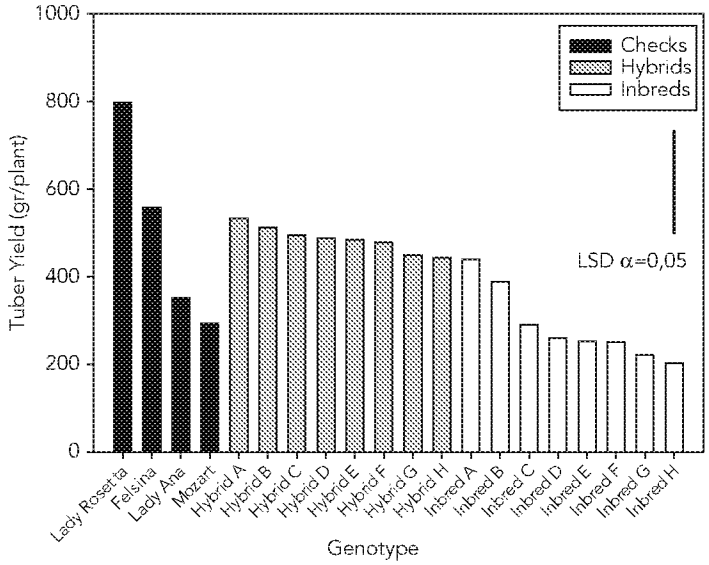


Figure 5 Hybrid performance of the first diploid experimental potato hybrids. Seed tubers were harvested from greenhouse-grown plants, raised in the winter from tubers (checks) and seedlings (hybrids and inbreds). The seed tubers were planted on 8 May 2015 in a trial field on sandy soil in Wageningen and harvested on 17 September 2015.

loci, hardly produced progenies and the seedlings were extremely weak. Therefore, new crosses were made between F_3 and F_5 inbred lines and selfing was started again from these F_1's to continue selecting parent lines that combine beneficial traits. As a result, performance of the inbreds improved over the following generations (Fig. 4).

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0569



Figure 6 Example of heterosis in diploid hybrid potato. The plants were from the same trial as in Figure 5. At the left the female F_3 parent and at the right the male F_5 parent, while the hybrid is in the middle.

In mature hybrid breeding systems, parents of the hybrids are selected based on general combining abilities (GCA), whereby molecular markers can be used to better predict the breeding value of the parents (Tobias et al., 2009). As no historic data were available on combining abilities of diploid potato parents, the selection of the first parents was based on the performance of the parents themselves.

Following the predictions of good performing inbred lines, crosses between these lines were made resulting in 45 potato hybrids which were tested in the field together with 20 inbred lines in two replicates of two plants per plot. The yields varied from 83 to 580 g/plant (Fig. 5). Thirteen hybrids scored higher than any of the inbreds, also outperforming the check variety Lady Anna, while some also showed a strong heterosis for yield (Fig. 6). The trials are being repeated in 2016 by using seed tubers raised from the field.

In addition, a new series of 216 experimental hybrids was generated in the winter-season 2014–15, mainly based on F_6 parent lines. The hybrid seeds were sown immediately after harvest and ten seedlings per hybrid were transplanted into the field in June, 2 months later than the usual seed tuber plantings. Still the yield of some hybrids was higher than 500 g/plant and the tubers were similar in size and shape to commercial seed tubers.

The results of the first experimental hybrids illustrate the potential of diploid hybrid potato varieties. As the first hybrids were randomly made without any a prior information about the combining abilities of the parents, it is expected that the next series of hybrids based on the results of these field trials will perform better and may show a stronger overlap with commercial controls.

4 Production of and commercialization of hybrid seed cultivars

The production of hybrid potato seeds is mainly done by hand pollinations. The seed yield per plant varies from hundreds to many thousands of seeds. Each successful hand pollination generates a berry with 50–150 seeds and each plant produces 5–50 berries. This

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0570

is similar to other vegetable *Solanum* crops, like tomato and pepper, where commercial seed is produced by hand pollination.

The emasculation of the flowers is a time-consuming part of the hand pollinations. Male sterility systems have been reported that make emasculations redundant and hence reduce seed production costs (Li, 2008). Alternatively, functional male sterility may be used, whereby pollen is prevented to land on the stigma of the same flower as the exerted stigma may be manually pollinated by pollen of the male parent before the own pollen may reach the stigma (Lössl et al., 2000; Abrol et al., 2012).

The transport of potato seeds over the world is very restricted. This is because the dominant breeding systems are based on the production of seed tubers and hardly any rules are in place for potato seeds. As a consequence, most countries consider potato seeds as belonging to the highest risk classes. This is remarkable as seed tubers may harbour any of over 200 species of pathogens that attack potato (Delleman et al., 2004). In contrast, only six pathogens are seed-borne (Solomon-Blackburn and Barker, 2001). These are five viruses and a viroid, which are absent in major potato-growing regions like the Netherlands. So, potato seeds are very safe and regulations will need to be adjusted accordingly.

The registration process for breeders' rights poses a similar situation: in countries which are members of the International Union for the Protection of new Varieties of Plants (UPOV), the path to commercialization of a hybrid starts with the registration process to obtain breeders' rights, which is based on seed tubers. So, protection by breeders' rights of hybrid potato seed cultivars is not yet possible. The European Union (EU) is adjusting the legislation process, but it may still take several years before this is established. Other non-UPOV countries will likely follow later.

5 Inbred lines for genetic research

Inbred lines allow the generation of mapping populations such as F_2, BC_1 and BC_2. As the parents have limited allelic variation, the signal-to-noise ratio is much higher than in studies with heterozygous tetraploid populations. Moreover, putative quantitative trait loci (QTL) can effectively be confirmed in dedicated populations that are selected to segregate for the loci under investigation, and are fixed for other regions on the genome (Wang et al., 2008; Schmalenbach and Pillen, 2009; Fu et al., 2010). In addition, new genetic populations can be generated, which are very powerful for quantitative genetic studies, like nearly isogenic lines (NILs), recombinant inbred lines (RILs) and libraries of introgression lines (Young et al., 1988; Paran et al., 1995; Jeuken and Lindhout, 2004; Finkers et al., 2007; Zhang et al., 2005; Chen et al., 2010; Viquez et al., 2014).

Genetic studies in potato have been done at the tetraploid level and at the diploid level. Tetraploids may support simple genetics like the mapping of resistance genes (Solomon-Blackburn and Barker, 2001), but quantitative studies are less reliable as the genetic noise of the numerous highly heterozygous loci is high (unexplained error). Genome-wide association studies (GWAS) at the tetraploid level will always generate hundreds of potential leads, but only a few hits may be meaningful (Li et al., 2010; D'Hoop et al., 2014). More accurate and reliable quantitative studies were done at the diploid level, initially by crossing heterozygous parents (Prasher et al., 2014) and, more recently, in a diploid F_2 population (Endelman and Jansky, 2016).

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0571

The possibility of using self-compatible, homozygous inbred lines for genetic studies offers three powerful advantages:

1 Only one allele per homozygous locus is present.
2 Only two alleles per heterozygous locus are present.
3 Backcrosses and selfings are feasible for confirmation studies.

For more than five decades, mutant studies have uncovered new alleles of important genes and have helped to confirm or determine the function of genes. Although advanced technologies like the CRISPR/Cas system (Belhaj et al., 2013) are likely to replace the methods by which mutants are made, mutants will remain powerful tools to discover unknown phenotypic traits or to study induced alleles that also have the advantage to be free of deregulation rules.

5.1 The first completely homozygous self-compatible diploid inbred line in potato

Most *Solanum* species that are crossable with cultivated potato are self-incompatible. An exception is *S. chacoense* (Hosaka and Hanneman, 1998a; Hawkes, 1990; Jansky et al., 2014) and introducing the *Sli*-gene from *S. chacoense* into cultivated diploid potato resulted in fertile self-compatible offspring (Lindhout et al., 2011a). After several rounds of inbreeding, highly homozygous self-compatible inbreds were generated. The level of homozygosity was assessed by using SNP markers to investigate the effects of inbreeding (Fig. 7). A strong correlation was observed between the overall level of homozygosity and reduced self-compatibility. By new series of crosses, selections and selfings, the agronomic

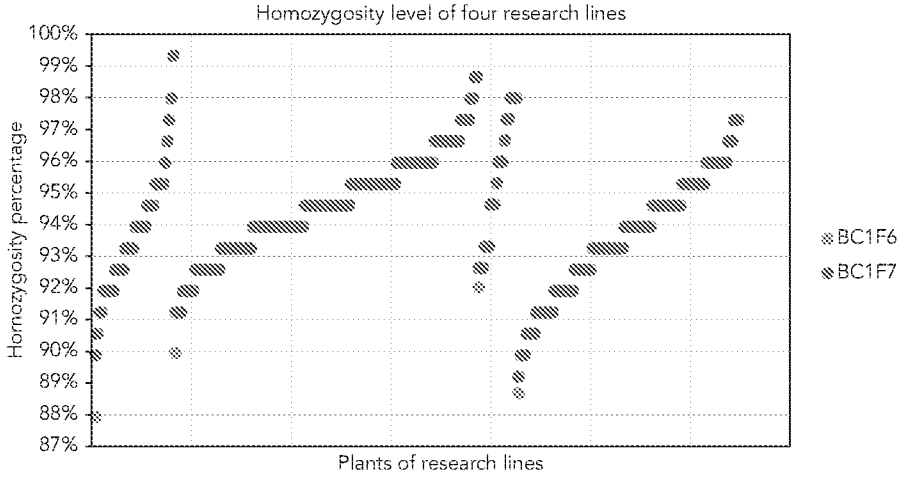


Figure 7 The increase of the level of heterozygosity in the offspring (BC_1F_7) of four BC_1F_6 plants obtained by selfing of the BC_1(D1 × F_1[DS × D1]) TQ: Please check the usage of brackets is as per style. (Table 1). The width of the bar indicates the number of plants in the progeny within the class with plants of the same homozygosity level. The calculations were based on a total of 150 markers.

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0572

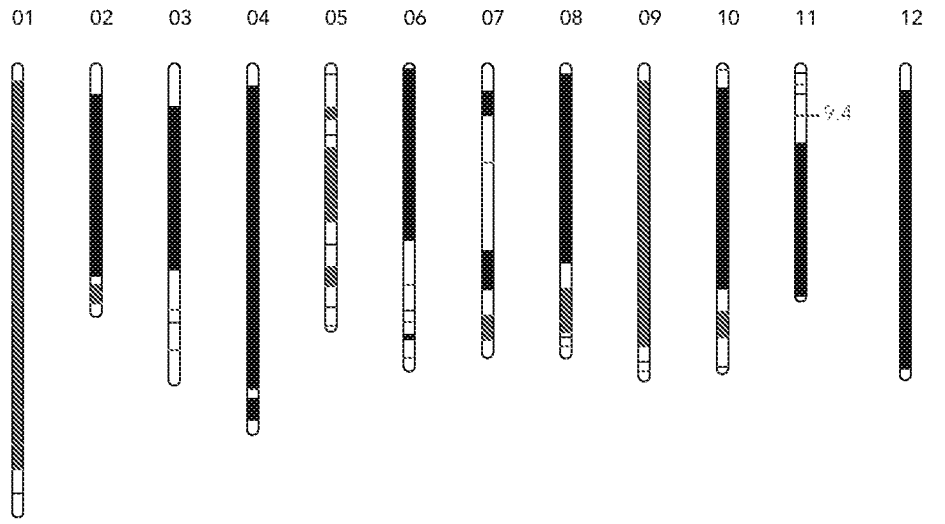


Figure 8 The first essentially homozygous self-compatible diploid potato. The position of the SNP markers is based on the published sequence (PSGC, 2011). The 12 vertical bars indicate the 12 chromosomes. Red bars indicate homozygous D1, blue bars indicate homozygous DS (Table 1). The green line on chromosome 11 indicates a heterozygous scored marker.

performance of the inbred lines continuously improved. After six generations of selfing, a homozygous self-compatible line was generated with only 1 out of 150 SNP markers still heterozygous (Fig. 8). Genotyping by sequencing techniques make it now possible to check the level of homozygosity in more detail.

5.2 Genetic studies in segregating diploid F_2 populations

Recently, Endelman and Jansky (2016) published the first results of a mapping study in an F_2 population of diploid potato. This was based on a cross between the doubled monoploid potato DM1-3 and M6, which is an S_7 inbred line derived from the self-compatible wild relative *S. chacoense*. A single F_1 plant was then self-pollinated and an F_2 population of 109 genotypes was grown, genotyped (>2200 SNPs) and phenotyped. Meijer et al. (2016) analysed an F_2 population (108 markers) based on a cross between two clones, namely DS (a homozygous diploid *S. chacoense* clone containing the self-incompatibility overcoming *Sli*-gene) and D2 (a partly heterozygous diploid *S. tuberosum* clone; see also Table 1). The results of both studies are comparable: tuber shape is associated with a region on chromosome 10, flesh colour with a region on chromosome 3 and tuber and pigment colour on chromosomes 2, 10 and 11. These QTLs were identified at the same loci as described in literature (van Eck et al., 1993, 1994). In both studies, additional QTLs were identified. Furthermore, there is an overwhelming reservoir of potential useful QTLs in the potato germplasm (Bradshaw et al., 2007) and thus also in the dihaploids that can be made. Such QTLs can now be more reliably studied at the diploid level and this will ultimately lead to the identification of the underlying genes. A limited subset of the diploid germplasm may already harbour many important traits for potato breeding (Table 1). Relevant genes

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0573

for these traits can be introgressed into vigorous and fertile diploid genotypes to develop progenies with maximum genetic fixation to minimize genetic noise. Such genotypes are unique materials for further detailed genetic studies.

5.3 Marker-assisted backcrossing

Another application for inbred lines in potato is introgression breeding. This requires knowledge of diagnostic markers for the gene of interest, preferably inside the gene, markers for the recurrent parent genome and a self-compatible homozygous backcrossing parent. There are dozens of well-studied resistance genes in potato that can be used for introgression breeding. This paves the way for a marker-assisted backcrossing (MAB) programme in potato (Frisch and Melchinger, 2005).

To this end, homozygous inbred lines are crossed with a diploid donor carrying a specific gene of interest. In two backcrosses, NILs can be generated by selection with diagnostic markers for the gene of interest and against markers in its flanking regions, combined with selection for markers well distributed over the potato genome (whole background selection). Such NILs can harbour over 98% of the recurrent genome in combination with the specific gene. Both parents of a hybrid may have an introgressed gene, resulting in a double stack hybrid. To introgress specific genes in a homozygous parental line will take 2–3 years. These MAB programmes are routinely used in other crops and are also feasible in potato (Mallick et al., 2015; Jeong et al., 2015).

6 Cropping systems based on true seeds

The production of commercial tubers in most parts of the world starts with seed tubers. These have a large reservoir of nutrients for the growing shoots, allow a rapid initial plant growth and fast leaf coverage of the soil, which is one of the most critical factors for potato yield. In contrast, potato seeds are extremely tiny, about 2500 seeds per gram. As a result, during the first period after germination the young seedlings are very vulnerable for abiotic stresses like drought, frost and heat. Field emergence has been reported between 50% and 80% with acceptable tuber yields under different experimental conditions (El-Bedewy et al., 1994; Renia, 1995). However, without a protective environment, the risk is very high that an emerging seedling will not survive, even when it is pelleted or primed.

This chapter describes alternative strategies to circumvent the exposure of week seedlings to harsh conditions.

6.1 Production of seedling tubers in greenhouse

Seedling tubers can be produced under greenhouse conditions by sowing in a medium with sufficient water supply and at optimum germination temperature of 15–20°C (Struik and Wiersema, 2012). As soon as seedlings reach 5–10 cm in length, they are transplanted in pots. The desired tuber size, the available space in the greenhouse and the length of the growing period will determine the pot size, nutrient supply, day/night temperature and light regime (for a detailed protocol see Struik and Wiersema, 2012). Densities of 80–170 plants/m^2 are common in greenhouses (Lommen, 1995; Tierno et al., 2014). There is a trade-off between number of tubers, size of tubers, planting density and time to harvest.

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0574

Depending on the exact conditions, end-product requirements and production costs, the optimal combination is chosen. Alternatively, hydroponic techniques are available, whereby the roots are grown in a dark, humid and soilless environment in two layers, one for nutrient uptake and the other layer for producing mini-tubers (Lommen, 2007). The seedling tubers are picked at regular intervals and the total yield per plant may reach dozens of tubers. The soilless culture assures clean seed tubers. The conditions and picking regime are set to have optimal numbers and tuber sizes.

6.2 Production of seedlings for commercial crop

Greenhouse-grown seedlings can also be used to start the cultivation of a commercial crop. This system is equivalent to the one used for lettuce, leek, cabbage and onion (Leskovar et al., 2014). Technically, commercial potato production from seedlings is feasible and maximizes the benefits of true seeds. When potato transplants are grown as a ware potato crop, a whole new cultivation system must be developed. Important elements are: transplanting systems, plant spacing including ridging or bedding, the use of soil coverage, weed control, irrigation and harvesting methods. Further mechanization and dedicated cultivation systems will be optimized for cropping systems that start with potato seedlings (Roy et al., 2015). In Kenya, tuber yields of 30 tonnes/ha were obtained, whereby seedling transplants were used as starting material for a commercial cultivation (Muthoni et al., 2014). This already represented 50% higher yield than average in Kenya (Wang'om and van Dijk, 2013).

6.3 Seedling tubers as starting materials for a commercial crop

Seedling tubers are equivalent to mini-tubers that are produced from tissue culture, which is routinely done to start a new multiplication round with clean basic seeds (Amin et al., 2014). They are certified as G1 material. The great advantage of seedlings are the reduced costs, compared to *in vitro* grown plants, and the flexibility to start the production whenever and wherever needed, as seeds can easily be stored and transported. As the cost to produce mini-tubers from *in vitro* grown plants is very high, in the present potato system at least three rounds of field multiplications are needed to dilute these high costs over many seed tubers. For a hybrid seed system, the cost of producing seedling tubers is much lower and hence fewer propagation rounds are needed. Therefore, seedling tubers should be multiplied only 1 year and then released to commercial farmers. Such a system also fits better to the fast introduction of new cultivars, which is typical for a hybrid breeding system.

6.4 Production of seed tubers from seedlings in field

At present, the production of seedling tubers is mostly done under tropical conditions. Seeds are sown in a simple greenhouse or in the field under plastic cover with plant densities of 80–100 plants/m^2 (Kumar, 2014; Struik and Wiersema, 2012; Fig. 9). When the seedlings have reached 5–10 cm in length, they are transplanted to the field, in ridges, at a defined plant density. Additional hilling will increase the number of seed tubers per plant (Wiersema, 1986). To decrease the risk of root damage, the complete substrate is transplanted with the seedling. Plantlets need some time to adapt to outdoor conditions before transplanting directly to the soil (Gopal, 2004). In South Asia, transplants are

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0575



Figure 9 The first seedlings of the first diploid hybrid potato hybrids in Democratic Republic of Congo.

planted on the sides of the ridges to benefit from the shade and higher soil humidity. The planting distance may be adjusted to compensate for a shorter growing season compared to seed tubers, if appropriate. The cultivation conditions are similar to traditional potato cultivation systems. In Egypt, seed tuber yields of 40–60 tonnes/ha were obtained in such a system, based on tetraploid TPS populations (El-Yazied et al., 2004).

Seedlings are more sensitive to frost and drought than seed tubers. Thus, transplanting is done in a frost-free season and with irrigation. Compared to the traditional systems of producing seed tubers, whereby the tubers are planted far before the last night with frost, the length of the growing season of transplants may be 2 months shorter. In addition, the plant development may be further delayed due to a transplanting shock and weak initial growth. Hence, tuber numbers are lower and tuber sizes are smaller compared to seed tuber grown plants, causing severe yield reductions. Plant density may be increased to compensate for these reduced yields per plants.

7 Case studies

7.1 Combatting *Phytophthora infestans*

Late blight, caused by the oomycete *Phytophthora infestans*, was responsible for the Irish famine of 1845–47 (Fry, 2008). All potato cultivars were susceptible to the disease and suffered severe yield losses that led to food shortage. Since then, breeders have selected cultivars with field resistance and from the early twentieth century onwards, have introduced specific *R*-genes, often sourced from wild relatives. However, cultivar Pentland Dell, which

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0576

carries three *R*-genes, already turned out susceptible to a new virulent race of the fungus even before its widespread use and just 4 years after its introduction (Malcomsum, 1969).

At present, the optimal way to control *P. infestans* is a combined approach of clean seed tubers, clean soils, early and preventive chemical protection and destruction of crop debris after harvest. The global annual cost of cultivation measures and yield loss is estimated at US$5 billion (Duncan, 1999).

P. infestans reproduces mainly clonally. With hundreds of billions of spores produced per hectare in an infected crop (Skelsey et al., 2010) and a mutation rate of $1:10^9$, mutations in any given gene of *P. infestans* are likely to occur in a disease-infected field. Sexual recombination, combined with the redundancy of several effectors that are recognized by *R*-genes, explains why *P. infestans* easily mutates effector genes and develops virulence (Jiang and Tyler, 2012).

As a result of the high genetic variation in potato, dozens of resistance sources have been identified and are available for breeding (Park et al., 2009). Whereas single genes are easily defeated by virulent races to *P. infestans*, combinations of *R*-genes are more effective, although the Pentland Dell case indicates that a more dynamic approach may be needed (Niks et al., 2011).

Ideally, isogenic cultivars are developed that only differ in the combination of *R*-genes. This would allow to deploy the most suitable cultivar, dependent on the epidemiology of *P. infestans*. However, the introduction of one gene from a wild related species into a tetraploid cultivar by traditional breeding already takes several decades, and to combine different *R*-genes in a breeding programme is simply too complicated.

Since 1990, many *R*-genes to *P. infestans* have been mapped and cloned (Ballvora et al., 2002; Huang et al., 2005; Park et al., 2009). These all belong to the so-called class of 'NBS/LRR genes' and have a cytoplasmic interaction with effector genes of *P. infestans*, resulting in defence responses that block the growth of the pathogen (Jones and Dangl, 2006). A genetic modification (GM) approach to develop a series of isogenic cultivars with different *R*-genes from crossable species is being pursued (Haverkort et al., 2016, Jacobsen and Schouten, 2008). These so-called cisgenic plants might fall under the highly costly and complex GM legislation, which would hamper their commercial opportunities.

The hybrid breeding system offers a clear path towards resilient resistance to *P. infestans*: *R*-genes can be stacked in a potato hybrid via marker-assisted introgression (Park et al., 2009). Two genes can be combined via the two parents in 2–3 years, and additional *R*-genes can be added within a year to generate multi-stack resistance hybrids. In this way, series of *R*-gene isogenic hybrids can be generated as a dynamic resource to select the best combination of *R*-genes to protect the crop against the prevailing races of *P. infestans*.

7.2 Hybrid potato breeding for East Africa

Hybrid potato cultivars will bring great benefits, not only to modern commercial farmers in the developed world, but they may even have a greater social impact in tropical regions where the population rely on potato as a major source of energy and nutrition (FAOstat, 2016). It is very challenging to start an initiative to develop hybrid potato for these regions (Thomas-Sharma et al., 2016; Kumara et al., 2015). When legal and physical protection of the breeding germplasm is not secured in these regions, the development of inbred lines and the hybrid crosses are done elsewhere. The implementation of hybrid potato cultivars in these regions requires considerable investments and strong cooperation of committed

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0577

Table 2 Potato area and production in six East-African countries in 2014 (FAOstat, 2016)

Country	Area (000 ha)	Production (000 tonnes)	Yield (tonnes/ha)
Burundi	24.4	181.2	7.4
Kenya	115.6	1626.0	14.1
Rwanda	166.4	2225.1	13.4
Tanzania	211.5	1761.0	8.3
Uganda	39.0	188.0	4.8
Ethiopia	67.4	921.8	13.7

AQ: We have changed 'T' to 'tonnes' in "Table 2". Please check if this is correct?

public and private partners. As an example, the implementation of hybrid potato cultivars for East Africa is briefly described below.

The highlands of East Africa are traditionally important production areas for potato because the crop is an important component of the local diet (Table 2). However, yields remain very low (Table 2). A range of traditional varieties is used from local sources (Kaguongo et al., 2008) as well as improved material from the International Centre for Potato (CIP). Seed tubers are produced by farmers and storage conditions are far from optimal (Kaguongo et al., 2008; Gildemacher et al., 2009). Janssens et al. (2013) concluded that bacterial wilt, lack of clean seed tubers and poor storage are the most prominent production constraints. Gildemacher et al. (2009) showed that only 3% of the seed tubers sold were free of viruses.

True hybrid potato seeds are devoid of contaminating pathogens and therefore offer an excellent opportunity to potato production improvement in East Africa. Such hybrids should be attuned to the needs of the farmers, who grow their crop at the typical local conditions like a short growing cycle of 90–100 days, high temperatures and tuber development under short days. The prerequisites for establishing dedicated hybrid potato breeding system for East Africa are:

- A (private) organization executing a breeding programme tailored to the needs of the region.
- Secured supply of hybrid seeds for the region.
- Regulations supporting imports and exports of seeds, seed tubers and commercial tubers.
- Formal registration system for breeders' rights protection.
- Local organizations testing new experimental hybrids.

Such breeding programme can only become sustainable if the complete downstream part of potato food chain is also well organized. This includes the following:

- Production systems of disease-free seed tubers from seedlings.
- An efficient supply system for farmers to obtain hybrid cultivars.
- Efficient farmers' cropping systems to produce high quality potato tubers for the target markets.
- Efficient logistics to transport farm produce to consumers and processors.

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0578

- A well-developed consumer market.
- A business plan over the whole potato chain, whereby all stakeholders benefit.

Solynta is already testing the first experimental hybrids in the highlands of the Albertine Rift in Ituri Province, Democratic Republic of Congo. These are experimental hybrids derived from the European hybrid breeding programme. At a local farm, established by the Lake Albert Foundation, seeds have been sown and seedlings have been transplanted into the field (Fig. 9). These hybrids are evaluated in good cooperation with local farmers and the results are shared with the Solynta breeding programme. These data are used to adjust the selection of inbred lines to the needs in East Africa and to continuously generate new experimental hybrids, which will be tested at the Congo farm again. This iterative and interactive process may already select the first dedicated hybrids for East Africa in 2–3 years.

This breeding programme is accompanied by research on cropping systems for the region. In addition, training programmes for research institutions, agronomists, local staff and interested farmers in the regions will make the farmers' communities and relevant institutions familiar with the new concepts of hybrid potato cultivars. Also market studies are needed to identify and secure stable and sustainable markets for the farmers' potato products. This market may comprise other countries in the Great Lakes Region (South Sudan, Uganda, Burundi and Rwanda).

The support for this programme by national authorities and development agencies – also in the neighbouring countries like Uganda – is required, but it will take time before the concept of hybrid potato cultivars is fully understood and accepted.

8 Conclusion

Since the first crosses in 2008, the Solynta research efforts have been focused on the development of a hybrid potato breeding system. This research has now reached the stage where hybrid potato breeding will become reality. This has recently been supported by two leading potato breeding companies in EU, KWS and HZPC, who have also expressed their conviction that hybrid potato breeding will be future main breeding system (KWS, 2016). In addition, 21 leaders in the industry and potato science in the United States have expressed their opinion on 'Reinventing potato as a diploid inbred line-based crop' with a scientific base for diploid hybrid potato breeding (Jansky et al., 2016).

We have made great advances in the development of useful homozygous inbred lines and the first field evaluations of experimental hybrids have shown the potential of hybrid cultivars to harvest heterosis.

The technologies to develop new cropping systems adapted to various climate zones and agronomic practices, which allow the production of commercial seed tubers from seedlings, are already available.

We envision that future hybrid potato varieties, similar to modern tomato hybrids, will harbour up to 15 resistance genes. In addition, the lifetime of new potato hybrid cultivars will be reduced to less than 5 years as is the experience in sugar beet, where the lifetime of new cultivars is only 2–3 years since the first diploid hybrids have been introduced into the market.

Our inbred lines will also be of great value for research purposes as they allow the development of sophisticated populations that are very helpful in genetic and genomic

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

studies as has been shown in other crops. This will give a boost in the exploration and exploitation of the genetic variation in the potato germplasm.

The self-compatible potato inbred lines will greatly stimulate quantitative research on the genetic control of complex traits such as interaction with mycorrhiza, biotic stresses, nutrient uptake, nutrition value and processing quality.

The application of hybrid potato breeding technology will not be restricted to the most advanced research groups in the public or private institutions. New players in the scientific and applied breeding field of the potato business will arise and new cooperations will be established to take full advantage of the hybrid breeding technology in science and product development.

Finally, hybrid potato breeding will require the skills of the breeder as well as the knowledge and tools of scientists. As a result, a new generation of potato breeding teams will be established combining the skills of the breeder with the in-depth knowledge of plant scientists.

9 Where to look for further information

This chapter describes the first implementation of a hybrid potato breeding strategy and the progress that is made since 2008. We direct the interested reader to the papers of Almekinders (1996, 2009) to learn about 'conventional TPS'. As a textbook on plant breeding, we suggest Brown and Caglari (2011), while more advanced potato genetics and genomics is found in Bradeen and Kole (2011). The history and mechanism of *Phytophthora* attack is well described by Fry (2008). Finally, FAO (FAOstat, 2016) statistics provide numerous data on potato cultivation.

Leading centres of research include Wageningen University in The Netherlands, James Hutton Institute in the United Kingdom, the International Potato Centre in Peru, Wisconsin University and Michigan State University both in the United States, whereby the most recent paper of Jansky et al. (2016) can be considered a must for anybody interested in hybrid potato breeding.

10 Acknowledgements

We are grateful to Jan Leemans and Herman Fleer for critically reading and reviewing this manuscript.

11 References

Abrol, D. P. (2012). Pollination for hybrid seed production. In *Pollination Biology. Biodiversity Conservation and Agricultural Production*. Publisher Springer Netherlands, pp. 397–411.

Almekinders, C. J. M., Chujoy, E. and Thiele, G. (2009). The use of true potato seed as pro-poor technology: The efforts of an international agricultural research institute to innovating potato production. *Pot. Res.* 52, 275–93.

Almekinders, C. J. M., Chilver, A. S. and Renia, H. M. (1996). Current status of the TPS technology in the world. *Pot. Res.* 39, 289–303.

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Amin, N., Amin, A. R., Roy, T. S., Ali, M. A., Rashid, M. M., Hossain, M. M. and Hasan, N. (2014). Bulking behavior of seedling tubers derived from true potato seed as affected by its size and harvesting time. *App. Sci. Rep.* 8, 1–8.

Ballvora, A., Ercolano, M. R., Weiss, J., Meksem, K., Bormann, C. A., Oberhagemann, P., Salamini, F. and Gebhardt, C. (2002). The *R1* gene for potato resistance to late blight (*Phytophthora infestans*) belongs to the leucine zipper/NBS/LRR class of plant resistance genes. *Plant J.* 30, 361–71.

Belhaj, K., Chaparro-Garcia, A., Kamoun, S. and Nekrasov, V. (2013). Plant genome editing made easy: targeted mutagenesis in model and crop plants using the CRISPR/Cas system. *Plant Meth.* 9, 39.

Birchler, J. A., Yao, H., Chudalayandi, S., Vaiman, D. and Veitia, R. A. (2010). Heterosis. *Plant Cell* 22, 2105–12.

Bradeen, J. M. and Kole, C. (2011). *Potato Genetics: Genetics, Genomics and Breeding of Potato.* CRC Press, USA

Bradshaw, J. E., Hackett, C. A., Pande, B., Waugh, R. and Bryan, G. J. (2007). QTL mapping of yield, agronomic and quality traits in tetraploid potato (*Solanum tuberosum* subsp. *tuberosum*). *Theor. Appl. Genet.* 116, 193–211.

Brown, J. and Caligari, P. (2011). *An Introduction to Plant Breeding.* Wiley-Blackwell, USA, p. 224.

Cappadocia, M. (1990). Wild Potato (*Solanum chacoense* Bitt.). In Y. P. S. Bajaj (ed.), *Vitro Production of Haploids. Biotechnology in Agriculture and Forestry, Vol. 12 Haploids in Crop Improvement I*, pp. 514–29. Springer-Verlag, Berlin, Heidelberg.

Chani, E., Veilleux, R. E. and Boluarte-Medina, T. (2000). Improved androgenesis of interspecific potato and efficiency of SSR markers to identify homozygous regenerants. *Plant Cell Tissue Organ Cult.* 60, 101–12.

Charlesworth, D. and Willis, J. H. (2009). The genetics of inbreeding depression. *Genetics* 10, 783–96.

Chauvin, J. E., Souchet, C., Dantec, J. P. and Ellisseche, D. (2003). Chromosome doubling of 2x *Solanum* species by oryzalin: method development and comparison with spontaneous chromosome doubling *in vitro*. *Plant Cell Tissue Organ Cult.* 73, 65–73.

Chen, X., Niks, R. E., Hedley, P. E., Morris, J., Druka, A., Marcel, T. C., Vels, A. and Wauh, R. (2010). Differential gene expression in nearly isogenic lines with QTL for partial resistance to *Puccinia hordei* in barley. *Genomics* 11, 629.

Crow, J. F. (1998). 90 Years ago: The beginning of hybrid maize. *Genetics* 148, 923–8.

CSAC (2005). Initial sequence of the chimpanzee genome and comparison with the human genome. *Nature* 437, 69–87.

D'hoop, B. B., Keizer, P. L. C., João Paulo, M., Visser, R. G. F., Van Eeuwijk, F. A. and Van Eck, H. J. (2014). Identification of agronomically important QTL in tetraploid potato cultivars using a marker-trait association analysis. *Theor. Appl. Genet.* 127, 731–48.

De Jong, H. and Rowe, P. R. (1971). Inbreeding in cultivated diploid potatoes. *Pot. Res.* 14, 74–83

De Mainea, M. J. (1982). An evaluation of the use of dihaploids and unreduced gametes in breeding for quantitative resistance to potato pathogens. *J. Agric. Sci.* 99, 79–83.

Delleman, J., Mulder, A. and Turkensteen, L. J. (2004). *Potato Diseases: Diseases, Pests and Defects.* Potatoworld and NIVAP, The Hague, the Netherlands.

Douches, D. S., Maas, D. J., Astrzebski, K. and Chase, R. W. (1996). Assessment of potato breeding progress in the USA over the last century. *Crop Sci.* 36, 1544–52.

Duncan, J. M. (1999). *Phytophthora*-an abiding threat to our crops. *Microbiol. Today* 26, 114–16.

Duvick, D. N. (2005). The contribution of breeding to yield advances in maize (Zea mays L.). *Adv. Agron.* 86, 83–145.

El-Bedewy, R., Crissman, C. and Cortbaoui, R. (1994). Progress report. Egypt's seed system based on true potato seed. *CIP Circular* 20, 5–8.

El-Yazied, A., Elminiawy, S. E., Hamoud, N. K. and El-Kheima, S. (2004). Seed tuber production of some hybrids using true potato seed. Mansoura University. *J. Agric. Sci.* 32, 1329–41.

Endelman, J. B. and Jansky, S. H. (2016). Genetic mapping with an inbred line-derived F_2 population in potato. *Theor. Appl. Genet.* 1–9

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0581

FAO, IFAD and WFP. (2015). The State of Food Insecurity in the World 2015. Meeting the 2015 international hunger targets: taking stock of uneven progress. Rome, FAO.

FAOstat. (2016). http://faostat3.fao.org/home/E

Finkers, R., Van Heusden, A. W., Meijer-Dekens, F., Van Kan, J. A. L. and Lindhout, P. (2007). The construction of a *Solanum habrochaites* LYC4 introgression population and the identification of QTLs for resistance to *Botrytis cinerea*. *Theor. Appl. Genet.* 114, 1071–80.

Frisch, M. and Melchinger, A. E. (2005). Selection theory for marker-assisted backcrossing. *Genetics* 170, 909–17.

Fry, W. (2008). Plant diseases that changed the world *Phytophthora infestans*: the plant (and *R* gene) destroyer. *Molec. Pl. Path.* 9, 385–402.

Gildemacher, P. R., Demo, P., Barker, I., Kaguongo, W., Woldegiorgis, GT., Wagoire, W. W., Wakahiu, M., Leeuwis, C. and Struik, P. C. (2009). A Description of seed potato systems in Kenya, Uganda and Ethiopia. *Am. J. Pot. Res.* 86, 373–82.

Gopal, J. (2004). True potato seed: Breeding for hardiness. In A. Haneafi (ed.), Sixth Triennial Congress of the African Potato Association. *Proc. APA Congr.*, 5–10 April, Agadir, Morocco, pp. 39–57.

Gopal, J. (2014). Heterosis breeding in potato. *Agric. Res.* 3, 204–17.

Haldane, J. (1930). Theoretical genetics of autopolyploids. *J. Genet.* 22, 359–72.

Haverkort, A. J., Boonekamp, P. M., Hutten, R., Jacobsen, E., Lotz, L. A. P., Kessel, G. J. T., Vossen, J. H. and Visser, R. G. F. (2016). Durable late blight resistance in potato through dynamic varieties obtained by cisgenesis: Scientific and societal advances in the DuRPh project. *Pot. Res.*, 59, 35–66.

Hawkes, J. G. (1990). *The Potato: Evolution, Biodiversity, and Genetic Resources*. Belhaven Press, London.

Hosaka, K. and Hanneman, R. E. (1998a). Genetics of self-compatibility in a self-incompatible wild diploid potato species *Solanum chacoense*. 1. Detection of an *S* locus inhibitor (*Sli*) gene. *Euphytica* 99, 191–7.

Hosaka, K. and Hanneman, R. E. (1998b). Genetics of self-compatibility in a self-incompatible wild diploid potato species *Solanum chacoense*. 2. Localization of an *S* locus inhibitor (*Sli*) gene on the potato genome using DNA markers. *Euphytica* 103, 265–71.

Hua, J., Xing, Y., Wu, W., Xu, C., Sun, X., Yu, S. and Zhang, Q. (2003). Single-locus heterotic effects and dominance by dominance interactions can adequately explain the genetic basis of heterosis in an elite rice hybrid. *Proc. Natl. Acad. Sci. USA* 100, 2574–9.

Huang, S., Van der Vossen, E. A., Kuang, H., Vleeshouwers, V. G., Zhang, N., Borm, T. J., Van Eck, H. J., Baker, B., Jacobsen, E. and Visser, R. G. (2005). Comparative genomics enabled the isolation of the *R3a* late blight resistance gene in potato. *Plant J.* 42, 251–61.

Hutten, R. C. B. (1994). Basic Aspects of Potato Breeding Via the Diploid Level. PhD Thesis, Wageningen University, p. 93.

Hutten, R. C. B., Schippers, M. G. M., Hermsen, J. G. Th. and Jacobsen, E. (1994). Comparative performance of diploid and tetraploid progenies from 2x.2x crosses in potato. *Euphytica* 81, 187–92.

Jacobsen, E. and Schouten, H. J. (2008). Cisgenesis, a new tool for traditional plant breeding, should be exempted from the regulation on genetically modified organisms in a step by step approach. *Pot. Res.* 51, 75.

Jansky, S. H. and Peloquin, S. J. (2006). Advantages of wild diploid *Solanum* species over cultivated diploid relatives in potato breeding programs. *Genet. Res. Crop Evol.* 53, 669–74.

Jansky, S. H., Charkowski, A. O., Douches, D. S., Gusmini, G., Richael, C., Bethke, P. C., Spooner, D. M., Novy, R. G., De Jong, H., De Jong, W. S., Bamberg, J. B., Thompson, A. L., Bizimungu, B., Holm, D. G., Brown, C. R., Haynes, K. G., Sathuvalli, V. R., Veilleux, R. E., Miller Jr., J. C., Bradeen, J. M. and Jiang, J. M. (2016). Reinventing potato as a diploid inbred line-based crop. *Crop Sci.* 56, 1–11.

Jansky, S. H., Chung, Y. S. and Kittipadakul, P. (2014). M6: A diploid potato inbred line for use in breeding and genetics research. *J. Plant Registr.* 8, 195–9.

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Janssens, S. R. M., Wiersema, S. G., Goos, H. and Wiersema, W. (2013). The value chain for seed and ware potatoes in Kenya; Opportunities for development LEI. Memorandum 13-080, p. 57.

Jeong, H-S., Jang, S., Han, K., Kwon, J-K. and Kang, B-C. (2015). Marker-assisted backcross breeding for development of pepper varieties (*Capsicum annuum*) containing capsinoids. *Molec. Breeding* 35, 226.

Jeuken, M. J. W. and Lindhout, P. (2004). The development of lettuce backcross inbred lines (BILs) for exploitation of the *Lactuca saligna* (wild lettuce) germplasm. *Theor. Appl. Genet.* 109, 394–401.

Jiang, R. H. Y. and Tyler, B. M. (2012). Mechanisms and evolution of virulence in oomycetes. *Ann. Rev. Phytop.* 50, 295–318.

Jones, J. D. G and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–9.

Kaguongo, W., Gildemacher, P., Demo, P., Wagoire, W., Kinyae, P., Andrade, J., Forbes, G., Fuglie, K., and Thiele, G. (2008). Farmer practices and adoption of improved potato varieties in Kenya and Uganda. International Potato Center (CIP), Lima, Peru. Social Sciences Working Paper 2008–5. 85 p.

Kumar, V. (2014). True potato seed technology – Prospects and problems. In N. K. Pandey, D. K. Singh and R. Kumar (eds), *Current Trends in Quality Potato Production, Processing and Marketing*, pp. 175–82. Central Potato Research Institute, India.

Kumara, N. S., Govindakrishnan, P. M., Swarooparani, D. N., Nitin, Ch. Surabhi, J. and Aggarwal, P. K. (2015). Assessment of impact of climate change on potato and potential adaptation gains in the Indo-Gangetic Plains of India. *Intern. J. Pl. Prod.* 9 (1), 151–70.

KWS press release (2016). http://www.kws.com/aw/KWS/company-info/Products/Potatoes/News-Articles/~hjqx/KWS-to-fully-focus-on-hybrid-potato-bree/

Leskovar, I. D., Crosby, M. K., Palma, A. M. and Edelstein, M. (2014). Vegetable crops: Linking production, breeding and marketing. In R. G. Dixon and E. D. Aldous (eds), *Horticulture: Plants for people and places, Volume 1: Production Horticulture*, pp. 75–96. Springer Netherlands, Dordrecht.

Li, L., Paulo, M.-J., Van Eeuwijk, F., and Gebhardt, C. (2010). Statistical epistasis between candidate gene alleles for complex tuber traits in an association mapping population of tetraploid potato. *Theor. Appl. Genet.* 121, 1303–10.

Li, X.-Q. (2008). Male sterility systems for hybrid seed production in Brassica crops. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources* 3, 1–14.

Lindhout, P., Meijer, D., Schotte, T., Hutten, R. C. B., Visser, R. G. F. and Van Eck, H. J. (2011a). Towards F1 hybrid seed potato breeding. *Pot. Res.* 54, 301–12.

Lindhout, W. H., Schotte, T. P., Visser, R. G. F., Van Eck, H. J. and Hutten, R. C. B. (2011b). Hybrid seed potato breeding. European Patent Office nr EP 2514303 A1

Lippman, Z. B. and Zamir, D. (2007). Heterosis: revisiting the magic. *Trends Genet.* 23, 60–6.

Lommen, W. J. M. (1995). Basic studies on the production and performance of potato minitubers. PhD Thesis, Wageningen Agricultural University, Wageningen, The Netherlands, p. 181.

Lommen, W. J. M. (2007). The canon of potato science: Hydroponics. *Pot. Res.* 50, 315–18.

Lössl, A., Götz, M., Braun, A. and Wenzel, G. (2000). Molecular markers for cytoplasm in potato: Male sterility and contribution of different plastid-mitochondrial configurations to starch production. *Euphytica* 116, 221–30.

Malcolmson, J. F. (1969). Races of *Phytophthora infestans* occurring in Great Britain. *Trans. Br. Mycol. Soc.* 53, 417–23.

Mallick, N., Vinod, Sharma, J. B., Tomar, R. S., Sivasamy, M. and Prabhu, K. V. (2015). Marker-assisted backcross breeding to combine multiple rust resistance in wheat. *Plant Breed.* 134, 172–7.

Meijer, D. A., Abdullah, S., Rothengatter, R., Van Eck, H. J., Visser, R. G. F., Lindhout, P. and Van Heusden, S. (2016). An F_2 QTL mapping study in a cross between *S. tuberosum* and *S. chacoense*. *Theor. Appl. Genet.* (submitted).

Muthoni, J., Shimelis, H., Melis, R. and Kinyua, Z. M. (2014). Response of potato genotypes to bacterial wilt caused by *Ralstonia solanacearum* (Smith) (Yabuuchi et al.) in the tropical highlands. *Am. J. Pot. Res.* 91, 215–32.

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Niks, R. E., Parlevliet, J. E., Lindhout, P. and Bai, Y. (2011). *Breeding Crops with Resistance to Diseases and Pests*. Wageningen Academic Publishers, p. 200.

Paran, I., Goldman, I., Tanksley, S. D. and Zamir, D. (1995). Recombinant inbred lines for genetic mapping in tomato. *Theor. Appl. Genet.* 90, 542–8.

Park, T. H., Vleeshouwers, V. G. A. A., Jacobsen, E., Van der Vossen, E. and Visser, R. G. F. (2009). Molecular breeding for resistance to *Phytophthora infestans* (Mont.) de Bary in potato (*Solanum tuberosum* L.): a perspective of cisgenesis. *Plant Breed.* 128, 109–17.

PGSC (2011). Genome sequence and analysis of the tuber crop potato. *Nature* 475, 189–94.

Phumichai, C. and Hosaka, K. (2006). Cryptic improvement for fertility by continuous selfing of diploid potatoes using *Sli* gene. *Euphytica* 149, 251–8.

Phumichai, C., Mori, M., Kobayashi, A., Kamijima, O. and Hosaka, K. (2005). Toward the development of highly homozygous diploid potato lines using the self-compatibility controlling *Sli* gene. *Genome* 48, 977–84.

Prashar, A., Hornyik, C., Young, V., McLean. K., Kumar Sharma, S., Dale, M. F. B. and Bryan, G. J. (2014). Construction of a dense SNP map of a highly heterozygous diploid potato population and QTL analysis of tuber shape and eye depth. *Theor. Appl. Genet.* 127, 2159–71.

Renia, H. (1995). True seed is a commercial reality in USA. *Pot. Rev.* 5, 48–51.

Rijk, B., van Ittersum, M. and Withagen, J. (2013). Genetic progress in Dutch crop yields. *Field Crops Res.* 149, 262–8.

Rowe, P. R. (1967). Performance and variability of diploid and tetraploid potato families. *Am. Pot. J.* 44, 263–71.

Roy, T. S., Baque, M. A., Chakraborty, R., Haque, M. N. and Suter, P. (2015). Yield and economic return of seedling tuber derived from True Potato Seed as influenced by tuber size and plant spacing. *Univ. J. Agric. Res.* 3, 23–30.

Schmalenbach, I. and Pillen, K. (2009). Detection and verification of malting quality QTLs using wild barley introgression lines. *Theor. Appl. Genet.* 118, 1411–27.

Skelsey, P., Rossing, W. A. H., Kessel, G. J. T. and Van der Werf, W. (2010). Invasion of *Phytophthora infestans* at the landscape level: How do spatial scale and weather modulate the consequences of spatial heterogeneity in host resistance? *Phytopath.* 100, 1146–61.

Solomon-Blackburn, R. M. and Barker, H. (2001). A review of host major-gene resistance to potato viruses X, Y, A and V in potato: genes, genetics and mapped locations. *Heredity* 86, 8–16.

Struik, P. C. and Wiersema, S. (2012). *Seed Potato Technology*. Wageningen Academic Publishers, p. 383.

Su, C. F., Lu, W. G., Zhao, T. J. and Gai, J. Y. (2009). Verification and fine-mapping of QTLs conferring days to flowering in soybean using residual heterozygous lines. *Chin. Sci. Bull.* 6, 499–508.

Thomas-Sharma, S., Abdurahman, A., Ali, S., Andrade-Piedra, J. L., Bao, S., Charkowski, A. O., Crook, D., Kadian, M., Kromann, P., Struik, P. C., Torrance, L., Garrett, K. A. and Forbes, G. A. (2016). Seed degeneration in potato: the need for an integrated seed health strategy to mitigate the problem in developing countries. *Plant Path.* 65, 3–16.

Tierno, R., Carrasco, A., Ritter, E. and Ruiz de Galarreta, J. I. (2014). Differential growth response and minituber production of three potato cultivars under aeroponics and greenhouse bed culture. *Amer. J. Pot. Res.* 91, 346–53.

Tobias, A., Schrag, T. A., Möhring, J., Melchinger, A. E., Kusterer, B., Dhillon, B. S., Piepho, H-P. and Frisch, M. (2009). Prediction of hybrid performance in maize using molecular markers and joint analyses of hybrids and parental inbreds. *Theor. Appl. Genet.* 120, 451–61.

Troyer, A. F. (2006). Adaptedness and heterosis in corn and mule hybrids. *Crop Sci.* 46, 528–43.

Uijtewaal, B. A., Huigen, D. J. and Hermsen, J. G. Th. (1987a). Production of potato monohaploids (2n = x = 12) through prickle pollination. *Theor. Appl. Genet.* 73, 751–8.

Uijtewaal, B. A., Jacobsen, E. and Hermsen, J. G. Th (1987b). Morphology and vigour of monohaploid potato clones, their corresponding homozygous diploids and tetraploids and their heterozygous diploid parent. *Euphytica* 36, 745–53.

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0584

Uitdewilligen, J. G. A. M. L., Wolters, A. M. A, D'hoop, B. B., Borm, T. J. A., Visser, R. G. F. and Van Eck, H. J. (2013). A next-generation sequencing method for genotyping-by-sequencing of highly heterozygous autotetraploid potato. *PLoS ONE* 8(5), e62355.

Van Breukelen, E. W. M., Ramanna, M. S. and Hermsen, J. G. Th. (1977). Pathenogenetic monohaploids (2n = 2x = 12 from *Solanum tuberosum* L. and *S. verrucosum* Schlechtd. and the production of homozygous potato diploids. *Euphytica* 26, 263–71.

Van Eck, H. J., Jacobs, J. M. E., van den Berg, P. M. M. M., Stiekema, W. J. and Jacobsen, E. (1994), The inheritance of anthocyanin pigmentation in potato (*Solanum tuberosum* L.) and mapping of tuber skin colour loci using RFLPs. *Heredity* 73, 410–21.

Van Eck, H. J., Jacobs, J. M. E., van Dijk, J., Stiekema, W. J. and Jacobsen, E. (1993) Identification and mapping of three flower colour loci of potato (*S. tuberosum* L.) by RFLP analysis. *Theor. Appl. Genet.* 86, 295–300.

Viquez-Zamora, M., Caro, M., Finkers, R., Tikunov, Y., Bovy, A., Visser, R. G. F., Bai, Y. and Van Heusden, S. (2014). Mapping in the era of sequencing: high density genotyping and its application for mapping TYLCV resistance in *Solanum pimpinellifolium*. *BMC Genomics* 15, 1152.

Visser, R. G. F., Bachem, C. W. B., Borm, T., de Boer, J., Van Eck, H. J., Finkers, R., Van der Linden, G., Maliepaard, C. A., Uitdewilligen, J. G. A. M. L., Voorrips, R., Vos, P. and Wolters, A. M. A. (2014). Possibilities and challenges of the potato genome sequence. *Pot. Res.* 57, 327–30.

Vos, P. G., Uitdewilligen, J. G. A. M. L, Voorrips, R. E., Visser, R. G. F. and Van Eck, H. J. (2015). Development and analysis of a 20K SNP array for potato (*Solanum tuberosum*): an insight into the breeding history. *Theor. Appl. Genet.* 128, 2387–401.

Wang, C. M., Lo, L. C, Feng, F., Zhu, Z. Y. and Yue, G. H. (2008). Identification and verification of QTL associated with growth traits in two genetic backgrounds of Barramundi (*Lates calcarifer*). *Animal Genet. Vol.* 39, 34–9.

Wang'om, W. G. and van Dijk, M. P. (2013). Low potato yields in Kenya: do conventional input innovations account for the yields disparity? *Agric. Food Sec.* 2, 14.

Wiersema, S. G. (1986). A method of producing seed tubers from true potato seed. *Pot. Res.* 29, 225–37.

Young, N. D., Zamir, D., Ganal, M. W. and Tanksley, S. D. (1988). Use of isogenic lines and simultaneous probing to identify DNA markers tightly linked to the *tm-2a* gene in tomato. *Genetics* 120, 579–85.

Zhang, Y-M., Mao, Y., Xie, C., Smith, H., Luo, L. and Xu, S (2005). Mapping Quantitative Trait Loci using naturally occurring genetic variance among commercial inbred lines of maize (*Zea mays* L.). *Genetics* 169, 2267–75.

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0585

ON THE NATURE OF THINGS: ESSAYS

New Ideas and Directions in Botany

How do we address the disconnect between genetic and morphological diversity in germplasm collections?[1]

Shelley H. Jansky[2,3,4], Julie Dawson[3], and David M. Spooner[2,3]

KEY WORDS diversity; genebanks; germplasm; potato; predictivity

The world's food production systems must keep up with an ever-increasing demand in spite of the challenges caused by climate change and environmental degradation. To meet this challenge, plant breeders must have access to genetic diversity for crop improvement. However, genetic homogeneity in the handful of major crops that feed the world limits options for breeding progress (Swaminathan, 2009). Wild and landrace relatives of crop plants provide much needed diversity and are maintained in gene banks worldwide (McCouch, 2013). This germplasm resource is underused by breeders, mainly because genotypic and phenotypic data are limited. However, using rapidly developing genomics resources, breeders are unlocking the genetic potential in gene banks and making remarkable advances (Tanksley and McCouch, 1997).

Morphological traits and neutral genetic markers are commonly used to characterize germplasm collections, with the goal of improving the value of these collections to breeders (Brown, 1989). Morphological and neutral marker data provide the foundation for the assembly of a core collection, defined as a subset of a germplasm collection intended to represent the entire collection. The concept of a core collection assumes that the core has some utility for plant improvement. That is, the core collection is maximally diverse and is useful for breeding. Evaluating the core collection for key traits should also have some level of predictability for the rest of the collection based on the relatedness of core accessions to other accessions in the collection.

It has been recognized for many years that morphological traits do not always provide a good measure of genetic values and may not accurately reveal the genetic variation in a germplasm collection (Tanksley and McCouch, 1997). Consequently, neutral molecular markers have become popular because they are thought to more accurately reflect genetic relationships in germplasm collections (Ebana et al., 2008). But genetic relatedness measures based on neutral markers may not predict similarity in trait values or parental performance. The patterns of allelic variation in a species may be very different for neutral markers compared with genes under selection. Based on a meta-analysis, McKay and Latta (2002) argued that allele frequencies at neutral and selected loci are not correlated because evolutionary forces act differently on them. A meta-analysis by Reed and Frankham (2001) showed only weak correlation between neutral molecular markers and quantitative measures of variation. Reeves, Panella, and Richards (2012) found that genetic variation at loci of agronomic interest in core collections assembled using neutral diversity may be lower than in collections assembled at random. In addition, neutral genetic markers identified in single populations or breeding lines may have significant ascertainment bias, which makes them unrepresentative of the full range of functional genetic diversity in the species (Moragues et al., 2010).

Our breeding research in potato has revealed a remarkably unexpected result that has led us to question the value of morphological diversity as a proxy for overall genetic diversity. We assessed an F_2 population created by self-pollinating an F_1 clone from a cross between two diploid ($2n = 2\times = 24$) potato clones: DM, a completely homozygous clone derived from somatically doubling an androgenic monoploid of a cultivated potato, and M6, a highly inbred clone derived from seven generations of self-pollination of the wild diploid potato relative *Solanum chacoense*. We evaluated the F_2 population for a variety of morphological features including tuber size, shape, and eye depth; skin and flesh color; and dry matter content. Phenotypic segregation in this F_2 population is astounding (Fig. 1). The tuber shape and color variability derived from self-pollinating a single diploid F_1 plant approaches that of the landrace collections maintained in potato genebanks (Spooner and Hetterscheid, 2005) (Fig. 1).

[1] Manuscript received 6 May 2015; revision accepted 11 June 2015.

[2] USDA-Agricultural Research Service, Vegetable Crops Research Unit, University of Wisconsin, 1575 Linden Drive, Madison, Wisconsin; and

[3] Department of Horticulture, University of Wisconsin-Madison, 1575 Linden Drive, Madison, Wisconsin 53706-1590 USA

[4] Author for correspondence: (e-mail: shelley.jansky@ars.usda.gov)

doi:10.3732/ajb.1500203

 This article is a U.S. Government work and is in the public domain in the USA. © Botanical Society of America (Excluding U.S. Government Works)

Aardevo Exhibit 1010
Page 0586



FIGURE 1 Tuber shapes and colors in the F_2 population of DM1-3 × M6.

The International Potato Center (Centro Internacional de la Papa, CIP) maintains the world's largest collection of potato, with 4354 landrace accessions from all the cultivated species and 2520 accessions of wild relatives of potato. The catalog of potato varieties maintained by the International Potato Center (https://research.cip.cgiar.org/red_varie/pages/home.php; accessed 04/06/2015) provides a list of morphological descriptors used for potato, and tuber morphology is a major character to infer diversity among potato landraces.

The phenotypic variation seen in our F_2 population originated from a single diploid plant, derived from a completely homozygous genotype (DM) and a nearly complete homozygous clone (M6). Consequently, a maximum of two alleles per locus contributed to this variation. Multiple alleles and diverse genotypes exist in collections of landraces. However, we have demonstrated that this level of genetic diversity is not needed to generate the tuber phenotypic variability often used to characterize landraces. Because these traits are controlled by a few major genes that are widely distributed across landraces, using them as an overall measure of diversity carries the risk of failing to preserve rare alleles that are not as readily apparent. Many traits used for characterization are qualitative,

Aardevo Exhibit 1010
Page 0587

rather than quantitative, making them easy to use but perhaps too limited in their ability to capture the underlying variation in a diverse germplasm collection. While our F_2 population exhibits tremendous phenotypic diversity for tuber traits, it obviously does not capture the wide range of allelic diversity in wild relatives for other traits of interest to breeders, such as disease resistance and stress tolerance.

The results from our study and those cited already present a dilemma for managers of germplasm collections. The case of potato is clearly extreme in the range of tuber morphological diversity present within a single F_2 population, but other species also harbor genetic variation that is not captured by morphological or neutral marker characterization of genebank accessions. There is real value in collections, and the germplasm collections maintained worldwide have proven utility for plant breeding, especially in breeding for improved pest and pathogen resistances and changing environmental and market conditions. In situations where breeders are faced with agricultural challenges for which elite lines are lacking, collections can be a major source of relevant genetic variation. However, these collections are not used to their full potential by plant breeders because of difficulties in identifying useful accessions to incorporate into breeding programs. The optimal strategy for characterizing and organizing information on collections to make them more accessible to plant breeders is not yet known.

Two lines of research seem particularly promising for the characterization and conservation of functional diversity. The first is already being pursued for several genebanks, including the potato collection at the International Potato Center (Ellis, 2014). Accessions are being genotyped using high-density genomic markers, such as genotyping by sequencing (GBS). This approach identifies genetic diversity for both neutral genomic regions and loci that affect traits of interest for plant improvement because of the full genome coverage of the GBS markers. GBS shows little to no ascertainment bias because markers are developed directly on the population being genotyped. The usefulness of these genotypic data, however, is highly dependent on the quality of the corresponding phenotypic data generated by field screening programs.

The second line of research focuses on the more effective use of in situ conservation methods to maintain genetic diversity. The availability of large numbers of inexpensive molecular markers has made it possible to study genetic diversity in situ, and recent studies have improved our knowledge of the patterns of genetic diversity conserved on-farm and the impacts of farmer seed exchanges, incorporation of new varieties, and phenotypic selection on genetic diversity for important crop species (Thomas et al., 2012).

The association of high-density genomic markers with phenotypic data is likely to be most immediately useful for plant breeders. However, the long-term creation and maintenance of genetic diversity for plant breeding will depend on better ties to on-farm and in situ conservation and selection. Because morphological descriptors, neutral genetic diversity, and geographic origin alone do not predict agronomic performance, it is imperative to move toward understanding and supporting the processes that create and maintain useful variation for functional traits and to develop strategies to identify and select valuable phenotypes.

LITERATURE CITED

Brown, A. H. D. 1989. Core collections: A practical approach to genetic resources management. *Genome* 31: 818–824.

Ebana, K., Y. Kojima, S. Fukuoka, T. Nagamine, and M. Kawase. 2008. Development of mini core collection of Japanese rice landrace. *Breeding Science* 58: 281–291.

Ellis, D. 2014. Could genebanks be a pot of gold at the end of the rainbow? *In* W. Tracy and M. Sligh [eds]. *In* Proceedings of the Summit on Seeds and Breeds for 21st Century Agriculture, March 2014, 129–142, Washington, D.C., USA. Rural Advancement Foundation International, Pittsboro, North Carolina, USA.

McCouch, S. 2013. Feeding the future. *Nature* 499: 23–24.

McKay, J., and R. G. Latta. 2002. Adaptive population divergence: Markers, QTL and traits. *Trends in Ecology & Evolution* 17: 285–291.

Moragues, M., J. Comadran, R. Waugh, I. Milne, A. Flavell, and J. Russell. 2010. Effects of ascertainment bias and marker number on estimations of barley diversity from high-throughput SNP genotype data. *Theoretical and Applied Genetics* 120: 1525–1534.

Reed, D. H., and R. Frankham. 2001. How closely correlated are molecular and quantitative measures of genetic variation? A meta-analysis. *Evolution* 55: 1095–1103.

Reeves, P. A., L. W. Panella, and C. M. Richards. 2012. Retention of agronomically important variation in germplasm core collections: Implications for allele mining. *Theoretical and Applied Genetics* 124: 1155–1171.

Spooner, D. M., and W. L. A. Hetterscheid. 2005. Origins, evolution, and group classification of cultivated potatoes. *In* T. J. Motley, N. Zerega, and H. Cross [eds.], Darwin's harvest: New approaches to the origins, evolution, and conservation of crops, 285–307. Colombia University Press, New York, New York, USA.

Swaminathan, M. S. 2009. Gene banks for a warming planet. *Science* 325: 517.

Tanksley, S. D., and S. R. McCouch. 1997. Seed banks and molecular maps: Unlocking genetic potential from the wild. *Science* 277: 1063–1066.

Thomas, M., E. Demeulenaere, J. C. Dawson, A. R. Khan, N. Galic, S. Jouanne-Pin, C. Remoue, C. Bonneuil, and I. Goldringer. 2012. On-farm dynamic management of genetic diversity: The impact of seed diffusions and seed saving practices on a population-variety of bread wheat. *Evolutionary Applications* 5: 779–795.

Aardevo Exhibit 1010
Page 0588

ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/322778347

The Evolution of Potato Breeding

Chapter *in* Plant breeding reviews · January 2018

DOI: 10.1002/9781119414735.ch4

CITATIONS
6

READS
2,970

2 authors, including:



Shelley H. Jansky
University of Wisconsin–Madison
134 PUBLICATIONS **2,422** CITATIONS

SEE PROFILE

Some of the authors of this publication are also working on these related projects:



Identification of genetic determinants of Verticillium wilt resistance in potato View project



Potato biodiversity View project

All content following this page was uploaded by Shelley H. Jansky on 30 November 2018.

The user has requested enhancement of the downloaded file.

4

The Evolution of Potato Breeding

Shelley H Jansky and David M Spooner
United States Department of Agriculture – Agricultural Research Service, and University of Wisconsin, Madison, WI

ABSTRACT

Potato cultivars worldwide are mostly tetraploid and clonally propagated. For over a century, the breeding strategy has been phenotypic recurrent selection. The polyploid nature of the crop, however, prevents breeders from eliminating deleterious alleles and assembling positive ones for the large array of traits necessary in a successful cultivar. Potato breeders have assumed that cultivars must be tetraploid, but some diploids produce tuber yields equivalent to tetraploid cultivars. In South America, most wild potato relatives are diploid and landraces exist at multiple ploidy levels, with no clear phenotypic distinction due to ploidy. The next step in the evolution of potato breeding may be to return to the diploid level and implement an inbred line based strategy. This will allow potato breeders to reduce the genetic load through inbreeding and then combine desirable traits through hybridization.

KEYWORDS: Cultivar; hybrid; inbred; landrace; polyploidization; polyploidy; *Solanum tuberosum;* tuber crop.

OUTLINE

ABBREVIATIONS
I. INTRODUCTION
II. CLASSIFICATION OF CULTIVATED POTATO
III. ORIGIN OF THE CULTIVATED POTATO
IV. DYNAMICS OF POTATO LANDRACE EVOLUTION
V. ORIGIN OF THE EUROPEAN POTATO
VI. NINETEENTH CENTURY POTATO BREEDING
VII. EARLY TWENTIETH CENTURY POTATO BREEDING

Plant Breeding Reviews, Volume 41, First Edition. Edited by Irwin Goldman.
© 2018 John Wiley & Sons, Inc. Published 2018 by John Wiley & Sons, Inc.

Aardevo Exhibit 1010
Page 0590

VIII. CONVENTIONAL POTATO BREEDING
IX. LATE TWENTIETH CENTURY POTATO BREEDING
X. TWENTY-FIRST CENTURY POTATO BREEDING
A. Is Tetraploidy Necessary for High Tuber Yield in Potato?
B. What Are The Advantages Of Moving to The Diploid Level and Developing Inbred Lines?
C. Is It Possible to Develop Diploid Inbred Lines in Potato?
XI. CONCLUSIONS
LITERATURE CITED

ABBREVIATIONS

DM	Doubled monoploid
FAO	Food and Agriculture Organization
RFLP	Restriction fragment length polymorphism
SNP	Single nucleotide polymorphism
SolCAP	Solanaceae Coordinated Agriculture Project

'...an ever-present interest in evolution – an ever-eager mind to peer backward and forward – is essential not only to the practice of plant improvement, but even to the barest understanding of it.'

Luther Burbank, quoted in Whitson *et al.* (1914)

I. INTRODUCTION

Potato is the third most important food crop worldwide. The Food and Agriculture Organization (FAO) of the United Nations declared 2008 the 'Year of the Potato' to highlight its value as a global food. The potato is a major player in addressing food security, because it is grown globally, it produces a large amount of food on a small amount of land, it is nutritious, and worldwide demand continues to grow. In the developing world, where more than half of the world's potato crop is harvested, its ease of cultivation and high energy production make it an important cash crop. Unlike grain crops, most potatoes are not traded globally, so prices received by farmers are not heavily influenced by factors outside the control of the local market. In addition to being a good source of energy, potatoes produce high-quality protein and are high in fibre, antioxidants, vitamin C and potassium (Kolasa, 1993; Camire *et al.*, 2009; Navarre, 2009).

Potato is considered to be superior to all other crops in terms of the wealth of its genetic resources and the ease with they can be introgressed

Aardevo Exhibit 1010
Page 0591

into cultivars (Ross, 1986). All wild and cultivated potatoes are classified in the genus *Solanum* L., which includes about 1,400 species (Anonymous, 2016). *Solanum* is one of the largest and most economically important flowering plant genera worldwide, and includes such foods as potato and tomato, lesser-known tropical fruits such as pepino and tree tomato, ornamentals such as *S. laxum* Spreng, and plants used in the pharmaceutical industry. Potato and its immediate tuber-bearing wild relatives are a subset of *Solanum*, classified as *Solanum* section *Petota* Dumort.

Following the latest comprehensive taxonomic conspectus by Spooner *et al.* (2007, 2014) and Ovchinnikova *et al.* (2011), potato includes:

(1) 107 wild species;
(2) four landrace (synonymous to native, ancestral, traditional, or indigenous) cultivated species; and
(3) modern improved cultivars grown worldwide.

All advanced cultivars of potato are collectively classified under the single species *S. tuberosum* L. The wild species of section *Petota* are broadly distributed in the American continent, from the southwestern United States to the Southern Cone of South America, and the landraces are found in the Andean mountains in South America, from western Venezuela south to northern Argentina. Then, after a 550 km gap, there are additional landraces in the lowlands of south central Chile (Hawkes and Hjerting, 1969, 1989; Ochoa, 1990, 1999; Spooner *et al.*, 2014).

Spooner *et al.* (2007, 2014) and Ovchinnikova *et al.* (2011) more than halved the number of potato species from the 228 wild species and seven cultivated species recognized by Hawkes (1990) to the 107 wild and four cultivated landrace species mentioned above, using data from field studies and integrated morphological and molecular studies, as reviewed in Spooner *et al.* (2014) and Spooner (2016).

II. CLASSIFICATION OF CULTIVATED POTATO

Ploidy has historically been an important criterion for the classification of cultivated potato. Rybin (1929, 1933) first determined that cultivated potatoes exist in a polyploid series that includes diploid ($2n = 2x = 24$), triploid ($3x$), tetraploid ($4x$), and pentaploid ($5x$) forms. Wild potatoes exist at all of these levels, in addition to hexaploid ($6x$). Early taxonomic treatments by Russian taxonomists recognized anywhere from

13 to 18 cultivated potato species (Juzepczuk and Bukasov, 1929; Bukasov, 1937, 1978; Lekhnovich, 1971). They and others, continuing up to Hawkes (1990), used ploidy as a major taxonomic character, with each ploidy level characteristic of a particular species or group of species.

In recent years, the use of ploidy as a sole taxonomic criterion has been abandoned by some taxonomists. The widely used taxonomy of Hawkes (1990) recognized seven cultivated species and seven subspecies: *S. ajanhuiri* Juz. and Bukasov (2*x*), *S. chaucha* Juz. and Bukasov (3*x*), *S. curtilobum* Juz. and Bukasov (5*x*), *S. juzepczukii* Juz. (3*x*), *S. phureja* Juz. and Bukasov subsp. *estradae* (L. López) Hawkes (4*x*), *S. phureja* subsp. *hygrothermicum* (Ochoa) Hawkes (4*x*), *S. phureja* subsp. *phureja* (2*x*), *S. stenotomum* Juz. and Bukasov subsp. *stenotomum* (2*x*), *S. stenotomum* subsp. *goniocalyx* (Juz. and Bukasov) Hawkes (2*x*), *S. tuberosum* subsp. *andigenum* Hawkes (4*x*), and *S. tuberosum* subsp. *tuberosum* (4*x*).

Based on morphological (Huamán and Spooner, 2002; Gavrilenko *et al.*, 2010) and molecular (Spooner *et al.*, 2005, 2007) data, Spooner *et al.* (2007) and Ovchinnikova *et al.* (2011) also recognized *S. ajanhuiri*, *S. curtilobum*, and *S. juzepczukii* as distinctive cultivated-wild species hybrids adapted to colder climates in the high Andes. Ovchinnikova *et al.* (2011) compiled all 602 basionyms (original names) of cultivated potatoes, located their type specimens, and placed these names in synonymy with this new classification. However, they combined all of the diploids, triploids and tetraploids (*S. chaucha*, *S. phureja* subsp. *phureja*, *S. stenotomum* subsp. *stenotomum*, *S. stenotomum* subsp. *goniocalyx*, and *S. tuberosum*) into a single species, *S. tuberosum*, divided into two 'cultivar groups', following a taxonomic philosophy similar to Dodds (1962).

The Andigenum group contains all cultivated potatoes at diploid, triploid, and tetraploid levels, from the high Andes through Venezuela and Argentina. The second group of cultivated potatoes, the Chilotanum group, is comprised of tetraploids in lowland south-Central Chile.

Classifications of potato landraces as distinct species versus cultivar groups reflect differing hypotheses about their evolutionary dynamics. The cultivar group hypothesis, described above and adopted here, was presented by Ugent (1970). It postulates that extensive gene flow occurs within and among ploidy levels of cultivated and wild species, preventing the maintenance of distinct cultivated species. Conversely, Hawkes (1990) postulated that gene flow could lead to the production of hybrids, but that the disruption of adaptive gene complexes ('hybrid

breakdown') would cause sterility and maintain species identity. Regardless, it is clear that ploidy is not a static feature that can be used to place cultivated potato groups into distinct and stable categories.

'In order to move forward a little, we must work backward ages and ages'
(Whitson *et al.*, 1914).

III. ORIGIN OF THE CULTIVATED POTATO

As discussed above, cultivated potato is divided into two groups, the Andigenum group in the high Andes of northern and central South America, and the Chilotanum group in the lowlands of southern Chile. The Andigenum group contains a range of ploidy levels, from diploid to tetraploid, while all members of the Chilotanum group are tetraploid. Polyploid wild and cultivated potato species are thought to have arisen from sexual polyploidization facilitated by $2n$ gamete production (Camadro and Peloquin, 1980; Iwanaga and Peloquin, 1982; Watanabe and Peloquin, 1991; Carputo *et al.*, 2003; Spooner *et al.*, 2010).

Based on ease of crossing ability, morphological similarity, shared distributions, and population dynamics in Andean fields (Dodds, 1962; Ugent, 1970; Huamán, 1975; Grun, 1990; Rabinowitz *et al.*, 1990; Spooner *et al.*, 2007), it appears that the Andigenum group of cultivated potato had its origin somewhere in a group of wild species informally referred to as the '*Solanum brevicaule* Bitter complex', widely distributed from southern Peru south throughout Bolivia to northern Argentina.

Because members of the *Solanum brevicaule* complex and potato landraces in the Andigenum group co-exist extensively, and because they intercross so easily, the prevailing opinion until recently was that cultivated potato in the Andigenum group had many and separate origins (Brucher, 1964; Ugent, 1970; Bukasov, 1978; Grun, 1990; Hawkes, 1990; Ochoa, 1990, 1999; Huamán and Spooner, 2002). The molecular study by Spooner *et al.* (2005), however, supported a single origin of the Andigenum group in southern Peru and immediately adjacent northern Bolivia. The progenitors originated from northern representatives of the *S. brevicaule* group, all of which were diploid in this region. This is in contrast to wild relatives, which are diploid and polyploid in the southern range of the *Solanum brevicaule* complex in Bolivia and Argentina. Hence, it is likely that the first cultivated potatoes were diploid, and the triploid and tetraploid members of the Andigenum group arose later.

While the first cultivated potatoes were likely members of the Andigenum group in the Andes of equatorial South America, there is a long history of disagreement about whether the Chilotanum group evolved from Andigenum or arose independently. This is discussed at length in Spooner *et al.* (2014), and the following provides an abstracted summary of these ideas.

The 'Russian School' (Juzepczuk and Bukasov, 1929; Bukasov, 1933, 1938, 1939; Vavilov, 1935, 1939; Juzepczuk, 1937) developed the multiple origin hypothesis, in which the Chilean and Andean landraces evolved independently of each other from indigenous wild species. The Russian scientists based their multiple origin idea on morphology, chromosome numbers, geography, and the physiological characters of frost tolerance, photoperiodic response, plant maturity and tuber dormancy. They proposed that the Andigenum group arose from diploid wild species in the Andes, while the Chilotanum group arose from tetraploid wild species in Chile (*S. fonckii* Phil. ex Reiche, *S. leptostigma* Juz., and *S. molinae* Juz.). Alternatively, Ugent *et al.* (1987) also proposed a Chilean origin of the Chilotanum group, but from the diploid wild species *S. maglia* Schltdl. *Solanum maglia* is currently found in coastal Chile and a few other nearby places inland and in a single valley in Argentina. It is, however, not found within 1,000 km of Chiloé Island, where the Chilotanum landraces grow today.

The 'English School' (Salaman, 1946; Hawkes, 1956, 1990; Simmonds, 1964) advanced a restricted origin hypothesis, proposing that potato domestication took place in the South American Andes of Peru and Bolivia, originally from diploid wild species (in agreement with the Russian school), followed by polyploidization to produce the range of ploidy levels in modern cultivated potato. Landraces then expanded into new ecological conditions north to Columbia and Venezuela and south to coastal Chile. The first landraces would have been adapted to a short day photoperiod, and then would have had to adapt to longer photoperiods as they moved north and south. However, there are no well-documented data supporting these movements.

The main contradiction between the multiple and restricted origin hypotheses concerns the origin and taxonomic status of tetraploid Chilean landraces. Unlike the Russians, who considered independent origins of tetraploid Chilean and Andean landraces in separate regions, the English school proposed that Andigenum group potatoes were transported by migrating humans to Chile and then evolved into Chilotanum group potatoes, which are adapted to produce tubers under the long photoperiods of southern Chile.

Aardevo Exhibit 1010
Page 0595

The restricted origin hypothesis proposed by the English group is based on two facts:

(1) At present, in the region of southern and central Chile, all the landraces are tetraploid, and there are no wild species from which *S. tuberosum* could have originated (Hawkes, 1944, 1956). Rather, the English scientists suggested that the putative wild species progenitors proposed by the Russians (*S. fonckii*, *S. leptostigma*, *S. molinae*) are simply wild escaped forms of the Chilotanum group.
(2) Long-term selection experiments were reported to have rapidly adapted Andean tetraploids to a Chilotanum-like form (referred to as Neo-Tuberosum), especially regarding adaptation to long photoperiods (Salaman, 1946; Simmonds, 1966, 1969; Rasco *et al.*, 1980).

As discussed above, Andigenum potatoes, which grow near the equator, produce tubers under a short (12 hour) photoperiod, while Chilotanum potatoes are adapted to tuberize under the longer photoperiods of temperate latitudes (Mendoza and Haynes, 1976; Kittipadakul *et al.*, 2012; Sarkar, 2010). Neo-Tuberosum refers to potato adapted to long photoperiods, and a syndrome of related morphological and physiological traits developed by recurrent selection of short day-adapted potatoes of the Andigenum group. Based on these hypotheses, Hawkes (1956) grouped all tetraploid potatoes (Chilean and Andean) under *S. tuberosum*, considering this species to be a complex of polymorphic forms. Grun (1990) proposed a modified scenario, whereby the Chilean landraces originated from hybridizations of Andean landraces with a wild species, possibly *S. chacoense* Bitter.

Recent studies investigated points in the restricted origin hypothesis. In one study, Ghislain *et al.* (2009) demonstrated, with nuclear microsatellite DNA markers, that Neo-Tuberosum germplasm is related to the Chilotanum group, not the Andigenum group. This is significant, because one of the arguments of the restricted origin hypothesis is based on the results of Simmonds (1966), who developed Neo-Tuberosum. The rapid selection of Neo-Tuberosum from putative Andigenum group germplasm suggested that this process could occur naturally to produce Chilotanum group germplasm. Ghislain *et al.* (2009) interpreted their unexpected result to be caused by strong rapid selection against the original Andigenum clones, after unintended hybridization with Chilotanum group germplasm that occurred in nearby experimental fields during the development of Neo-Tuberosum. This result did not discount the idea of artificial selection of Andigenum group germplasm over longer times, but questioned another hypothesis,

namely that the European potato was derived from the Andigenum group (Salaman, 1937). Instead, Ghislain's study supported an alternative and earlier hypothesis that the European potato was derived from the Chilotanum group (Juzepczuk and Bukasov, 1929).

Other studies have addressed the hypothesis of Ugent *et al.* (1987), that the Chilotanum group arose from the diploid wild species *S. maglia* Schltdl. Rodríguez *et al.* (2010), using DNA sequence data of the *waxy* gene, found that two of three *S. maglia* accessions had alleles grouping with both *S. maglia* and a clade containing the Andigenum group and related wild species. This supported *S. maglia* as a hybrid contributor to the Chilotanum group.

A study by Spooner *et al.* (2012), using nuclear microsatellites, also grouped *S. maglia* with the Chilotanum group. These results were, however, ambiguous, because *S. maglia* lacked the 241 bp plastid deletion shared by most accessions of the Chilotanum group. Hence, the involvement of *S. maglia* in the origin of the Chilotanum group, and a possible independent origin of this germplasm, remains unresolved.

IV. DYNAMICS OF POTATO LANDRACE EVOLUTION

Landraces evolved after selection among cultivated potatoes for specific environments and uses by native farmers in the Andes. Landrace potato fields in South America are markedly different from most commercial fields worldwide. A typical commercial potato field in Europe or North America, for example, contains a single cultivar, selected for adaptation to the local environment and suitable for the regional market. In contrast, a single landrace potato field in South America contains multiple cultivars of different cultivated species and ploidy levels (Ochoa, 1958; Huamán, 1975; Jackson *et al.*, 1980; Brush *et al.*, 1981; Johns and Keen, 1986; Johns *et al.*, 1987; Quiros *et al.*, 1990, 1992; Zimmerer, 1991; de Haan *et al.*, 2010).

For example, in a well-documented study of potato cultivar diversity in central Peru, de Haan *et al.* (2010) showed that, at the regional level, diploid, triploid, and tetraploid members of the Andigenum group were present while, at higher altitudes, the frost-resistant pentaploids were also present. Morphological characterization of landraces in this study documented 557 unique cultivars, while simple sequence repeat (SSR) genotyping identified 406 unique cultivars. This pattern of high regional and intra-field potato landrace diversity in the Andes matches that of earlier studies (Del Rio *et al.*, 1997; Ochoa, 1999).

Aardevo Exhibit 1010
Page 0597

The first potato breeders were likely Andean farmers, and they continue their work today. They harvest open-pollinated berries from landraces and sow the seed, often to improve tuber yield when tuber 'degeneration' occurs, likely due to virus infection (Quiros *et al.*, 1992). They then select for tuber size, number, and quality. These farmers maintain high landrace diversity in native fields for at least three reasons:

(1) to enhance their diet by providing a mixture of flavours, textures, shapes and colours;
(2) to assure the survival and harvest of at least some plants in case of disease, pests, or abiotic stress; and
(3) to employ specific cultivars for consumption in different potato foods and preparations, or for ceremonial rituals (Quiros *et al.*, 1992).

de Haan *et al.* (2010) also pointed out that indigenous farmers typically prefer cultivar mixtures for home consumption, as these are associated with quality traits and long-established culinary preferences. They concluded that the indigenous food system is at the heart of farmer-driven conservation of native germplasm.

In addition to sowing open-pollinated berries to rejuvenate the tuber crop, indigenous farmers have established production practices that allow for the evolution of landraces. In the Peruvian Andes, a potato crop is typically followed, in the next production year, by another potato crop (Brush *et al.*, 1981; Jackson *et al.*, 1980). Some seeds produced by open-pollination of the potato landraces will germinate. Tubers from those potato seedlings will be harvested, along with the second tuber crop, separated from the crop, selected, and saved for propagation (Jackson *et al.*, 1980). In Western Bolivia, the potato crop is often followed by a crop other than potatoes. In the second year of that production system, volunteer potatoes from the previous year are harvested and selected as well. Consequently, new genotypes are constantly introduced into the landrace system and selected by indigenous farmers (Johns and Keen, 1986; Quiros *et al.*, 1992).

The ethnobotanical practice of collecting open-pollinated seed from landraces does not simply maintain potato landrace diversity, but also increases it. For example, Watanabe and Peloquin (1989, 1991) showed that $2n$ gametes are common in South American wild and cultivated species, allowing gene transfer from diploid to tetraploid cultivars within fields, and between tetraploid cultivars and diploid wild potato species growing adjacent to farmers' fields. In addition, Huamán (1975)

showed evidence of natural crosses between the diploid wild species *S. boliviense* Dunal (as *S. megistacrolobum* Bitter) and the diploid members of the Andigenum group. Rabinowitz *et al.* (1990), Celis *et al.* (2004) and Scurrah *et al.* (2008) documented gene flow in the Andes between diploid wild species and potato cultivars. Lawson (1983) documented multi-year survival of botanical seed of members of the *S. tuberosum* Chilotanum group and germination of these to form weed potatoes in the UK, and these almost certainly occur in the landraces of the Andean fields as well.

Hence, natural gene flow occurs among wild and cultivated species wherever they grow together. Adding to this natural gene flow, Quiros *et al.* (1992) documented intentional crosses by Andean farmers who plant botanical seed not only to rejuvenate the tuber seed system as described above, but also to generate new genetic combinations, with the purpose of selecting new landraces.

Hybridization in landrace cultivars is probably not only likely but necessary, for the vitality of the potato crop in the long term. According to Grun (1990), the Andigenum group acts like a '*genetic sponge, absorbing genes via introgression from any wild or cultivated species with which it hybridize[s].*' This has allowed it to expand to include a continuous array of phenotypic and genotypic variants through hybridization, recombination, and segregation. Similarly, Quiros *et al.* (1992) have suggested that '*the Andean potato is a large and plastic gene pool being increased and renovated by outcrossing.*'

V. ORIGIN OF THE EUROPEAN POTATO

Landrace potatoes are clearly indigenous to South America. However, in 1567, the potato appeared in the Canary Islands, off the northwestern coast of Africa (Hawkes and Francisco-Ortega, 1993; Ríos *et al.*, 2007) and, in 1573, it appeared in continental Spain (Hawkes and Francisco-Ortega, 1992; Romans, 2005). The potato then spread throughout Europe and worldwide. It was slow to be adopted as a major food crop until about 100 years later and, in some European countries, it was rejected as an acceptable food well into the late 1700s (Ames and Spooner, 2008).

The Russian school suggested an origin of the 'European' potato from lowland Chile, based on morphological similarity and long-day adaptation of both germplasm sets. However, an alternative Andean origin hypothesis became widely accepted. Although all modern potato cultivars have predominantly Chilean germplasm, the English school

suggested that these cultivars originated from breeding with Chilean landraces, subsequent to the late blight epidemics of the mid-19th century. Additionally, they pointed to the ability of Andigenum group germplasm to adapt rapidly to long days in the Neo-Tuberosum experiments, and suggested that this rapid selection could have occurred after transport of the Andigenum group to Europe.

There were problems with these arguments, however. As discussed above, the nuclear microsatellite and plastid deletion data of Ghislain *et al.* (2009) showed that Neo-Tuberosum was incorrectly interpreted to have an Andigenum group origin; rather, it was of Chilotanum group origin. Separately, plastid DNA data from herbarium specimens dating back to the early 16th century provided the first direct evidence of these introductions, documenting that, although the Andigenum group was predominant in the 1700s, Chilotanum group germplasm was introduced into Europe as early as 1811, and was well established in Europe long before the late blight epidemics (Ames and Spooner, 2008). As detailed by Hawkes (1967, 1990), conflicting and fragmentary reports make it difficult to date the first introduction of potato in various countries, with a likely date of 1590 for England and the early 1600s for North America. By the late 1600s, potato had reached a near-worldwide distribution.

> 'The whole life history of a plant is stored away in its seeds. If we plant enough of the seeds, in enough different environments, we are sure to have that life history with all of its variations, all of its hardships, all of its improvements and retrogressions, uncovered before us.'
>
> Luther Burbank, quoted in Whitson *et al.* (1914).

VI. NINETEENTH CENTURY POTATO BREEDING

The potato spent more than a century on the European continent before any thoughts of breeding were suggested. In 1807, the Englishman Thomas Andrew Knight proposed making cross-pollinations and, in 1810, he said that he had successfully done so in potato (Glendinning, 1983). However, his efforts apparently did not gain widespread acceptance in Europe or North America. There was no serious effort to breed improved potato cultivars until the mid-1800s. The potato crop at that time was propagated by retaining a few tubers at harvest for use as seed in the following growing season. Since the seed and food crop were the same entity, most farmers used the inferior tubers as seed, saving the best for food. Tuber yields were typically low until improved cultural practices were implemented in the 1830s. These practices included the use of

high-quality seed and planting early to extend the growing season. They resulted in tuber yield increases of up to eightfold (Glendinning, 1983).

In Europe and North America, the late blight epidemics of the mid-1840s accelerated an interest in potato breeding. The scientific basis for these breeding efforts was not sound, but the outcomes were positive and had a major impact on genetic improvement strategies for decades to come. During the epidemics, the prevailing idea was that centuries of asexual propagation had led to poor vigour and, consequently, susceptibility to the late blight oomycete pathogen (Goodrich, 1863). Potato specialists thought they could restore the vigour that was lost due to centuries of asexual propagation by returning to the home of the potato and collecting sexually propagated wild potatoes.

Consequently, the Reverend Chauncey E. Goodrich of Utica, NY, imported potatoes from Chile in 1851. For 20 years, he practised potato breeding by planting alternate rows of US-grown cultivars with the Chilean cultivars, and then collecting the open-pollinated berries and growing the seeds (Dodge, 1970). These berries likely contained a large proportion of self-pollinated seeds, as studies have shown that selfing is common in tetraploid potato (Glendinning, 1976; Brown, 1993; Simmonds, 1997).

Most new genetic combinations were inferior to cultivars, but some were not. The best seedlings were probably derived from heterozygous plants. Goodrich selected the best plants and repeated the process year after year. The most well-known cultivar he selected was 'Garnet Chili,' an open-pollinated offspring of one of his original importations, 'Rough Purple Chili.' An open-pollinated offspring of 'Garnet Chili' was selected by Albert Breese, and released as 'Early Rose' in 1867. Breese also selected 'Early Ohio' from a population of 'Early Rose' open-pollinated seeds.

Luther Burbank's claim to fame in potato also resulted from selection in an open-pollinated population. He found a berry on an 'Early Rose' plant in his mother's potato patch and extracted seeds, of which 23 grew into seedlings in 1871. Most were not adapted or were of poor quality, but one was superior to the others in yield and had large, smooth, white tubers. The 'Burbank Seedling' was released by a seed company in 1876 (Bethke *et al.*, 2014). The name was shortened to 'Burbank', and a russet sport, 'Russet Burbank,' became the predominant potato cultivar in the US and Canada in the mid-20th century; it continues to be the most important cultivar today, occupying 22% of certified seed acreage in 2014. The Chilean cultivar 'Daber', introduced into Europe in 1830, is a prominent parent of many European cultivars (Glendinning, 1983).

Aardevo Exhibit 1010
Page 0601

Thus, while most northern hemisphere breeders in the mid-19th century did not make controlled pollinations, they had the insight to realize that potato seeds are genetically different from each other; in order to make gains, it is necessary to introduce new germplasm, generate seeds, and evaluate segregating populations. They also observed a phenomenon that their successors in potato breeding would quickly learn – the vast majority of seedlings in a hybrid potato population are inferior to their parents.

> 'But plant potato seeds, and you tap a mine of heredity, infinite in its uncertainly, but infinite, too, in its possibility.'
>
> (Whitson *et al.*, 1914)

While most potato improvement efforts of the 19th century were based on open-pollinated seeds, some breeders were making crosses. The first European to embark on a systematic effort to hybridize potatoes for cultivar development was probably William Patterson in Dundee, Scotland. His best known cultivar, 'Victoria,' was released in 1856, 30 years after his first attempts to breed potatoes (Stuart, 1928). In the Netherlands, Geert Veenhuizen initiated a successful potato breeding programme that began to release cultivars in 1888 (de Haan, 1958). One of his most notable releases was 'Eigenheimer', which was a significant cultivar for both human consumption and industrial uses. As late as the 1950s, cultivars released by Veenhuizen occupied over half of the potato production land in the Netherlands.

The first North American known to breed potatoes through hybridization was Cyrus Guernsey Pringle, of Charlotte, Vermont (Stevenson and Clark, 1937; Dodge, 1970). He was interested in hybridizing many types of plants, including potatoes. In 1876, he crossed 'Ruby' ('Early Rose') with 'Excelsior', and selected 'Snowflake' from that population.

It is interesting to note that Pringle made numerous attempts to cross potato cultivars with *S. fendleri* A. Gray (= *S. stoloniferum* Schltdl.), a wild species found in the southwest US, which is the northern edge of the distribution of wild *Solanum* species on the North American continent. Although he expressed confidence that he would eventually succeed, he likely never did, since *S. fendleri* is not sexually compatible with cultivated potato. Perhaps his work was inspired by the observation that another wild potato species native to the Southwest US, *S. jamesii* Torr., was used by native people as a food crop. In fact, in southern Utah, the Escalante Valley was known as 'Potato Valley' when it was settled by Europeans in 1875 (Louderbeck, 2014).

The importance of wild potato as a food crop for native North Americans of the southwest US is well documented and discussed by

Bamberg *et al.* (2003). Back on the East Coast, as potato breeding efforts expanded in the late 1800s, North American breeders focused on appearance, quality, and yield. In contrast, European breeders added disease resistance and starch content to their lists of traits of interest (Stuart, 1928).

> 'But years of cultivation have removed from the potato the necessity of bearing seeds for the preservation of its race. The potato plant, so certain, now to reproduce itself through subdivision of its bulb or tuber – so reliant on man for its propagation – has little use for the seed upon which its ancestors depended for perpetuation before men relieved it of this burden.'
>
> (Whitson *et al.*, 1914)

A serious limitation for potato breeders was, and to some extent continues to be, low male fertility (Krantz, 1924; Stevenson and Clark, 1937). Ovule abortion is rare, but the production of inviable pollen is common (Krantz, 1946). Krantz (1924) reported that most cultivars did not produce viable pollen, and those that were male-fertile were of poor commercial quality. Therefore, potato breeders were left with no option but to use inferior male parents in their crossing blocks: '*A further drawback to progressive work in potato breeding is due to what might be termed the irony of fate, in that the few varieties which do produce ample viable pollen are more or less unsatisfactory from a commercial standpoint, with the result that the seedlings derived from the use of such pollen inherit many of the undesirable characteristics of their male parent*' (Stuart, 1928).

Krantz (1924) suggested that potato breeders face two problems. First, the few male fertile parents available carry dominant undesirable traits; and, second, undesirable recessives hidden in the parents are revealed in offspring. However, in large populations generated by self-pollinating clones with low male fertility, some fertile offspring can be observed. In fact, male fertility was reported to increase markedly, following self-fertilization of nearly male-sterile clones for two or three generations (Krantz, 1929).

Poor fertility is likely due to a number of factors, including: 1) polyploidy; 2) selection of clones with poor berry set; 3) cytoplasmic-genetic interactions; and 4) inbreeding depression. An expansion of each of these points is important.

(1) Poor fertility in polysomic polyploids is well-documented, and due to complications associated with pairing and segregation of multiple sets of chromosomes (Comai, 2005; Otto, 2007). The negative effect of polyploidy on fertility in potato was demonstrated

by Rowe (1967a), who observed that somatic doubling of fertile diploid potato clones produced tetraploids with significantly reduced fertility.

(2) Selection for lack of berry production was carried out by early potato breeders, because some evidence pointed to competition for resources between berries and tubers (Knight 1807). Subsequent research has supported this observation (Bartholdi, 1942; Jansky and Thompson, 1990). Bartholdi (1942) suggested an interesting paradox: '*If ... competition exists in the potato between fruit formation and tuber development then non-fruitfulness would be a desirable character to retain in the breeding of improved varieties.*' So, breeders likely selected against highly fertile plants, either intentionally or unintentionally.

(3) Cytoplasmic-genetic male sterility may have been introduced into Northern Hemisphere germplasm when sensitive cytoplasm from 'Rough Purple Chili' and other South American landrace germplasm interacted with dominant genes from European and North American cultivars (Bradshaw and MacKay, 1994).

(4) Inbreeding resulting from crosses between related parents, or from self-pollination during the production of open-pollinated seed, can reveal recessive alleles that inhibit normal gametophyte development.

Despite the challenges resulting from low fertility in parental clones, potato breeders in Europe and North America in the latter half of the 19th century were very active. US breeders released a staggering 350 cultivars in that period (Hougas and Ross, 1956). There was then a notable lull in the development of new North American cultivars for about 40 years, beginning in 1890, while improved cultivars continued to be released in Europe. The difference was that, during this time period, North American potato breeders changed their emphasis from sexual breeding to clonal selection, while Europeans continued to make crosses (Krantz, 1924). It is likely that clonal selection mainly identified plants with lower levels of tuber seed-borne diseases. Selections were never higher yielding than the same cultivar grown from clean tuber seed (Krantz, 1924). Then, in the 1930s in the US, there was a shift from private to public efforts, and potato breeding moved in a new direction (Hougas and Ross, 1956).

> 'It is not too much to say that the introduction of the variety Katahdin marks the beginning of a new era in potato breeding.'
>
> (Reddick, 1940)

VII. EARLY TWENTIETH CENTURY POTATO BREEDING

In 1910, the US Department of Agriculture (USDA) initiated a potato breeding programme with a focus on breeding for virus resistance. Then, in 1929, a National Potato Breeding Programme was established in the US. This movement toward a new, concerted potato breeding effort was critical in eliminating the stagnation that had occurred during the previous decades of clonal selection. One impetus for developing a national effort was that, while breeders in many states were interested in the genetic improvement of potato, most could not generate true seed (Stevenson and Clark, 1937). Only researchers in cool locations were consistently successful at true seed production.

After the implementation of the national breeding effort, a handful of programmes produced true seed and seedling populations for all members of the programme to evaluate. Each project then focused on selecting for traits relevant to its needs. The transition from individual breeding efforts to a national effort had a synergistic effect, leading to the production of dozens of improved potato cultivars. As noted by Stevenson and Clark (1937), '*The potato-breeding project as it now stands and the results up to the present time demonstrate clearly the necessity and the advantages of cooperation in scientific enterprises.*' The release of the full-sib cultivars 'Katahdin' and 'Chippewa', in 1933, indeed marked the beginning of modern potato breeding in the US.

The University of Minnesota potato breeder Frank Krantz was influenced by his corn-breeding colleagues, who were spearheading a revolution by developing inbred lines and then hybridizing the inbreds (East, 1908, 1909; Shull, 1909, 1946). Krantz followed their lead by self-pollinating potatoes with the hope of removing deleterious alleles and assembling positive ones in inbred lines (Krantz, 1924). '*The desirable features of our commercial varieties must be separated from the undesirable; then, by a building-up process individuals of the desirable types must be constructed. Inbreeding is the genetic method of analysis. It exposes characters and shows in what degree of purity these characters exist within the individual, thus enabling a selective process to separate the desirable elements into pure line groups. By crossing, these groups may then be split up and recombined to meet specific requirements.*' (Krantz, 1929).

Krantz observed tremendous segregation for vine and tuber traits in S_1 populations, with many off-types produced, as expected. He was, however, surprised at the ease with which self-pollination revealed undesirable traits, which could then be selected against. While self-pollination of a diploid easily reveals harmful alleles by the production

of homozygous recessive individuals, those alleles are not so readily removed in tetraploids. For example, self-pollination of a triplex (AAAa) plant will not reveal any recessive phenotypes, unless the rare products of double reduction in both male and female gametes unite at fertilization.

Krantz (1924) was encouraged by this new direction in potato breeding, and reported that S_1 plants were better as parents than the original clones. However, by the mid-20th century, his enthusiasm was dampened by observations of the effects of further inbreeding on parental value. Crossing partial inbreds to cultivars did not, in general, lead to high-yielding offspring (Krantz, 1946). Large differences in combining ability were observed among parents, and these masked the effects of inbreeding. In addition, comparisons of cultivar × F_1 versus cultivar × inbred clone (S_1, S_2, S_3, S_4) families revealed no effect of inbreeding on the performance of progeny (Mullin and Lauer, 1966).

During inbreeding of tetraploid cultivars, the decline in vigour was greatest in the S_1 and S_2 generations, and was smaller in S_3 and S_4 populations (Krantz, 1929). S_3 and S_4 populations began to become fixed for some traits and revealed some inter-line differences. The decline in vigour varied, however, among lines, even from the same cultivar. By necessity, only the most vigorous, fertile plants were carried to the next generation. Consequently, there was likely a bias toward heterozygous individuals during selection. Yield and vigour continued to decrease through six generations of self-pollination. Mean S_6 yields were 19% of those of the initial heterozygous parents (Krantz, 1946). F_1 seedlings (from crossing two heterozygous tetraploid parents) were more vigorous than those from self-pollinating either parent (Krantz, 1924). However, it is interesting to note that an S_3 population from a Mexican cultivar (name unknown) was as vigorous as a typical F_1 population (Krantz, 1924).

In Europe, researchers were also interested in the concept of developing inbred lines in potato. Feistritzer (1952) discussed the possibility of self-pollination for genetic improvement in potato, and Engel (1957) observed inbreeding depression upon selfing and heterosis, following outcrossing in his programme. Rudorf (1958) was able to self some tetraploids to the S_5 generation, but sterility and poor vigour prevented many lines from advancing beyond the S_2 generation.

While some potato breeders were self-pollinating their tetraploid germplasm, others were looking outward and introducing exotic germplasm into their programmes. Genetic variability among 20th century cultivars was low, as evidenced by low estimates of genotypic variance in tuber yield trials (reviewed by Mendoza and Haynes, 1974a).

Prior to the late blight epidemics of the mid-1800s, two genetic bottlenecks reduced the genetic base of potato outside its home territory – transport of a limited number of clones from South America to Europe and North America, and the loss of many of those clones due to poor adaptation and farming practices. However, the genetic base of potato after the late blight epidemic became even smaller, as many cultivars were destroyed by the disease. '*... there can be little doubt that Tuberosum (S. tuberosum* Chilotanum group*), the European (and North American) cultivated form of potato, is founded on a narrow genetic base relative to that available in its centres of origin and that, during its evolution in Europe up until the twentieth century, this genetic base will have been further eroded.*' (Bradshaw and MacKay, 1994).

The potato germplasm resource, however, is extensive, and includes both cultivated and wild relatives. This germplasm is readily available to breeders through genebanks worldwide (Jansky *et al.*, 2013). The infusion of germplasm from cultivated relatives has resulted in gains in total tuber yield (Glendinning, 1969; Haynes, 1972; Mendoza and Haynes, 1974a; Tarn and Tai, 1977; Sanford and Hanneman, 1982a). However, unacceptable tuber type (and consequently low marketable tuber yield), short tuber dormancy, and late vine maturity have limited the use of cultivated relatives for cultivar development (Jansky and Peloquin, 2005).

Efforts have also been made to use wild relatives in breeding programmes, mainly as sources of resistance to late blight, viruses, and nematodes (Bradshaw and Ramsay, 2005; Bradshaw, 2009). The greatest impact on cultivar development resulted from the use of the 'W races' from Germany, carrying late blight resistance derived from *S. demissum* Lindl. (Ross, 1986). In work parallel to European introgression efforts, Donald Reddick at Cornell University in the US began using *S. demissum* as a source of late blight resistance (Stevenson and Clark, 1937). However, he encountered challenges when working with this new germplasm, saying '*Owing to sterility, incompatibility, etc., the results thus far have been simply what could be got, not what it was planned to get*' (Stevenson and Clark, 1937).

Some successes on both continents were realized, however, and resistance was incorporated into cultivated germplasm. Resistance was often lost in subsequent backcrosses, though. In clones that retained resistance genes from *S. demissum*, the pathogen eventually overcame the *R* genes. Nonetheless, it is interesting to note that a heterotic benefit from the influx of new genes was observed in breeding clones derived from *S. demissum* hybrids (Ross, 1986). In spite of this observation,

Aardevo Exhibit 1010
Page 0607

potato breeders then and today use wild germplasm as donors of specific genes, usually for host plant resistance to pathogens rather than for base broadening. Hybridization of a wild species clone to a cultivated one is typically followed by several generations of backcrossing to cultivated germplasm. Consequently, the contribution of wild germplasm to broadening the genetic base of potato is likely small (Mendoza and Haynes, 1973; Brown, 2011).

Despite decades of breeding with wild germplasm, only a few species are found in the pedigrees of modern cultivars (Ross, 1986; Plaisted and Hoopes, 1989; Hawkes, 1990, 1994; Bradshaw, 2009). The most common are *S. acaule* Bitter, *S. demissum*, *S. spegazzinii* Bitter (= *S. brevicaule*), *S. stoloniferum*, and *S. vernei* Bitter and Wittm (Ross, 1986). All of these species were introduced with the goal of incorporating specific disease resistance genes.

Wild germplasm introduces negative traits, such as late maturity and extensive stolon development. If a wild species is crossed to a cultivar to introduce disease resistance, then rigorous selection for maturity and tuber yield in early clonal generations may eliminate resistant individuals; host plant resistance trials are not initiated until sufficient seed is generated, several years after the first selection cycle. Landrace germplasm may also be selected against because it introduces rough tuber shape and cytoplasmic-genetic male sterility (De Jong and Tai, 1977; Sanford and Hanneman, 1982a; Jansky and Peloquin, 2005).

The challenge of introgressing exotic germplasm into cultivars was eloquently stated by Bonierbale *et al.* (1993): '*In the development of modern cultivars, unadapted germplasm has often been used as a source for adding desirable qualitative traits, but it has been used less commonly for the improvement of quantitative characters. Genes for lack of adaptation often interfere with good performance in crosses between adapted and unadapted clones, thus creating problems in identifying superior recombinants; and while genetic variability is enhanced, mean performance is reduced.*' It is clear that potato breeders need a strategy to separate the positive from the negative alleles when introgressing wild germplasm.

It is encouraging to note, however, that since the mid-20th century, potato breeders have added new alleles to cultivars without a loss of existing alleles (Vos *et al.*, 2015). These new alleles are probably from introgressions of wild relatives. However, beneficial alleles introduced into modern cultivars have not become fixed, because breeders have not been able to effectively select against their deleterious counterparts (Vos *et al.*, 2015).

> '... the plant-breeder's ideal of breeding high-yielding potatoes resistant to disease and frost, rich in protein and vitamins, cannot be expected to be successfully achieved without proper understanding of the genetical basis of the characters involved.'
>
> (Pal and Pushkarnath, 1951)

Breeders were four decades into the 20th century before they realized that potato cultivars are tetrasomic tetraploids (Cadman, 1942). However, this knowledge did not affect their breeding strategy: '*Even then, potato breeding continued to remain empirical and unsophisticated, in essence a continuation and intensification of the approach adopted by Knight almost 200 years ago.*' (Bradshaw and MacKay, 1994).

Several decades passed before breeders began to understand the challenges associated with breeding a crop that exhibits polysomic inheritance: genetic studies are difficult or impossible, because the segregation pattern of a gene depends on its position on the chromosome, and the unpredictable behaviour of chromosomes at meiosis; Mendelian ratios do not apply to genes distal to the centromere; double reduction creates unexpected homozygotes; very large families are needed to find transgressive segregants; a deleterious recessive allele has multiple opportunities to hide behind a functional allele; and continuous variation is observed even with single gene traits (Little, 1945, 1952; Chase, 1963a, 1963b; Rowe, 1967a; Sanford, 1983; Gopal, 2015). In addition, epistatic interactions are magnified, gene dosage effects are often important, and interactions with the environment can be complex (Jansky, 2009). All of these phenomena lead to slow genetic gains, poor response to selection, and the inability to identify even major genes.

Polyploidy is considered to be a mutation on a major scale. As such, it leads to a reduction in fertility and vigour, as was described earlier in this chapter. Asexual reproduction can compensate for the reduction in fertility experienced by polysomic polyploids, but it does not advance breeding efforts. In addition, because polysomic tetraploids potentially have twice as many genetic variants as diploids, they undergo twice as many mutations. Consequently, the mutation load in tetraploids is twice that of diploids (Otto, 2007).

Polyploidy has happened to most plants at some point in their evolutionary history. The response to polyploidy over evolutionary time is to diploidize the genome. So, while the extra genome copies are valuable sources of new genetic variability, the negative features of polyploidy, such as reduced fertility, are not tolerated over evolutionary time.

Aardevo Exhibit 1010
Page 0609

'So, in working out any ideal in plant improvement, the first factor and the last one is selection.'

(Whitson *et al.*, 1914)

VIII. CONVENTIONAL POTATO BREEDING

Conventional breeding programmes in Europe and North America follow remarkably similar practices. The scheme is outlined in Table 4.1, and has been described by many (Ross, 1986; Tarn *et al.*, 1992; Bradshaw, 2009; Gopal, 2015).

Basically, crosses are made between tetraploid parents that complement each other for traits of interest to the breeder. While bi-parental crosses form the foundation for the development of potato breeding populations, occasionally an interspecific hybrid will be backcrossed to the cultigen for one or two generations. Tubers are then produced from seedlings, often in a greenhouse. Little or no selection is carried out during this tuber production step. Within each family, one tuber per plant is then harvested and placed in a family bag. The family bag is planted in single hill (plant) units in the field. Selection is severe at this stage, with 90% or more single hills discarded, based on appearance.

Since there is a very low probability of re-assorting genes of heterozygous tetraploid parents into an even better combination of genes than in those selected parents, conventional breeding relies on evaluating large numbers of progeny. It is not unusual for a breeding programme to evaluate 100,000 single hills every year. An important distinction between this clonal breeding strategy and inbred line production in seed propagated crops is that, in potato, no additional sexual cycles are carried out after the initial cross is made. That is, there are no more opportunities for meiosis to break up linkages and rearrange allelic combinations.

Table 4.1. Typical schedule for a conventional potato breeding programme.

Year	Activity
1	Bi-parental crosses made
2	Seedlings grown for first generation tuber production
3	Single hill (plant) field plantings for selection; seed increase
4	Small non-replicated plots for selection; seed increase
5–7	Replicated trials on research farms; seed increase
8–12	Replicated multi-state trials; seed increase
13–14	Cultivar release

Aardevo Exhibit 1010
Page 0610

In subsequent years, asexual propagation continues, and the number of clones evaluated decreases, while the plot size increases. Asexual propagation leads to only an eightfold increase in seed (tubers) per year (Bradshaw and MacKay, 1994). Consequently, replicated trials and extensive phenotyping do not begin until about the fifth year after the cross is made. Of course, the breeding programme maintains clones at all stages of the breeding cycle every year, so extensive land and labour resources are required.

The accumulation of pathogens (especially viruses) is a problem in two ways. First, the breeding programme needs a system to maintain clean seed to provide to the seed certification system when a cultivar is released. That seed must be maintained disease-free for a decade or more, while populations are being evaluated for the identification of new cultivars. Second, seed must be maintained disease-free while it is being bulked up for phenotyping trials. The use of diseased tuber-seed in phenotyping trials will produce data that are not reflective of the true potential of a clone.

Using the conventional potato breeding strategy, approximately nine years are required to collect sufficient data on a clone to decide whether it should be used as a parent in another cycle of crossing and selection (Bradshaw and MacKay, 1994; Gopal, 2015). Consequently, only a handful of meiotic events separate modern cultivars from those of the mid-20th century. Major potato cultivars have a long life – often 30 years or more (Bradshaw, 2009). The use of successful cultivars as parents throughout this long life span lengthens breeding cycle times and, consequently, slows genetic gain.

Conventional potato breeding programmes focus on two processes – selection of parents for bi-parental crosses, and selection in first generation progeny for highly heritable traits initially, and for less heritable traits in later replicated clonal trials. Phenotypic selection for most commercially important traits in potato is ineffective, especially in early generations of a breeding programme (Bradshaw and MacKay, 1994). However, as mentioned above, selection is intense in single hill families in the first field generation.

A general rule of thumb in potato field trials is that the magnitude of the genotype by environment interaction is similar to that of the genotype effect (Bradshaw and MacKay, 1994). Thus, the gain from selection in the single hill environment is limited. Gopal (2015) suggested that '*In early generations of progeny evaluations for the selection of superior clones, the selection pressure needs to be nil to low ...*' This is not the general practice. As mentioned above, selection is severe, beginning with the first field generation.

IX. LATE TWENTIETH CENTURY POTATO BREEDING

To address the narrow genetic base of tetraploid potato in Europe, breeders in Scotland began a programme in the 1950s to recreate European cultivated potato from Andigenum (tetraploid clones of *S. tuberosum* Andigenum group). This programme developed the so-called Neo-Tuberosum germplasm, which was mentioned early in this chapter (Glendinning, 1975a, 1975b, 1975c, 1976). Four generations of phenotypic recurrent mass selection produced adapted clones similar to European cultivars in yield and maturity. A similar programme was carried out North America in the 1980s, but using bulked pollen rather than open-pollination (Munoz and Plaisted, 1981). Tuber yield heterosis was observed in crosses to cultivars (Tarn and Tai, 1973, 1977). Tubers were, however, too rough to be commercially acceptable (Bradshaw, 2009).

Mass selection of diploid cultivated potatoes was also carried out in the second half of the 20th century. Hybrids between the cultivated groups Phureja and Stenotomum (short dormancy and normal dormancy diploid clones of *S. tuberosum* Andigenum group, respectively) were selected for adaptation in Europe (Carroll, 1982) and North America (Haynes, 1972). Selection was effective, and tuber size increased in a few generations. These populations were developed with the goal of capturing desirable genetic diversity and eventually transferring it to the tetraploid level. Clones that produce 2*n* gametes have been crossed to cultivars and are currently represented in advanced breeding clones (K. Haynes, pers. comm.). The North American recurrent selection germplasm is still being used as source for valuable traits (Haynes *et al.*, 1989; Haynes and Christ, 1999; Christ and Haynes, 2001; Haynes, 2001).

Progress in 20th century potato breeding was limited by both a narrow genetic base and conservative breeding strategies (Bradshaw and MacKay, 1994). The mass selection efforts described above attempted to introduce new germplasm. However, phenotypic recurrent selection at the tetraploid level remained the major breeding strategy. Some scientists began to wonder whether a new direction could be fruitful. Among these was Kazimierz Świeżyński (1985), who stated that '*Both the great number of characters and the rare appearance of desired recombinants in some characters hinders the advance in potato breeding by means of conventional methods. This may even become worse when the number of characters to be taken into account keeps growing. Eventually in potato breeding the situation may arise that further general advance is hardly possible. There are limitations to the amount of breeding material a breeder can handle. Detecting small differences in*

important characters like yielding ability is difficult and time consuming ... A break-through in methods is badly needed.'

Another scientist to question conventional potato breeding methods was Sherret Chase, a corn breeder with the DeKalb Agricultural Association. Chase (1963a, 1963b) suggested that plant breeding requires two dissimilar efforts – intragenomic repatterning and intergenomic repatterning. The former is accomplished in many diploid crops by creating elite inbred lines, while the latter results from the development and evaluation of hybrids between those inbred lines. Because these two phases cannot be easily accomplished in polysomic tetraploids, breeding progress is slow. Chase proposed a diploid analytic breeding scheme in potato, using new genetic diversity from wild potato relatives (Chase, 1963a). This scheme is carried out in three steps: 1) ploidy is reduced, 2) hybridization and breeding is carried out at the diploid level, and then 3) selected diploids are brought back to the tetraploid level. Several breeding programmes experimented with this strategy in the second half of the twentieth century:

(1) *Step 1: Ploidy reduction.* Potato dihaploids can be generated through parthenogenesis, following crosses between tetraploid clones and selected Phureja Group pollinators (Hougas *et al.*, 1958; Peloquin *et al.*, 1960; Singsit and Hanneman, 1987; van Suchtelen, 1966). Most are male-sterile, and many are not vigorous. The extraction of a dihaploid from a polysomic tetraploid leads to an inbreeding coefficient equivalent to three generations of self-pollination (Yeh *et al.*, 1964). The inbreeding depression exhibited by dihaploids, expressed as poor vigour and low fertility, has limited their use in breeding (Bradshaw, 2009). Sanford and Hanneman (1982b) suggested that inter-mating dihaploids does not improve tuber yield, because new genetic diversity is not added to the breeding system (Sanford and Hanneman, 1982b). However, van Suchtelen (1966) and Świeżyński (1985) reported that inter-mating dihaploids improved male fertility and vigour. Importantly, they also found that some of these so-called secondary dihaploids were self-compatible. It is worth noting that some tetraploids produce more vigorous dihaploids than others. For example, dihaploids derived from the cultivar 'Atzimba' were reported to produce tuber yields comparable to tetraploid cultivars (Watanabe *et al.*, 1996).

(2) *Step 2: Hybridization to exotic diploids.* As discussed above, the importance of adding genetic diversity to breeding stocks is recognized by potato breeders, and a wealth of such diversity is available in wild potato relatives, most of which are diploid (Ross, 1966;

Hawkes, 1990; Hanneman, 1999; Jansky, 2000; Bradshaw and Ramsay, 2005). The logical implementation of this step, then, is to cross dihaploids with wild relatives, and select for traits of interest. The production of dihaploid-wild species hybrids and their evaluation for value as parents was the focus of numerous studies in the late 20th century (Ross *et al.*, 1964; Hanneman and Ruhde, 1978; Hermundstad and Peloquin, 1985, 1986; Hilali *et al.*, 1987; Yerk and Peloquin, 1989, 1990; Jansky *et al.*, 1990; Ortiz and Peloquin, 1991; Novy and Hanneman, 1991; Thill and Peloquin, 1994; Carputo *et al.*, 1996; Serquen and Peloquin, 1996; Tucci *et al.*, 1996; Buso *et al.*, 1999a; Santini *et al.*, 2000; Oltmans and Novy, 2002; Hamernik *et al.*, 2009; Weber and Jansky, 2012). This effort has been effective at introducing exotic alleles for specific traits into the cultivated potato at the diploid level.

(3) *Step 3: Polyploidization.* Tetraploids can be obtained by somatic doubling of diploids, or by sexual polyploidization using 2*n* gametes. As discussed earlier, a number of studies have demonstrated that somatic doubling of diploids does not lead to superior tetraploids (Rowe, 1967b; Stupar *et al.*, 2007; Aversano *et al.*, 2015). Compared with their diploid progenitors, the tetraploids typically grow more slowly, are not as vigorous, take longer to mature, produce smaller tuber yields, and are less fertile. When doubled diploids were crossed with each other, and their families compared to intercrosses between their diploid progenitors, there was no yield difference between ploidy levels (Rowe, 1967a). When comparing a heterozygous diploid with monoploids derived from anther culture and diploids and tetraploids derived from somatic doubling, the heterozygous diploid outperformed the homozygous tetraploids (Uijtewaal *et al.*, 1987). These studies reveal that increasing ploidy without adding new genetic variation does not accomplish the goals of analytic breeding, and that an increase in ploidy *per se* does not lead to increased productivity.

The second method of developing tetraploids in potato is through sexual polyploidization via 2*n* gametes. Naturally occurring meiotic mutations in wild and cultivated potato lead to gametes with the somatic chromosome number (2*n* gametes). These mutations, which result in the production of 2*n* pollen and, through different genetic pathways, 2*n* eggs, are likely responsible for the evolution of polyploidy in potato (Mok and Peloquin, 1972; den Nijs and Peloquin, 1977; Camadro and Peloquin, 1980; Carputo *et al.*, 2003). 2*n* gametes transmit heterozygosity to offspring, often resulting in tuber yield heterosis.

The value of sexual polyploidization for potato breeding has been demonstrated repeatedly and reviewed often (Mendiburu and Peloquin, 1977; De Jong, 1977; De Jong *et al.*, 1981; Sanford and Hanneman, 1982b; Darmo and Peloquin, 1990; Werner and Peloquin, 1991; Hutten *et al.*, 1995; Ortiz, 1998; Buso *et al.*, 1999a, 1999b, 2000; Carputo *et al.*, 2003; Alberino *et al.*, 2004; Carputo and Barone, 2005; Ortiz *et al.*, 2009; Khan *et al.*, 2010). The assumption has been made that improved productivity following sexual polyploidization is due to an increase in genome-wide allelic diversity. Experiments have not been conducted, however, to determine whether overall diversity or diversity in particular genetic regions is more important. A hint that specific regions are important is provided by research showing that chromosomal regions near the centromere contribute to high yield in products of sexual polyploidization (Buso *et al.*, 1999b).

Despite the theoretical and demonstrated advantages of analytic breeding for potato, this system has not been widely adopted by breeders. There are several explanations for this observation. Considerable effort is required to generate and identify dihaploids, and an even larger effort is needed to develop a collection of fertile, adapted, vigorous dihaploids to use as parents. Despite decades of work by many breeding programmes to generate dihaploids and use them for breeding, they are not found in the pedigrees of commercially important cultivars. Maris (1990) was clearly frustrated with the dihaploid breeding method when making the statement that '*... the use of dihaploids in potato breeding in the last thirty years has not yielded a single superior cultivar in which only dihaploids are involved, and no other diploid species. To avoid loss of more time and money, therefore, it seems advisable to abandon continued use of dihaploids in potato breeding.*' (Maris, 1990).

It is important to note, though, that a major North American cultivar, 'Yukon Gold,' is the product of sexual polyploidization. Its female parent is a diploid hybrid between a Phureja clone and a Tuberosum dihaploid (Johnston and Rowberry, 1981).

Another challenge with the analytic breeding programme is that it is resource-intensive to develop and maintain superior diploids in addition to tetraploid parents in a breeding programme. It is difficult to combine all the traits needed for commercial success with $2n$ gamete production (a recessive trait) and fertility. Even when this is accomplished, seed set in crosses between tetraploids and $2n$ gamete-producing diploids is low, so significant resources are needed to produce the large families needed for selection at the tetraploid level. Finally, the performance of tetraploid offspring is often difficult to predict based on parent performance, and may need to be determined through progeny testing (De Jong and Tai, 1991).

Aardevo Exhibit 1010
Page 0615

> 'It is not obvious that crosses between current cultivars will give big increases in farm yields…'
>
> (Bradshaw, 2009).

So, despite efforts in the late 20th century to change the breeding system to an analytical one based on improvement at the diploid level, followed by sexual polyploidization, the early 21st century breeding system is still fundamentally unchanged. It is based on making crosses between complementary tetraploid parents, and carrying out phenotypic selection (MacKay, 2005). '*… the breeding of varieties per se has changed little in essence except in its scale and technology. It remains by and large empirical and genetically unsophisticated, relying primarily on phenotypic recurrent selection as exemplified by potato breeding programmes worldwide*' (Bradshaw and MacKay, 1994).

It is not surprising that breeding efforts to date have not been able to increase the frequencies of the large set of desirable alleles required by the potato industry. '*This is because in a single cross, the frequency of clones combining all of the desirable characteristics of the two parents is extremely low. Without efficient recurrent selection, breeders will simply be shuffling genes with each generation of crosses without making real progress…*' (Bradshaw, 2009).

In addition to sexual polyploidization, other modifications to the potato breeding scheme adopted by some programmes include: selecting parents that are multiplex for dominant resistance genes; selecting parents for good general combining ability; estimating breeding values; and progeny testing (Bradshaw and MacKay, 1994; Slater *et al.*, 2014a, 2014b; Gopal, 2015). Other novel potato breeding approaches include: *in vitro* selection of protoplasts for disease resistance (Shepard, 1982; Shepard *et al.*, 1980); somatic fusion of diploids as an alternative to sexual polyploidization (Deimling *et al.*, 1988); and the production of triploid clones similar to those produced in sugar beets (van Suchtelen, 1976). Finally, Hermsen (1980) suggested pursuing apomictic potatoes. None of these strategies, however, overcome the inherent challenge of breeding a polysomic polyploid crop. That is, breeders still have no mechanism to separate the intragenomic repatterning step from the intergenomic repatterning step, as described by Chase (1963a, 1963b).

The continued evolution of potato breeding should lead to a system that can take advantage of modern breeding methods, tools and genomics resources. Tuber yield gains in potato have been mainly due to production practices, rather than to improved cultivars (Douches *et al.*, 1996; Jansky *et al.*, 2014a). This is despite the belief that '*The genetic*

potential for yield is far from being exhausted.' (Ross, 1986). It is important to note that new potato cultivars have been improved for maturity, appearance, and processing quality. These are, however, relatively simple inherited traits that are easy to score phenotypically. Genetic gain for complex quantitative traits has been more difficult to achieve.

Perhaps the evolution of a potato breeding system is leading back to the diploid level. Earlier in this chapter, it was shown that ploidy is not a distinguishing feature in landrace cultivars, and it is no longer used by taxonomists to differentiate among cultivated forms. Ploidy may not be as significant a feature of cultivated potato as breeders have assumed it is. The development of diploid potato cultivars can take advantage of the breeding strategies and genetics techniques that have been refined in diploid crops.

Taking the concept a step further, developing diploid inbred line-based cultivars will allow potato breeders to do what maize breeders did a century ago. While maize breeders needed to create new breeding strategies as they developed inbred lines, those efforts can now be leveraged with emerging genomics tools to leap forward in potato breeding. If the next step in the evolution of potato breeding is a return to the diploid level, then some fundamental questions must be answered.

X. TWENTY-FIRST CENTURY POTATO BREEDING

A. Is Tetraploidy Necessary for High Tuber Yield in Potato?

In the late 20th century, the prevailing thought was that maximum heterozygosity (four alleles per locus) was critical for high yield in polysomic polyploids (Bingham, 1980; Hermsen, 1984). Statements such as this one likely perpetuated this assumption: '*It seems that the highly heterozygous tetraploids would have a genetic structure predominantly trigenic and tetragenic*' (Mendoza and Haynes, 1973).

Therefore, methods to increase the number of alleles per locus at as many loci as possible were expected to lead to tuber yield gains. However, two studies carried out to test the maximum heterozygosity hypothesis failed to do so. In the first study, hybrids containing three adapted genomes (Chilotanum, Andigenum, Phureja) did not out-yield those carrying germplasm from two genomes (Chilotanum, Phureja) (Sanford and Hanneman, 1982a). Consequently, a presumed increase in triallelic loci did not lead to higher yield. In the second study, when crosses were made between adapted (Chilotanum) and unadapted (Neo-Tuberosum, Phureja) parents, there was no relationship between estimated heterozygosity (based on RFLP markers) and tuber yield

(Bonierbale *et al.*, 1993). In crosses involving only adapted germplasm, there was, however, a negative relationship between tuber yield and marker homozygosity.

Genomic data are also leading geneticists to question the maximum heterozygosity hypothesis in potato. At least at the single nucleotide level, tri-allelic and tetra-allelic loci are rare in major potato cultivars (Uitdewilligen *et al.*, 2011; Hirsch *et al.*, 2013). Perhaps this is not surprising, considering that many successful cultivars are partially inbred. In any case, increased levels of heterozygosity *per se* do not necessarily translate to higher yield.

Breeding for optimal heterozygosity at certain loci may be more productive than breeding for maximum heterozygosity. Based on the maximum heterozygosity study described above, Bonierbale *et al.* (1993) suggested that the presence of certain alleles in a heterozygous state is more important than overall heterozygosity for improving tuber yield, especially when exotic germplasm is the source of new genetic diversity. In support of this idea, genome-wide SNP evaluations of a large set of cultivars in the SolCAP project revealed that '*potato breeding during the last century resulted in changes to allele composition at select loci to address the needs of specific market classes rather than genome-scale alterations of heterozygosity*' (Hirsch *et al.*, 2013).

Natural and artificial evolutionary forces do not necessarily lead from diploids to superior tetraploids. In fact, as discussed above, potato landraces in South America range in ploidy from diploid to pentaploid, with tuber yields unrelated to ploidy (Dodds, 1965). Furthermore, an extensive study of ploidy variation in cultivated potatoes led to the conclusion that '*the question of broader inherent adaptation capacity of polyploids is difficult to resolve in cultivated potatoes*' (Spooner *et al.*, 2010).

In diploid and tetraploid potatoes, gene action and heterozygosity may have a greater effect on plant vigour and productivity than ploidy level (Rowe, 1967a). The presumed yield superiority of tetraploids must now be questioned in light of the observation that diploids have been repeatedly shown to have high tuber yield potential (Rowe, 1967a; Carroll, 1982; Świeżyński, 1985; Maris, 1990; Hutten, 1994; De Maine, 1996; Watanabe *et al.*, 1996; Simmonds, 1997). In fact, in a study comparing crosses between diploids with crosses between their somatically doubled counterparts, the highest yielding 10% included 27 tetraploid and 13 diploid clones (Rowe, 1967a). In another trial, diploid landraces produced yields equivalent to tetraploid cultivar checks (Watanabe *et al.*, 1996).

Since there appears to be no obvious advantage to breeding tetraploid cultivars, can breeders revert back to the diploid level? The evolution

of cultivated potato has moved from diploid progenitors to landraces with an array of ploidy levels, followed by the fixation of tetraploidy in North America and Europe, perhaps as a chance event rather than selection for an optimal genetic system. As discussed above, the cultivated potato of the northern hemisphere likely originated from Chilean landraces, all of which are tetraploid.

2*n* gametes provide a mechanism to produce polyploids, and are believed to be the main mechanism by which polyploid potato species have arisen (Iwanaga and Peloquin, 1982; Carputo *et al.*, 2003). However, recent reviews have concluded that polyploidy is not inherently superior to diploidy (Comai, 2005; Otto, 2007). In contrast to meiotic mutations that lead to polyploidy, natural systems that reduce ploidy are not common. Parthenogenesis is rare in potato, and leads to dihaploids with poor vigour and fertility, as described earlier. While it is easy to increase ploidy in natural systems, ploidy reductions are difficult to accomplish. So, the answer to the question of tetraploid superiority is no. Tetraploidy does not appear to be necessary for high yield.

> 'This preservation of favourable variations and the rejection of injurious variations, I call Natural Selection.'
>
> (Darwin, 1859)

B. What are the Advantages of Moving to the Diploid Level and Developing Inbred Lines?

Darwin's definition of natural selection also happens to clearly state the ultimate goal of plant breeders – to select desirable combinations of alleles, and discard deleterious ones. This has proved to be an impossible task in potato. In fact, as mentioned earlier, it is typical for a potato breeder to discard more than 90% of progeny from crosses between phenotypically desirable parents. Allard (1999) observed that '*nearly all vegetatively propagated plants are open-pollinated, and highly heterozygous ... when they reproduce sexually, the extensive segregation and recombination that occur during sexual reproduction lead to very large numbers of novel genotypes, nearly all of which are inferior to the parental genotype. Thus, improvement tends to be slow in nature and in cultivation.*'

In fact, North American breeders recognized this phenomenon 60 years ago, and proposed a concerted effort to develop tetraploid inbred breeding lines (Rieman *et al.*, 1956). They calculated that, in order to assemble and identify a specific combination of 20 genes in potato, five million individuals would need to be evaluated.

Aardevo Exhibit 1010
Page 0619

Creating parental lines homozygous for traits of agronomic importance (and, therefore, not segregating) would allow breeders to work with smaller populations.

While genetic variation is the foundation for any breeding programme, variation *per se* is not necessarily desirable. *Useful* genetic variation is key. The challenge is to assemble combinations of beneficial alleles while eliminating the harmful ones (the intragenomic restructuring step of Chase's analytic scheme). The best way to do this is through self-pollination, because '*... inbreeding (especially selfing) is capable of binding the entire genotype together, including favourably interacting alleles of different loci located on different chromosomes. Hence, inbreeding by selfing is expected to be particularly efficient in organizing the entire gene pool into multiple favourably interacting and stable epistatic systems.*' (Allard, 1999).

Following self-pollination, the approach to homozygosity is much faster in diploids than tetraploids (Haldane, 1930). Therefore, unless tetraploids are inherently superior (and it appears that they are not), breeders will make faster progress by self-pollinating diploids.

In tetraploid potato, it has been difficult to breed for recessive traits. Obviously, it is more difficult to configure a genome to carry four rather than two recessive alleles. While a quarter of the progeny from a diploid dihybrid cross will be homozygous recessive, only 1/36 of the progeny from a cross between two duplex (AAaa) parents will be nulliplex (aaaa) (McCord *et al.*, 2012). A trait controlled by three recessives would be observed in 1/64 progeny of a diploid trihybrid cross, but the frequency in an analogous tetraploid ranges from 1/8,000 to 1/46,656 progeny, depending on the cytological behaviour of the chromosomes carrying the genes of interest (Little, 1945).

In addition, once a homozygous recessive clone is identified, the trait will be lost in subsequent crosses, unless the other parent is also homozygous recessive. Several agronomically important traits in potato are expressed as recessives, including deep yellow tuber flesh produced by the zeaxanthin epoxidase gene (*zep1*) (Wolters *et al.*, 2010), acyl sugar droplets on glandular trichomes, leading to broad spectrum insect resistance (Schilmiller *et al.*, 2012), and the production of leptine glycoalkaloids, which are toxic to insects (Ronning *et al.*, 1998). Efforts to incorporate recessive traits in potato have largely been abandoned in tetraploid breeding programmes. These traits would be relatively easy to manipulate in a diploid breeding programme.

Since potato breeding is based on the selection of clones rather than populations, the extremes of a population distribution are more important than the mean. The variation in a segregating diploid

population is estimated to be three times larger than that of a similar tetraploid population (Rowe, 1967a). That is, there are more individuals grouped around the mean in a tetraploid population. In a comparison between diploid populations and tetraploid populations generated by intercrossing somatically doubled diploids, the variance for the two components of yield, tuber number and tuber weight was higher in the diploids than the tetraploids (Rowe, 1967a). Selection of the desirable extremes should lead to higher gains in diploid populations than in tetraploids.

In 2011, the potato genome sequence was published (The Potato Genome Sequencing, 2011), and genomic resources are beginning to emerge, in large part due to the efforts of the SolCAP consortium (Felcher *et al.*, 2012; Hirsch *et al.*, 2013). Inbred lines (products of intra-genomic restructuring) and hybrid populations (products of inter-genomic restructuring) would allow researchers to take full advantage of the power of genome sequence data for breeding and genetics studies. For example, a diploid inbred line was recently used to create a series of mutation lines via the *Tnt1* retrotransposon (Duangpan *et al.*, 2013).

C. Is It Possible to Develop Diploid Inbred Lines in Potato?

A gametophytic self-incompatibility system is present in most diploid potatoes (Pushkarnath, 1942; Pandey, 1962). Two approaches have been used to develop inbred lines. One is anther culture to develop monoploid (1*x*) plants, which can be somatically doubled to produce homozygous diploids (Veilleux, 1990; Paz and Veilleux, 1999). This strategy generated the diploid *S. tuberosum* DM1-3 clone (DM is an abbreviation for doubled monoploid), which was the first potato clone to be sequenced (The Potato Genome Sequencing, 2011).

The second strategy takes advantage of natural self-incompatibility inhibitors in potato and its wild relatives. The best-documented system is the dominant allele of the *Sli* gene, which overcomes self-compatibility (Phumichai *et al.*, 2005). The *Sli* gene was discovered in the normally self-incompatible wild potato relative *S. chacoense*, which is sexually compatible with diploid cultivated potato.

In addition, self-compatibility has been identified in other plants of self-incompatible wild potato species (Cipar *et al.*, 1964). Self-compatibility also exists in diploid cultivated potato. The dihaploid US-W4 is male-fertile and self-compatible (De Jong and Rowe, 1971). It was derived from the partially inbred clone Minn. 20-20-34, developed by the Krantz programme. The significance of this germplasm for breeding was apparent to geneticists over four decades ago, when they indicated that

'*... some crosses involving haploids from the seedling Minn. 20-20-34 are self-fertile. This suggests the possibility of obtaining a faster advance toward homozygosity [than sib mating].*' (Mendoza and Haynes, 1973).

Using self-incompatibility inhibitor genes, inbred lines in potato have been developed. The first to be reported were created by Kazuyoshi Hosaka and co-workers in Japan using self-compatible hybrids between *S. chacoense* (carrying *Sli*) and diploid cultivated Phureja (Phumichai *et al.*, 2005). S_5 clones with more than 90% homozygosity were generated, and fertility decreased across generations. Although selection for tuber yield was not carried out during selfing, individuals with good tuber yield were observed in the S_4 generation.

Next, Pim Lindhout, in the Netherlands, reported on the development of diploid inbred lines with acceptable tuber shape and size (Lindhout *et al.*, 2011). F_3 plants were uniform for tuber quality and yield, so they were intercrossed to make F_1 hybrid prototypes. Some F_1 hybrids were uniform and exhibited good tuber quality. This proof of concept paper has set the stage for the development of diploid F_1 hybrids in potato.

Finally, in the US, one of the co-authors of this manuscript and her co-workers released M6, which is a diploid S_7 inbred line of *S. chacoense* (Jansky *et al.*, 2014b). This line is vigorous, highly fertile, and homozygous for a dominant self-incompatibility inhibitor. US-W4 has also been self-pollinated for five generations. The North American potato breeding community is interested in pursuing the idea of developing diploid inbred lines in potato, and has recently published a white paper outlining this vision (Jansky *et al.*, 2016).

Perhaps potato is actually more tolerant of inbreeding than most breeders assume it is (Brown, 2011). Recent SNP analyses of ten wild potato relatives revealed high levels of homozygosity (Hirsch *et al.*, 2013). Self-pollination, the most extreme form of inbreeding, has produced vigorous, fertile, homozygous clones of wild potatoes, and hybrids with cultivated clones (Phumichai *et al.*, 2005; Jansky *et al.*, 2014b). Native cultivars are selected from open-pollinated berries, which likely carry a high proportion of self-pollinated seeds, as discussed above.

In addition, North American and European cultivars are partially inbred (Mendoza and Haynes, 1974b; Glendinning, 1997). This is due to the bottlenecks described above, and to practices by potato breeders, such as selfing parental clones and using selected parents repeatedly. There are many examples, but one extreme is the cultivar 'Monona', released in 1965. Two full-sibs, 'Chippewa' and 'Katahdin,' were self-pollinated, and then crossed to develop the population from which 'Monona' was selected (Stevenson *et al.*, 1965).

One of the co-authors of this manuscript and her co-workers developed a diploid F_1 interspecific hybrid population by pollinating DM1-3 (described above) with M6 (also mentioned above). The F_1 hybrid population is phenotypically uniform, fertile, and self-compatible. A single F_1 plant was self-pollinated to obtain an F_2 population. Phenotypic segregation in this population is astonishing. The tuber shape and colour variability hidden within a single diploid plant approaches that of landrace collections from the Andes (Jansky *et al.*, 2015).

To our knowledge, this is the first inbred line-derived F_2 population in potato. It has been SNP genotyped and, since all segregating alleles can be traced to either DM1-3 or M6, and the population exhibits Mendelian genetic ratios, it is a powerful resource for mapping. Ten genes have been mapped in this F_2 population (Endelman and Jansky, 2016), and additional mapping studies are under way. In addition, this population is being self-pollinated to homozygosity to develop recombinant inbred lines.

The value of developing inbred lines from a diverse F_2 population was described by George Shull: '*Self-fertilization of the individuals in a hybrid progeny results, in the course of a few generations, in analyzing that hybrid into a group of pure lines which together may possess all of the hereditary elements present in the hybrid with which the self-fertilizations were initiated. If this original hybrid was very complex, each individual pure line derived from it may be unique in possessing a combination of hereditary elements different from that which characterizes each other pure line of the same derivation. When this stage of 'purity' has been established by repeated self-fertilizations, we have in hand the elements for the production of various kinds of hybrid corn.*' (Shull, 1946). Pure lines derived from potato F_2 populations are expected to provide a robust genetic resource for dissecting many traits important to potato improvement.

XI. CONCLUSIONS

A doubling of global crop demand is anticipated to occur by 2050 (Tilman *et al.*, 2011). In order to increase production while minimizing the environmental impact, scientists must focus on improving yield on existing croplands rather than bringing more land into cultivation. However, global climate change models predict potato tuber yield decreases of up to 32% by 2040 (Hijmans, 2003). Incremental improvements in tuber yield will not be sufficient to keep up with this combination of higher food demands and yield reductions. Consequently, we

cannot afford to ignore innovative breeding strategies. The assumption that cultivated potato must be tetraploid may have limited the realization of genetic gains for yield and other important traits, by narrowing options for breeding strategies, genetic analyses and genome manipulations. The evolution of the potato breeding system toward diploid inbred lines may offer a strategy to rapidly develop more productive, nutritious, and sustainable potato cultivars.

LITERATURE CITED

Alberino, S., D. Carputo, G. Caruso, M.R. Ercolano, and L. Frusciante (2004). Field performance of families and clones obtained through unilateral sexual polyploidization in potato (*Solanum tuberosum*). *Advances in Horticultural Science* **18**: 47–52.

Allard, R.W. (1999). History of plant population genetics. *Annual Review of Genetics* **33**: 1–27.

Ames, M., and D.M. Spooner (2008). DNA from herbarium specimens settles a controversy about origins of the European potato. *American Journal of Botany* **95**: 252–257.

Anonymous (2016). PBI *Solanum* Project. Solanaceae Source. http://www.solanaceaesource.org/.

Aversano, R., M.-T. Scarano, G. Aronne, I. Caruso, V. D'Amelia, V. De Micco, C. Fasano, P. Termolino, and D. Carputo (2015). Genotype-specific changes associated to early synthesis of autotetraploids in wild potato species. *Euphytica* **202**: 307–316.

Bamberg, J., A. del Rio, Z. Huamán, S. Vega, M. Martin, A. Salas, J. Pavek, S. Kiru, C. Fernandez, and D. Spooner (2003). A decade of collecting and research on wild potatoes of the Southwest USA. *American Journal of Potato Research* **80**: 159–172.

Bartholdi, W. (1942). Influence of flowering and fruiting upon vegetative growth and tuber yield in the potato. *Minnesota Agricultural Extension Station Technical Bulletin* **150**: 1–20.

Bethke, P.C., A.M.K. Nassar, S. Kubow, Y.N. Leclerc, X.-Q. Li, M. Haroon, T. Molen, J. Bamberg, M. Martin, and D.J. Donnelly (2014). History and origin of Russet Burbank (Netted Gem) a sport of Burbank. *American Journal of Potato Research* **91**: 594–609.

Bingham, E. (1980). Maximizing heterozygosity in autopolyploids. In: Lewis, W. (ed.), *Polyploidy: Biological relevance*, pp. 471–489. Plenum Press, New York.

Bonierbale, M., R. Plaisted, and S. Tanksley (1993). A test of the maximum heterozygosity hypothesis using molecular markers in tetraploid potatoes. *Theoretical and Applied Genetics* **86**: 481–491.

Bradshaw, J.E. (2009). Potato breeding at the Scottish Plant Breeding Station and the Scottish Crop Research Institute: 1920–2008. *Potato Research* **52**: 141–172.

Bradshaw, J.E., and G.R. MacKay (1994). Breeding strategies for clonally propagated crops. In: Bradshaw, J.E. and MacKay, G.R. (eds). *Potato genetics*, p. 467–497. CAB International, Wallingford, UK.

Bradshaw, J.E., and G. Ramsay (2005). Utilisation of the Commonwealth Potato Collection in potato breeding. *Euphytica* **146**: 9–19.

Brown, C.R. (1993). Outcrossing rate in cultivated autotetraploid potato. *American Potato Journal* **70**: 725–734.

Brown, C.R. (2011). The contribution of traditional potato breeding to scientific potato improvement. *Potato Research* **54**: 287–300.

Brücher, H. (1964). El origin de la papa. *Physis* **24**: 439–452.
Brush, S.B., H.J. Carney, and Z. Huamán (1981). Dynamics of Andean potato agriculture. *Economic Botany* **35**: 70–88.
Bukasov, S. (1933). The potatoes of South America and their breeding possibilities. *Trudy Po Prikladnoi Botanike, Genetike I Selektsii* **S58**: 1–192.
Bukasov, S. (1937). Potato breeding. In: Vavilov, N. (ed). *Theoretical principles of plant breeding*, Volume **3**, pp. 3–75. Kolos, Leningrad.
Bukasov, S. (1938). *Interspecific hybridization in the potato*. Izvestia Akademii nauk SSSR. Seria biologicheskaia, 711–732.
Bukasov, S. (1939). The origin of potato species. *Physis* **18**: 41–46.
Bukasov, S. (1978). Systematics of the potato. *Trudy Po Prikladnoi Botanike, Genetike I Selektsii* **62**: 3–35.
Buso, J.A., L.S. Boiteux, and S.J. Peloquin (1999a). Evaluation under long-day conditions of 4x-2x progenies from crosses between potato cultivars and haploid Tuberosum-*Solanum chacoense* hybrids. *Annual Review of Applied Biology* **135**: 35–40.
Buso, J.A., L.S. Boiteux, G.C.C. Tai, and S.J. Peloquin (1999b). Chromosome regions between centromeres and proximal crossovers are the physical sites of major effect loci for yield in potato: Genetic analysis employing meiotic mutants. *Proceedings of the National Academy of Sciences of the United States of America* **96**: 1773–1778.
Buso, J.A., L.S. Boiteux, and S.J. Peloquin (2000). Heterotic effects for yield and tuber solids and type of gene action for five traits in 4x potato families derived from interploid (4x–2x) crosses. *Plant Breeding* **119**: 111–117.
Cadman, C.H. (1942). Autotetraploid inheritance in the potato: Some new evidence. *Journal of Genetics* **44**: 33–52.
Camadro, E., and S.J. Peloquin (1980). Polyploid evolution of wild potatoes via genetically determined 2n gametes. *American Potato Journal* **57**: 473.
Camire, M.E., S. Kubow, and D.J. Donnelly (2009). Potatoes and human health. *Critical Reviews in Food Science and Nutrition* **49**: 823–840.
Carputo, D., and A. Barone (2005). Ploidy level manipulations in potato through sexual hybridization. *Annals of Applied Biology* **146**: 71–79.
Carputo, D., M. Speggiorin, P. Garreffa, A. Raio, and L.M. Monti (1996). Screening for resistance to tuber soft rot and blackleg in diploid *Solanum* species and *S. tuberosum* haploids. *Journal of Genetics and Breeding* **50**: 221–226.
Carputo, D., L. Frusciante, and S.J. Peloquin (2003). The role of 2n gametes and endosperm balance number in the origin and evolution of polyploids in the tuber-bearing Solanums. *Genetics* **163**: 287–294.
Carroll, C.P. (1982). A mass-selection method for the acclimatization and improvement of edible diploid potatoes in the United Kingdom. *Journal of Agricultural Science* **99**: 631–640.
Celis, C., M. Scurrah, S. Cowgill, S. Chumbiauca, J. Green, J. Franco, G. Main, D. Kiezebrink, R. Visser, and H. Atkinson (2004). Environmental biosafety and transgenic potato in a centre of diversity for this crop. *Nature* **432**: 2–5.
Chase, S.S. (1963a). Analytic breeding in *Solanum tuberosum* L. – A scheme utilizing parthenotes and other diploid stocks. *Canadian Journal of Genetics and Cytology* **5**: 389–395.
Chase, S.S. (1963b). Analytic breeding of amphipolyploid plant varieties. *Crop Science* **4**: 334–337.
Christ, B.J., and K.G. Haynes (2001). Inheritance of resistance to early blight disease in a diploid potato population. *Plant Breeding* **120**: 169–172.
Cipar, M.S., S.J. Peloquin, and R.W. Hougas (1964). Variability in the expression of self-incompatiblility in tuber-bearing diploid *Solanum* species. *American Potato Journal* **41**: 155–162.

Aardevo Exhibit 1010
Page 0625

Comai, L. (2005). The advantages and disadvantages of being polyploid. *Nature Reviews Genetics* **6**: 836–846.

Darmo, E., and S.J. Peloquin (1990). Performance and stability of nine 4x clones from 4x–2x crosses and four commercial cultivars. *Potato Research* **33**: 357–364.

Darwin, C. (1859). *On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life*. John Murray, London.

de Haan, H. (1958). Geert Veenhuizen (1857–1930), the pioneer of potato breeding in the Netherlands. *Euphytica* **7**: 31–37.

de Haan, S., J. Núñez, M. Bonierbale, and M. Ghislain (2010). Multilevel Agrobiodiversity and Conservation of Andean Potatoes in Central Peru. *Mountain Research and Development* **30**: 222–231.

de Jong, H. (1977). Self-compatibility in inbred cultivated diploid potatoes. *Incompatibility Newsletter* **8**: 16–17.

de Jong, H., and P.R. Rowe (1971). Inbreeding in cultivated diploid potatoes. *Potato Research* **14**: 74–83.

de Jong, H., and G.C.C. Tai (1977). Analysis of tetraploid-diploid hybrids in cultivated potatoes. *Potato Research* **20**: 111–121.

de Jong, H., and G. Tai (1991). Evaluation of potato hybrids obtained from tetraploid-diploid crosses. I. Parent-offspring relationships. *Plant Breeding* **107**: 177–182.

de Jong, H., G.C.C. Tai, W.A. Russel, G.R. Johnston, and K.G. Proudfoot (1981). Yield potential and genotype-environment interactions of tetraploid-diploid (4x–2x) potato hybrids. *American Potato Journal* **58**: 191–199.

De Maine, M.J. (1996). An assessment of true potato seed families of *Solanum phureja*. *Potato Research* **39**: 323–332.

Deimling, S., J. Zitzlsperger, and G. Wenzel (1988). Somatic fusion for breeding of tetraploid potatoes. *Plant Breeding* **101**: 181–189.

del Rio, A.H., J.B.B. Bamberg, Z. Huamán, A. Salas, and S.E. Vega (1997). Assessing changes in the genetic diversity of potato gene banks. 2. In situ vs ex situ. *Theoretical and Applied Genetics* **95**: 199–204

den Nijs, T.P.M., and S.J. Peloquin (1977). Polyploid evolution via 2n gametes. *American Potato Journal* **54**: 377–386.

Dodds, K. S. (1962). Classification of cultivated potatoes. p. 517–539. In: D. S. Correll (ed.), The potato and its wild relatives. Texas Research Foundation, Renner.

Dodds, K. (1965). The history and relationships of cultivated potatoes. In: Hutchinson, J. (ed). *Crop Plant Evolution*, pp. 123–141. Cambridge University Press, Cambridge.

Dodge, B.S. (1970). *Potatoes and People*, 1st edition. Little, Brown and Company, Boston.

Douches, D., D. Maas, K. Jastrzebski, and R. Chase (1996). Assessment of potato breeding progress in the USA over the last century. *Crop Science* **36**: 1544–1552.

Duangpan, S., W. Zhang, Y. Wu, S.H. Jansky, and J. Jiang (2013). Insertional mutagenesis using Tnt1 retrotransposon in potato. *Plant Physiology* **163**: 21–29.

East, E. (1908). *Inbreeding in corn*. Report of the Connecticut Agricultural Experiment Station, pp. 491–528.

East, E. (1909). The distinction between development and heredity in inbreeding. *American Naturalist*. **43**: 173–181.

Endelman, J.B., and S.H. Jansky (2016). Genetic mapping with an inbred line-derived F2 population in potato. *Theoretical and Applied Genetics* **129**: 935–943.

Engel, K.H. (1957). Grundlegende Fragen zu einem Schema fuer Arbeiten mit Inzuchten bei Kartoffeln (Basic questions about a scheme for work on inbreeding with potatoes). *Züchter (*now *Theoretical and Applied Genetics)* **27**: 98–124.

Aardevo Exhibit 1010
Page 0626

Feistritzer, W. (1952). Die Selbstungsanalyse, eine Voraussetzung fuer die Kreuzungszucht der Kartoffel (The selfing analysis, a prerequisite for the crossbreeding of the potato). *Zeitschrift für Pflanzenzuechtung (*now *Plant Breeding)* **31**: 173–195.

Felcher, K.J., J.J. Coombs, A.N. Massa, C.N. Hansey, J.P. Hamilton, R.E. Veilleux, C.R. Buell, and D.S. Douches (2012). Integration of two diploid potato linkage maps with the potato genome sequence. *PloS One* **7**(4): e36347.

Gavrilenko, T., O. Antonova, A. Ovchinnikova, L. Novikova, E. Krylova, N. Mironenko, G. Pendinen, A. Islamshina, N. Shvachko, S. Kiru, L. Kostina, O. Afanasenko, and D.M. Spooner (2010). A microsatellite and morphological assessment of the Russian National cultivated potato collection. *Genetic Resources and Crop Evolution* **57**: 1151–1164.

Ghislain, M., J. Núñez, M.D.R. Herrera, D.M. Spooner, and M. del Rosario Herrera (2009). The single Andigenum origin of Neo-Tuberosum materials is not supported by microsatellite and plastid marker analyses. *Theoretical and Applied Genetics* **118**: 963–969.

Glendinning, D.R. (1969). The performance of progenies obtained by crossing groups Andigena and Tuberosum of *Solanum tuberosum*. *European Potato Journal* **12**: 13–19.

Glendinning, D.R. (1975a). Neo-Tuberosum: New potato breeding material. 1. The origin, composition, and development of the Tuberosum and Neo-Tuberosum genepools. *Potato Research* **18**: 343–350.

Glendinning, D.R. (1975b). Neo-Tuberosum: New potato breeding material. 2. A comparison of Neo-Tuberosum with unselected Andigena and with Tuberosum. *Potato Research* **18**: 343–350.

Glendinning, D. (1975c). Neo-Tuberosum: New potato breeding material. 3. Characteristics and variability of Neo-Tuberosum, and its potential value in breeding. *Potato Research* **18**: 351–362.

Glendinning, D.R. (1976). Neo-Tuberosum: new potato breeding material. 4. The breeding system of Neo-Tuberosum, and the structure and composition of the Neo-Tuberosum. *Potato Research* **19**: 27–36.

Glendinning, D.R. (1983). Potato introductions and breeding up to the early twentieth century. *New Phytologist* **94**: 479–505.

Glendinning, D.R. (1997). Estimation of inbreeding in potato pedigrees. *Potato Research* **40**: 277–284.

Goodrich, C.E. (1863). The origination and test culture of seedling potatoes. *Transactions of the New York State Agricultural Society* **23**: 89–102.

Gopal, J. (2015). Challenges and way-forward in selection of superior parents, crosses and clones in potato breeding. *Potato Research* **58**: 165–188.

Grun, P. (1990). The evolution of cultivated potatoes. *Economic Botany* **44**: 39–55.

Guern, A.P. (1940). On self-pollinated strains of potato. *Proceedings of the Lenin Academy of Agricultural Sciences of the USSR* **7**: 29–36.

Haldane, J. (1930). Theoretical genetics of autopolyploids. *Journal of Genetics* **22**: 359–372.

Hamernik, A.J., R.E.J. Hanneman, and S.H. Jansky (2009). Introgression of wild species germplasm with extreme resistance to cold sweetening into the cultivated potato. *Crop Science* **49**: 529–542.

Hanneman, R.E. (1999). The reproductive biology of the potato and its implications for breeding. *Potato Research* **42**: 283–312.

Hanneman, R.E. Jr., and R.W. Ruhde (1978). Comparison of bulk populations of species and species-haploid hybrids for tuberization and yield. *American Potato Journal* **55**: 377.

Hawkes, J. (1944). *Potato collecting expeditions in Mexico and South America. II. Systematic classification of the collections*, pp. 1–142. Imperial Bureau of Plant Breeding and Genetics, Aberystwyth, UK.

Aardevo Exhibit 1010
Page 0627

Hawkes, J.G. (1956). Taxonomic studies on the tuber-bearing Solanums. 1: *Solanum Tuberosum* and the tetraploid species complex. *Proceedings of the Linnean Society of London* **166**: 97–144.

Hawkes, J. G. (1967). The history of the potato. *Journal of the Royal Horticultural Society* **92**: 207–302.

Hawkes, J.G. (1990). *The Potato: Evolution, Biodiversity, and Genetic Resources*. Smithsonian Institution Press, Washington, D.C.

Hawkes, J. (1994). Origins of cultivated potatoes and species relationships. In: Bradshaw, J. and Mackay, G. (eds). *Potato Genetics*, pp. 3–42. CAB International, Wallingford, UK.

Hawkes, J.G., and J. Francisco-Ortega (1992). The potato in Spain during the late 16th century. *Economic Botany* **46**: 86–97.

Hawkes, J.G., and J. Francisco-Ortega (1993). The early history of the potato in Europe. *Euphytica* **70**: 1–7.

Hawkes, J.G., and J.P. Hjerting (1969). *The Potatoes of Argentina, Brazil, Paraguay, and Uruguay*. Oxford University Press, Oxford.

Hawkes, J. G., and J. Hjerting (1989). *The potatoes of Bolivia: Their breeding value and evolutionary relationships*. Oxford University Press, Oxford.

Haynes, F. (1972). The use of cultivated diploid *Solanum* species in potato breeding. In: French, E. (ed). *Prospects for the potato in the developing world*, pp. 100–110. Intl. Potato Center, Lima, Peru.

Haynes, K.G. (2001). Variance components for yield and specific gravity in a diploid potato population after two cycles of recurrent selection. *American Journal of Potato Research* **78**: 69–75.

Haynes, K.G., and B.J. Christ (1999). Heritability of resistance to foliar late blight in a diploid hybrid potato population of *Solanum phureja* x *Solanum stenotomum*. *Plant Breeding* **118**: 431–434.

Haynes, K.G., F.L. Haynes, and W.R. Henderson (1989). Heritability of specific gravity of diploid potato under high temperature growing conditions. *Crop Science* **29**: 622–625.

Hermsen, J.G. Th. (1980). Breeding for apomixis in potato: Pursuing a utopian scheme. *Euphytica* **29**: 595–607.

Hermsen, J.G.Th. (1984). Nature, evolution and breeding of polyploids. *Iowa State Journal of Research* **58**: 411–412.

Hermundstad, S.A., and S. Peloquin (1985). Male fertility and 2n pollen production in haploid-wild species hybrids. *American Journal of Potato Research* **62**: 479–487.

Hermundstad, S.A., and S.J. Peloquin (1986). Tuber yield and tuber traits of haploid-wild species F_1 hybrids. *Potato Research* **29**: 289–297.

Hijmans, R.J. (2003). The effect of climate change on global potato production. *American Journal of Potato Research* **80**: 271–280.

Hilali, A., F.I. Lauer, and R.E. Veilleux (1987). Reciprocal differences between hybrids of *Solanum tuberosum* Groups Tuberosum (haploid) and Phureja. *Euphytica* **36**: 631–639.

Hirsch, C., C. Hirsch, K. Felcher, J. Coombs, D. Zarka, A. VanDeynze, W. DeJong, R. Veilleux, S. Jansky, P. Bethke, D. Douches, and C. Buell (2013). Retrospective view of North American potato (*Solanum tuberosum* L.) breeding in the twentieth and twenty-first centuries. *G3: Genes, Genomes, Genetics* **3**: 1003–1013.

Hougas, R.W., and R.W. Ross (1956). The use of foreign introductions in breeding American potato varieties. *American Potato Journal* **33**: 328–339.

Hougas, R.W., S.J. Peloquin, and R.W. Ross (1958). Haploids of the common potato. *Journal of Heredity* **49**: 103–106.

Huamán, Z. (1975). *The origin and nature of Solanum ajanhuiri* Juz. et Buk., *a South American cultivated diploid potato*. PhD Thesis, University of Birmingham.

Huamán, Z., and D.M. Spooner (2002). Reclassification of landrace populations of cultivated potatoes (*Solanum* sect. *Petota*). *American Journal of Botany* **89**: 947–965.

Hutten, R. (1994). *Basic aspects of potato breeding via the diploid level*. PhD Thesis, Wageningen: 93 pp.

Hutten, R.C.B., M.G.M. Schippers, J.G.T. Hermsen, and E. Jacobsen (1995). Comparative performance of diploid and tetraploid progenies from 2x.2x crosses in potato. *Euphytica* **81**: 187–192.

Iwanaga, M., and S.J. Peloquin (1982). Origin and evolution of cultivated tetraploid potatoes via 2n gametes. *Theoretical and Applied Genetics* **61**: 161–169.

Jackson, M.T., J.G. Hawkes, and P.R. Rowe (1980). An ethnobotanical field study of primitive potato varieties in Peru. *Euphytica* **29**: 107–113.

Jansky, S.H. (2000). Breeding for disease resistance in potato. *Plant Breeding Reviews* **19**: 69–155.

Jansky, S.H. (2009). Breeding, genetics, and cultivar development. In: Singh, J. and Kaur, L. (eds). *Advances in potato chemistry and technology*, pp. 27–61. Elsevier, San Diego, CA.

Jansky, S.H., and S.J. Peloquin (2005). Advantages of wild diploid *Solanum* species over cultivated diploid relatives in potato breeding programs. *Genetic Resources and Crop Evolution* **53**: 669–674.

Jansky, S.H., and D.M. Thompson (1990). The effect of flower removal on potato tuber yield. *Canadian Journal of Plant Science* **70**: 1223–1225.

Jansky, S., G. Yerk, and S. Peloquin (1990). The use of potato haploids to put 2x wild species germplasm into a usable form. *Plant Breeding* **104**: 290–294.

Jansky, S., H. Dempewolf, E. Camadro, M. Bonierbale, E. Zimnoch-Guzowska, D. Bisnognin, and S. Reinhard (2013). A case for crop wild relative preservation and use in potato (*Solanum tuberosum* L.). *Crop Science* **53**: 1–9.

Jansky, S., P. Bethke, and D.M. Spooner (2014a). Yield gains in potato: Contributing factors and future prospects. In: Smith, S., Diers, B., Carver, B., and Specht, J. (eds). *Yield gains in major U.S. field crops*, pp. 195–217. Crop Science Society of America, Madison, WI.

Jansky, S., Y. Chung, and P. Kittipadukal (2014b). M6: A diploid potato inbred line for use in breeding and genetics research. *Journal of Plant Registrations* **8**: 195–199.

Jansky, S.H., J. Dawson, and D.M. Spooner (2015). How do we address the disconnect between genetic and morphological diversity in germplasm collections? *American Journal of Botany* **102**: 1213–1215.

Jansky, S.H., A.O. Charkowski, D.S. Douches, G. Gusmini, C. Richael, C. Paul, D.M. Spooner, R.G. Novy, H. De Jong, W.S. De Jong, J.B. Bamberg, L. Thompson, B. Bizimungu, D.G. Holm, C.R. Brown, K.G. Haynes, R. Vidyasagar, R.E. Veilleux, J.C. Miller, J.M. Bradeen, and J.M. Jiang (2016). Reinventing potato as a diploid inbred line-based crop. *Crop Science* **11**: 1–11.

Johns, T., and S.L. Keen (1986). Ongoing evolution of the potato on the altiplano of western Bolivia. *Economic Botany* **40**: 409–424.

Johns, T., Z. Huamán, C.M. Ochoa, and P.E. Schmiediche (1987). Relationships among wild, weed, and cultivated potatoes in the *Solanum* ×*ajanhuiri* complex. *Systematic Botany* **12**: 541–552.

Johnston, G., and R. Rowberry (1981). Yukon Gold: A new yellow-fleshed, medium-early, high quality table and french-fry cultivar. *American Potato Journal* **58**: 241–244.

Juzepczuk, S. (1937). New species of the genus *Solanum* L. in the group Tuberarium Dun. *Izvestiya Akademii Nauk* SSR **2**: 295–311.

Juzepczuk, S., and S. Bukasov (1929). A contribution to the question of the origin of the potato. *Trudy Vsesoyuznogo S"zeda po Genetike i Selektsii Semenovodstvu i Plememnnomu Zhivotnovodstvu. Leningrad* **3**: 593–611.

Khan, N., R. Barba-Gonzalez, M. Ramanna, P. Arans, R. Visser, and J. van Tuyl (2010). Relevance of unilateral and bilateral sexual polyploidization in relation to intergenomic recombination and introgression in *Lilium* species hybrids. *Euphytica* **171**: 157–173.

Kittipadukal, P., P.C. Bethke, and S.H. Jansky (2012). The effect of photoperiod on tuberization in cultivated × wild potato species hybrids. *Potato Research* **55**: 27–40.

Knight, T. (1807). On raising of new and early varieties of the potato (*Solanum tuberosum*). *Transactions of the Horticultural Society of London* **1**: 57–59.

Kolasa, K.M. (1993). The potato and human nutrition. *American Potato Journal* **70**: 375–385.

Krantz, F.A. (1924). Potato breeding methods. *Minnesota Agricultural Extension Station Technical Bulletin* **25**: 3–32.

Krantz, F.A. (1929). Potato breeding methods II. Selection in inbred lines. *Minnesota Agricultural Extension Station Technical Bulletin* **58**: 3–26.

Krantz, F.A. (1946). Potato Breeding Methods III. *Minnesota Agricultural Extension Station Technical Bulletin* **173**: 1–24.

Lawson, H.M. (1983). True potato seeds as arable weeds. *Potato Research* **26**: 237–246.

Lekhnovich, V. (1971). Cultivated potato species. In: Bukasov, S. (ed). *Flora of Cultivated Plants*, 9th edition, p. 41–304. Kolos, Leningrad.

Lindhout, P., D. Meijer, T. Schotte, R.C.B. Hutten, R.G.F. Visser, and H.J. Eck (2011). Towards F_1 hybrid seed potato breeding. *Potato Research* **54**: 301–312.

Little, T. (1945). Gene segregation in autotetraploids. *Botanical Review* **11**: 60–85.

Little, T.M. (1952). Gene segregation in autotetraploids II. *Botanical Review* **24**: 318–339.

Louderbeck, L. (2014). *The ecology of human diets during the Holocene at North Creek Shelter, Utah*. PhD Thesis, University of Washington.

MacKay, G. (2005). Propagation by traditional breeding methods. In: Razdan, M. and Mattoo, A. (eds). *Genetic improvement of Solanaceae crops, Volume 1, Potato*, pp. 65–81. Science Publishers, Inc., Enfield, NH.

Maris, B. (1990). Comparison of diploid and tetraploid potato families derived from *Solanum phureja* x dihaploid *S. tuberosum* hybrids and their vegetatively doubled counterparts. *Euphytica* **46**: 15–33.

McCord, P., L. Zhang, and C. Brown (2012). The incidence and effect on total tuber carotenoids of a recessive zeaxanthin epoxidase allele (*Zep1*) in yellow-fleshed potatoes. *American Journal of Potato Research* **89**: 262–268.

Mendiburu, A.O., and S.J. Peloquin (1977). Bilateral sexual polyploidization in potatoes. *Euphytica* **26**: 573–583.

Mendoza, H., and F. Haynes (1973). Some aspects of breeding and inbreeding in potatoes. *American Potato Journal* **50**: 216–222.

Mendoza, H.A., and F.L. Haynes (1974a). Genetic basis of heterosis for yield in autotetraploid potato. *Theoretical and Applied Genetics* **45**: 21–25.

Mendoza, H.O., and F.L. Haynes (1974b). Genetic relationship among potato cultivars grown in the United States. *HortScience* **9**: 328–330.

Mendoza, H.O., and F.L. Haynes (1976). Variability for photoperiodic reaction among diploid and tetraploid potato clones from three taxonomic groups. *American Potato Journal* **53**: 319–332.

Mok, D.W.S., and S.J. Peloquin (1972). Three mechanisms of 2n pollen formation in diploid potatoes. *Canadian Journal of Genetics and Cytology* **49**: 217–225.

Aardevo Exhibit 1010
Page 0630

Mullin, R., and F. Lauer (1966). Breeding behavior of F_1 and inbred potato clones. *Proceedings of the American Society for Horticultural Science* **89**: 449–455.

Munoz, F.J., and R. Plaisted (1981). Yield and combining abilities in andigena potato after six cycles of recurrent phenotypic selection for adaptation to long day conditions. *American Journal of Potato Research* **58**: 469–479.

Navarre, D.A. (2009). Nutritional value of potatoes: Vitamin, phytonutrient, and mineral content. In: Singh, J. and Kaur, L. (eds). *Advances in potato chemistry and technology*, pp. 395–424. Elsevier, San Diego, CA.

Novy, R.G., and R.E. Hanneman Jr. (1991). Hybridization between Gp. Tuberosum haploids and 1EBN wild potato species. *American Potato Journal* **68**: 151–169.

Ochoa C.M. (1958). *Expedición Colectora de Papas Cultivadas a la Cuenca del Lago Titicaca (Ministerio de Agricultura, Lima, Perú)*, Vol **1**.

Ochoa, C.M. (1990). *The Potatoes of South America.* Cambridge University Press, Cambridge.

Ochoa, C. (1999). *Las papas de Sudamerica: Peru (parte 1).* International Potato Center, Lima, Peru.

Oltmans, S.M., and R.G. Novy (2002). Identification of potato (*Solanum tuberosum* L.) haploid x wild species hybrids with the capacity to cold chip. *American Journal of Potato Research* **79**: 263–268.

Ortiz, R. (1998). Potato breeding via ploidy manipulations. *Plant Breeding Reviews* **16**: 15–86.

Ortiz, R., and S.J. Peloquin (1991). Breeding for 2n egg production in haploid x species 2x potato hybrids. *American Potato Journal* **68**: 691–703.

Ortiz, R., P. Simon, S. Jansky, and D. Stelly (2009). Ploidy manipulation of the gametophyte, endosperm and sporophyte in nature and for crop improvement: a tribute to Professor Stanley J. Peloquin (1921–2008). *Annals of Botany* **104**: 795–807.

Otto, S.P. (2007). The evolutionary consequences of polyploidy. *Cell* **131**: 452–462.

Ovchinnikova, A., E. Krylova, T. Gavrilenko, T. Smekalova, M. Zhuk, S. Knapp, and D.M. Spooner (2011). Taxonomy of cultivated potatoes (*Solanum* section *Petota*: Solanaceae). *Botanical Journal of the Linnean Society* **165**: 107–155.

Pal, B., and Pushkarnath (1951). Potato breeding investigations in India. *Empire Journal of Experimental Agriculture* **19**: 87–103.

Pandey, K.K. (1962). Incompatibility in some *Solanum* crosses. *Genetica* **33**: 1–23.

Paz, M., and R. Veilleux (1999). Influence of culture medium and in vitro conditions on shoot regeneration in *Solanum phureja* monoploids and fertility of regenerated doubled monoploids. *Plant Breeding* **118**: 53–57.

Peloquin, S.J., R.W. Hougas, and A.C. Gabert (1960). The frequency of haploids in *Solanum tuberosum*. *American Potato Journal* **37**: 289–297.

Phumichai, C., M. Mori, A. Kobayashi, O. Kamijima, and K. Hosaka (2005). Toward the development of highly homozygous diploid potato lines using the self-compatibility controlling *Sli* gene. *Genome* **48**: 977–984.

Plaisted, R.L., and R.W. Hoopes (1989). The past record and future prospects for the use of exotic potato germplasm. *American Journal of Potato Research* **66**: 603–627.

Pushkarnath, P. (1942). Studies on sterility in potatoes. 1). The genetics of self- and cross-incompatibilities. *Indian Journal of Genetics and Plant Breeding* **2**: 11–36.

Quiros, C.F., S.B. Brush, D.S. Douches, K.S. Zimmerer, and G. Huestis (1990). Biochemical and folk assessment of variability of Andean cultivated potatoes. *Economic Botany* **44**: 254–266.

Quiros, C.F., R. Ortega, L. van Raamsdonk, M. Herrera-Montoya, P. Cisneros, and E. Schmidt (1992). Increase of potato genetic resources in their center of diversity: The role of natural outcrossing and selection by the Andean farmer. *Genetic Resources and Crop Evolution* **39**: 107–113.

Aardevo Exhibit 1010
Page 0631

Rabinowitz, D., C.R. Londer, R. Ortega, D. Begazo, H. Murguia, D.S. Douches, and C.F. Quiros (1990). High levels of interspecific hybridization between *Solanum sparsipilum* and *S. stenotomum* in experimental plots in the Andes. *American Potato Journal* **67**: 73–81.

Rasco, E.T., R.L. Plaisted, and E.E. Ewing (1980). Photoperiod response and earliness of *S. tuberosum* ssp. *andigena* after six cycles of recurrent selection for adaptation to long days. *American Potato Journal* **57**: 435–447.

Reddick, D. (1940). Problems in breeding for disease resistance. *Chronica Botanica* **6**: 73–77.

Rieman, G.H., W.J. Hooker, F.A. Krantz, and H.O. Werner (1956). Potato improvement through parental line breeding. *American Potato Journal* **33**: 319–323.

Ríos, D., M. Ghislain, F. Rodríguez, D.M. Spooner, D. Rios, and F. Rodriguez (2007). What is the origin of the European potato? Evidence from Canary Island landraces. *Crop Science* **47**: 1271–1280.

Rodríguez, F., M. Ghislain, A.M. Clausen, S.H. Jansky, and D.M. Spooner (2010). Hybrid origins of cultivated potatoes. *Theoretical and Applied Genetics* **121**: 1187–98.

Romans, A. (2005). *The Potato Book*. Frances Lincoln, London.

Ronning, C., L. Sanford, R. Kobayashi, and S. Kowalsld (1998). Foliar leptine production in segregating F_1, Inter F_1, and backcross families of *Solanum chacoense* Bitter. *American Journal of Potato Research* **75**: 137–143.

Ross, H. (1966). The use of wild *Solanum* species in German potato breeding of the past and today. *American Potato Journal* **43**: 63–80.

Ross, H. (1986). *Potato Breeding – Problems and Perspectives*. Verlag Paul Parey, Berlin and Hamberg.

Ross, R.W., S.J. Peloquin, and R.W. Hougas (1964). Fertility of hybrids from *Solanum phureja* and haploid *S. tuberosum* matings. *European Potato Journal* **7**: 81–89.

Rowe, P.R. (1967a). Performance and variability of diploid and tetraploid potato families. *American Potato Journal* **44**: 263–271.

Rowe, P.R. (1967b). Performance of diploid and vegetatively doubled clones of Phureja-haploid Tuberosum hybrids. *American Potato Journal* **44**: 195–203.

Rudorf, W. (1958). Zuchtmethoden. In Kappert, H. and Rudorf, W. (eds). *Handbuch der Pflanzenzuechtung vol. 3. Zuechtung der Knollen-und Wurzelfruchtarten. Kartoffel*, p. 156–167. Paul Parey. Handbook of Plant Breeding.

Rybin, V. (1929). Karyological investigation on some wild growing and indigenous cultivated potatoes of America. *Trudy Po Prikladnoi Botanike, Genetike I Selektsii* **20**: 655–720.

Rybin, V. A. (1933). Cytological investigation of the South American cultivated and wild potatoes, and its significance for plant breeding. *Trudy Po Prikladnoi Botanike, Genetike I Selektsii, Ser II* **2**: 3–100.

Salaman, R. (1937). The potato in its early home and its introduction into Europe. *Journal of the Horticultural Society* **62**: 61–266.

Salaman, R. (1946). The early European potato, its character and place of origin. *Journal of the Linnean Society (Botany)* **53**: 1–27.

Sanford, J.C. (1983). Ploidy manipulations. In: Moore, J.N. and Janick, J. (eds). *Methods in fruit breeding*, pp. 100–123. Purdue University Press, Purdue.

Sanford, J., and R.E. Hanneman, Jr (1982a). A possible heterotic threshold in potato and its implications for breeding. *Theoretical and Applied Genetics* **61**: 151–159.

Sanford, J., and R. Hanneman, Jr (1982b). Intermating of potato haploids and spontaneous sexual polyploidization – effects on heterozygosity. *American Journal of Potato Research* **59**: 407–414.

Santini, M., E.L. Camadro, O.N. Marcellán, and L.E. Erazzú (2000). Agronomic characterization of diploid hybrid families derived from crosses between haploids of the common potato and three wild Argentinean tuber-bearing species. *American Journal of Potato Research* **77**: 211–218.

Sarkar, D. (2010). Photoperiodic inhibition of potato tuberization: An update. *Plant Growth Regulation* **62**: 117–125.

Schilmiller, A.L., A.L. Charbonneau, and R.L. Last (2012). Identification of a BAHD acetyltransferase that produces protective acyl sugars in tomato trichomes. *Proceedings of the National Academy of Sciences of the United States of America* **109**: 16377–16382.

Scurrah, M., C. Celis-Gamboa, S. Chumbiauca, A. Salas, and R.G.F. Visser (2008). Hybridization between wild and cultivated potato species in the Peruvian Andes and biosafety implications for deployment of GM potatoes. *Euphytica* **164**: 881–892.

Serquen, F.C., and S.J. Peloquin (1996). Variation for agronomic and processing traits in *Solanum tuberosum* haploids x wild species hybrids. *Euphytica* **89**: 185–191.

Shepard, J.F. (1982). The regeneration of potato plants from leaf-cell protoplasts. *Scientific American* **246**: 154–166.

Shepard, J.F., D. Bidney, and E. Shahin (1980). Potato protoplasts in crop improvement. *Science* **208**: 17–24.

Shull, G. (1909). A pureline method of corn breeding. *American Breeders Association Report* **5**: 51–59.

Shull, G. (1946). Hybrid seed corn. *Science* **103**: 547–550.

Simmonds, N. (1964). Studies of the tetraploid potatoes I.The variability of the Andigena Group. *Journal of the Linnean Society (Botany)* **58**: 461–474.

Simmonds, N. (1966). Studies of the tetraploid potatoes. II. Factors in the evolution of the Tuberosum Group. *Journal of the Linnean Society (Botany)* **59**: 279–288.

Simmonds, N. (1969). Prospects of potato improvement. *Scottish Plant Breeding Station Annual Report* **48**: 18–38.

Simmonds, N. (1997). A review of potato propagation by means of seed, as distinct from clonal propagation by tubers. *Euphytica* **40**: 191–214.

Singsit, C., and R.E. Hanneman, Jr (1987). Haploids of tetraploid (2N=4X=48) Mexican potato species – their extraction, cytology and crossability. *American Potato Journal* **64**: 469–482.

Slater, A.T., N.O.I. Cogan, B.J. Hayes, L. Schultz, M.F.B. Dale, G.J. Bryan, and J.W. Forster (2014a). Improving breeding efficiency in potato using molecular and quantitative genetics. *Theoretical and Applied Genetics* **127**: 2279–2292.

Slater, A.T., G.M. Wilson, N.O.I. Cogan, J.W. Forster, and B.J. Hayes (2014b). Improving the analysis of low heritability complex traits for enhanced genetic gain in potato. *Theoretical and Applied Genetics* **127**: 809–820.

Spooner, D. M. (2016). Species delimitations in plants: Lessons learned from potato taxonomy by a practicing taxonomist. *Journal of Systematics and Evolution* **54**: 191–203.

Spooner, D.M., K. McLean, G. Ramsay, R. Waugh, and G.J. Bryan (2005). A single domestication for potato based on multilocus amplified fragment length polymorphism genotyping. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 14694–14699.

Spooner, D.M., J. Núñez, G. Trujillo, M.D.R. Herrera, F. Guzmán, M. Ghislain, J. Nunez, M. del Rosario Herrera, and F. Guzma (2007). Extensive simple sequence repeat genotyping of potato landraces supports a major reevaluation of their gene pool structure and classification. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 19398–19403.

Aardevo Exhibit 1010
Page 0633

Spooner, D.M., T. Gavrilenko, S.H. Jansky, A. Ovchinnikova, E. Krylova, S. Knapp, and R. Simon (2010). Ecogeography of ploidy variation in cultivated potato (*Solanum* sect. *Petota*). *American Journal of Botany* **97**: 2049–2060.

Spooner, D., S. Jansky, A. Clausen, M. del Rosaria Herrera, and M. Guislain (2012). The enigma of *Solanum maglia* in the origin of the Chilean cultivated potato, *Solanum tuberosum* Chilotanum Group. *Economic Botany* **66**: 12–21.

Spooner, D.M., M. Ghislain, R. Simon, S.H. Jansky, and T. Gavrilenko (2014). Systematics, diversity, genetics, and evolution of wild and cultivated potatoes. *The Botanical Review* **80**: 283–383.

Stevenson, F.J., and C.F. Clark (1937). *Breeding and genetics in potato improvement.* Yearbook of Agriculture, USDA: 405–444.

Stevenson, F.J., R.V. Akeley, G. Newton, and D. Isleib (1965). Monona: A new variety of potato, distinctive for excellent chip color after numerous storage treatments. *American Potato Journal* **42**: 253–255.

Stuart, W. (1928). *The Potato: Its culture, uses, history, and classification.* J.B. Lippincott, Philadelphia and London.

Stupar, R.M., P.B. Bhaskar, B.S. Yandell, W.A. Rensink, A.L. Hart, S. Ouyang, R.E. Veilleux, J.S. Busse, R.J. Erhardt, C.R. Buell, and J. Jiang (2007). Phenotypic and transcriptomic changes associated with potato autopolyploidization. *Genetics* **176**: 2055–2067.

Świeżyński, K. *et al.* (1985). Potato (*Solanum tuberosum* L.) improvement by breeding at the diploid level. *Ziemniak* **1985**, p. 5–23.

Tarn, T., and G. Tai (1973). Heterosis in F_1 hybrids between group Andigena and group Tuberosum potatoes. *American Potato Journal* **50**: 337.

Tarn, T.R., and G.C.C. Tai (1977). Heterosis and variation of yield components in F_1 hybrids between Group Tuberosum and Group Andigena potatoes. *Crop Science* **17**: 517–521.

Tarn, T.R., G. Tai, H. De Jong, R. Tarn, A. Murphy, and J. Seabrook (1992). Breeding potatoes for long-day, temperate climates. *Plant Breeding Reviews* **9**: 217–232.

The Potato Genome Sequencing Consortium (2011). Genome sequence and analysis of the tuber crop potato. *Nature* **475**: 189–195.

Thill, C.A., and S.J. Peloquin (1994). Inheritance of potato chip color at the 24-chromosome level. *American Potato Journal* **71**: 629–646.

Tilman, D., C. Balzer, J. Hill, and B.L. Befort (2011). Global food demand and the sustainable intensification of agriculture. *Proceedings of the National Academy of Sciences of the United States of America* **108**: 20260–20264.

Tucci, M., D. Carputo, G. Bile, and L. Frusciante (1996). Male fertility and freezing tolerance of hybrids involving *Solanum tuberosum* haploids and diploid *Solanum* species. *Potato Research* **39**: 345–353.

Ugent, D. (1970). The potato: What is the origin of this important crop plant, and how did it first become domesticated? *Science* **170**: 1161–1166.

Ugent, D., T. Dillehay, and C. Ramirez (1987). Potato remains from a late Pleistocene settlement in southcentral Chile. *Economic Botany* **41**: 17–27.

Uijtewaal, B. A, E. Jacobsen, and J.G.T. Hermsen (1987). Morphology and vigour of monohaploid potato clones, their corresponding homozygous diploids and tetraploids and their heterozygous diploid parent. *Euphytica* **36**: 745–753.

Uitdewilligen, J., A.-M. Wolters, V. der Vlies, R. Visser, and H. van Eck (2011). *SNP and haplotype identification from targeted next-generation re-sequencing in a set of 83 potato cultivars.* The 18th Triennial Conference of the European Association for Potato Research, p. 97.

Aardevo Exhibit 1010
Page 0634

van Suchtelen, N.J. (1966). Investigation of dihaploid potatoes in the Netherlands. *European Potato Journal* **9**: 64–68.

van Suchtelen, N.J. (1976). Triploids of the common potato *Solanum tuberosum* L. *Potato Research* **19**: 377–380.

Vavilov, N. (1935). II. Botaniko-geografi cheskie osnovy selektsii [Botanical and geographical bases of plant breeding]. In: Vavilov, N. (ed). *Teoreticheskie Osnovy Selektsii Rasteniy*, pp. 17–74. State Agricultural Publishing House, Moscow.

Vavilov, N. (1939). The great ancient centers of agriculture in pre-Columbian America and their interrelations. *Bulletin of the State Geographical Society* **10**: 1487–1515.

Veilleux, R. (1990). *Solanum phureja*: Anther culture and the induction of haploids in a cultivated diploid potato species. In: Bajaj, Y. (ed). *Biotechnology in Agriculture and Forestry 12: Haploids in Crop Improvement I*, p. 530–543. Springer-Verlag, New York.

Vos, P.G., J. Uitdewilligen, R.E. Voorrips, R.G.F. Visser, and H.J. van Eck (2015). Development and analysis of a 20K SNP array for potato (*Solanum tuberosum*): an insight into the breeding history. *Theoretical and Applied Genetics* **128**: 2387–2401.

Watanabe, K., and S.J. Peloquin (1989). Occurrence of 2n pollen and *ps* gene frequencies in cultivated groups and their related wild species in tuber-bearing *Solanums*. *Theoretical and Applied Genetics* **78**: 329–336.

Watanabe, K., and S.J. Peloquin (1991). The occurrence and frequency of 2n pollen in 2x, 4x, and 6x wild, tuber-bearing *Solanum* species from Mexico, and Central and South America. *Theoretical and Applied Genetics* **82**: 621–626.

Watanabe, K., M. Orrillo, S. Perez, and J. Crusado (1996). Testing yield of diploid potato breeding lines for cultivar development. *Breeding Science* **46**: 245–249.

Weber, B., and S. Jansky (2012). Resistance to *Alternaria solani* in hybrids between a *Solanum tuberosum* haploid and *S. raphanifolium*. *Phytopathology* **102**: 214–221.

Werner, J.E., and S.J. Peloquin (1991). Yield and tuber characteristics of 4x progeny from 2x x 2x crosses. *Potato Research* **34**: 261–267.

Whitson, J., R. John, and H. Williams (1914). *Luther Burbank: His Methods and Discoveries and Their Practical Application,* Volume 1. Luther Burbank Press, New York and London.

Wolters, A.-M.A., J. Uitdewilligen, B.A. Kloosterman, R.C.B. Hutten, R.G.F. Visser, and H.J. van Eck (2010). Identification of alleles of carotenoid pathway genes important for zeaxanthin accumulation in potato tubers. *Plant Molecular Biology* **73**: 659–671.

Yeh, B.P., S.J. Peloquin, and R.W. Hougas (1964). Meiosis in *Solanum tuberosum* haploids and haploid-haploid F_1 hybrids. *Canadian Journal of Genetics and Cytology* **6**: 393–402.

Yerk, G.L., and S.J. Peloquin (1989). Evaluation of tuber traits of 10, 2x(2EBN) wild species through haploid x wild species hybrids. *American Potato Journal* **66**: 731–739.

Yerk, G.L., and S.J. Peloquin (1990). Performance of haploid x wild species, 2x hybrids (involving five newly evaluated species) in 4x x 2x families. *American Potato Journal* **67**: 405–417.

Zimmerer, K.S. (1991). The regional biogeography of native potato cultivars in highland Peru. *Journal of Biogeography* **18**: 165–178.

Aardevo Exhibit 1010
Page 0635

ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/327077074

Hybrid potato breeding for improved varieties

Chapter · August 2018

DOI: 10.19103/AS.2018.0046.04

CITATIONS
8

READS
1,183

6 authors, including:



Michiel E. de Vries
Solynta
15 PUBLICATIONS **215** CITATIONS
SEE PROFILE



Ying Su
Solynta
1 PUBLICATION **8** CITATIONS
SEE PROFILE



Adriaan W van Heusden
Wageningen University & Research
127 PUBLICATIONS **2,968** CITATIONS
SEE PROFILE

Some of the authors of this publication are also working on these related projects:



Ornamental Research View project



Irrigated Rice in Africa View project

All content following this page was uploaded by Michiel E. de Vries on 09 September 2018.

The user has requested enhancement of the downloaded file.

Aardevo Exhibit 1010
Page 0636

Hybrid potato breeding for improved varieties

Pim Lindhout, Michiel de Vries, Menno ter Maat, Su Ying, Marcela Viquez-Zamora and Sjaak van Heusden, Solynta, the Netherlands

1 Introduction
2 The scientific basis for hybrid potato breeding
3 The state of the art of hybrid potato breeding
4 Production of and commercialization of hybrid seed cultivars
5 Inbred lines for genetic research
6 Cropping systems based on true seeds
7 Case studies
8 Conclusion
9 Where to look for further information
10 Acknowledgements
11 References

1 Introduction

The cultivated potato, *Solanum tuberosum*, can be reproduced generatively through seeds and vegetatively through tubers. This may have evolutionary advantages: seeds may provide better survival under extreme conditions, such as frost or drought, and can remain viable in the soil for years. When conditions are mild, tubers survive in a dormant state for a couple of months. When conditions become favourable again, their fast and strong sprouting provides a clear competitive advantage over other plants in the same ecological niche.

In traditional potato breeding, each breeding cycle starts with a cross between two genotypes, usually tetraploid varieties, followed by many years of selection and multiplication (see Chapters 2 and 3). The advantage of this approach is uniformity: the tubers are clones and thus genetically identical. The disadvantage is the low genetic gain in each lengthy breeding cycle, as the genetic composition of the two parental genotypes is just reshuffled, including alleles which negatively affect plant growth and development. As a result, potato yield has not significantly been improved over the past century (Douches

http://dx.doi.org/10.19103/AS.2016.0016.04
© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

AQ: Please check if 'is less than a factor 10 per season' is OK as is.

et al., 1996; Vos et al., 2015). In addition, the reproduction of seed tubers is less than a factor 10 per season. It takes many years to build up sufficient quantities of seed tubers for commercial production, and the risk of contamination by pathogens increases with each multiplication step.

True potato seed (TPS) has been promoted as an alternative for seed tubers because TPS is easy to store and devoid of most soil-borne pathogens. In South Asia, East Africa and the Andes, TPS is used mainly by subsistent farmers (Almekinders et al., 1996). TPS is produced by crossing parent plants that have been selected to produce a hybrid variety. The parents are propagated vegetatively, similar to seed tuber propagation. As the parents of a TPS variety are heterozygous, all seeds of a TPS cultivar are genetically different. This results in a highly variable crop that is not acceptable in most markets, such as the high value markets of Europe and North America.

Since the success of hybrid breeding in corn in the 1930s, breeders have adapted a hybrid breeding system for many crops (Fig. 1; Crow, 1998; Troyer, 2006; Hua et al., 2003). Typically, hybrid cultivars produce higher yields and show high crop uniformity (Rijk et al., 2013). In addition, the breeding system is fast and efficient and new traits can rapidly be introduced by marker-assisted introgression.

These advantages are also expected for potato: hybrid potato varieties will be higher yielding, will need less crop protection chemicals due to disease resistance and will have better quality for processors and consumers (FAO et al., 2015). A hybrid breeding system for potato offers two additional advantages: fast multiplication of hybrid seeds and easier logistics, as clean true seeds can easily be produced, transported and stored (Duvick et al., 2005).

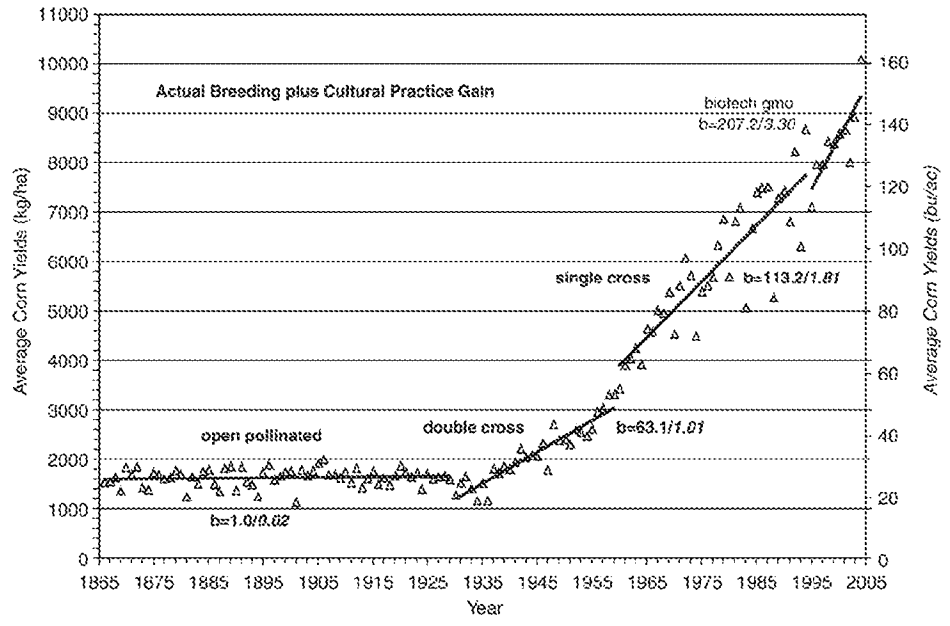


Figure 1 Fivefold increase in corn yields since the introduction of hybrids (Troyer, 2006).

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0638

However, self-incompatibility and inbreeding depression have for long hindered progress in hybrid potato breeding (De Jong and Rowe, 1971; Charlesworth and Willis, 2009; Jansky et al., 2016). These two limitations have recently been overcome by introducing a self-compatibility restorer gene and by large-scale and consistent breeding (Lindhout et al., 2011a).

This chapter describes the scientific principles and applied aspects of hybrid potato breeding. The successful introduction of the principle of hybrid potato breeding was described in 2011 (Lindhout et al., 2011a). Since then, we have focused on further developing the potato hybrid breeding system, especially genetic studies to establish a genetics-driven hybrid breeding system. In a recent paper, Jansky et al. (2016) have confirmed the possibilities of such approach.

This is the first publication on the state of the art of a hybrid potato breeding programme. The authors, all working at Solynta, want to emphasize that scientific papers on this topic are not available yet, and hence we have to rely entirely on the results of the Solynta breeding and research programme. Still, in presenting these results, we hope to contribute to a better understanding of the principles and applied aspects of hybrid potato breeding.

2 The scientific basis for hybrid potato breeding

2.1 The principle of hybrid breeding

The basic idea of hybrid breeding is to combine the genes of two parent genotypes, both of which may harbour suboptimal alleles, resulting in weaker performance. If parents have different suboptimal alleles, hybrid offspring can show increased vigour and yield, designated 'heterosis', as the suboptimal alleles in one parent may be compensated by the favourable genes from the other parent (Birchler et al., 2010; Gopal, 2014; Fig. 6). If the parents are completely homozygous, the resulting hybrid offspring will be partially heterozygous and genetically uniform. By testing many hybrid offspring under relevant cultivation conditions, the best combining parents are identified. These are maintained and propagated in separate groups as 'heterotic pools' for further breeding (Brown and Cagliari, 2011).

Thus, hybrid breeding has two distinct processes: development of homozygous parent lines and production and testing of experimental hybrids.

2.2 Diploids are more efficient than tetraploids for hybrid breeding

Homozygous diploids are faster to generate than homozygous tetraploids. For instance, seven generations of selfing are required to obtain 50% homozygous loci starting from a tetragenic tetraploid heterozygote (carrying four different alleles). The same homozygosity level is reached starting from a heterozygous diploid by only one generation of selfing (Haldane, 1930; Fig. 1). For this reason, hybrid potato breeding is more efficient at the diploid level.

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0639

2.3 Generation of homozygous diploid potato genotypes via haploidization and via inbreeding

Haploid genotypes can be generated from an egg cell (gynogenesis) or from pollen, often via anther culture (androgenesis). In potato, some haploids have been generated by anther cultures. The resulting haploids were converted into diploids by chromosome doubling. However, the resulting homozygous diploids were very weak and sterile (van Breukelen et al., 1977; Uijtewaal et al., 1987b), hampering their usage in breeding.

Haploidization has been more successful in crossable species like *S. chacoense* (Cappadocia, 1990; Phumichae et al., 2005; Phumichae and Hosaka, 2006) and *S. phureja* (Chani et al., 2000).

A reason for the failure to produce vigorous doubled haploids may be the transition to complete homozygosity in one step. Inbreeding depression may be so severe that homozygous plants are too weak to survive. Repeated selfing, on the other hand, might lead to a more gradual improvement of homozygosity. However, in potato, inbreeding is seriously limited by self-incompatibility, which prevents self-fertilization. Still some rare examples of homozygous diploid plants have been generated by inbreeding but again the homozygous diploid plants always showed a strong inbreeding depression, which limited their usage in breeding (De Jong and Rowe, 1971; Charlesworth and Willis, 2009).

2.4 Large genetic variation in potato causes inbreeding depression

The tetraploid and outcrossing nature of commercial potato is likely responsible for the large genetic variation. In a study on the allelic composition of 800 genes in 83 potato cultivars, an average frequency of 3,2 alleles per locus within a genotype was identified (Uitdewilligen et al., 2013). Among the 83 cultivars, often more than ten alleles per locus

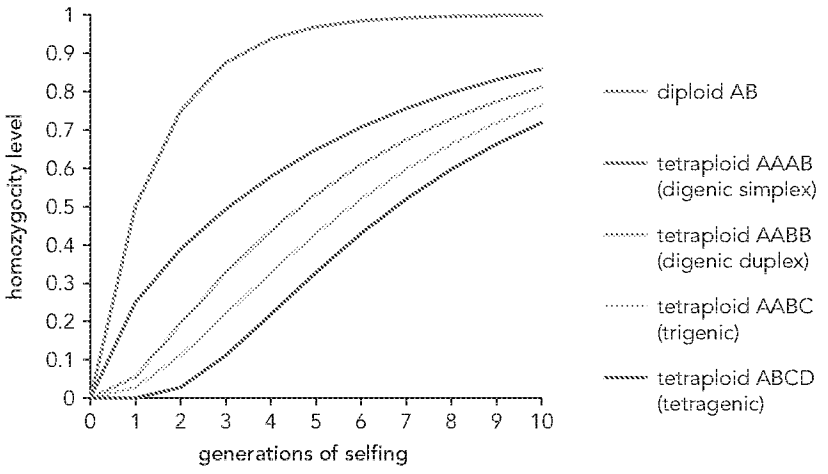


Figure 2 The theoretical increase in homozygosity in diploids and tetraploids through inbreeding, adjusted from Haldane et al. (1930).

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0640

were observed. In addition, the frequency of single nucleotide polymorphisms (SNPs) in potato is 1 in 15–30 base pairs (PGCS, 2011; Visser et al., 2014). This means that the genetic distance between the two sets of chromosomes within one diploid potato genotype is four times larger than the genetic distance between the genomes of man and chimpanzee (CSAC, 2005).

The high frequency of allelic variation has the inevitable consequence that 'weak alleles' that have a negative effect on plant fitness are maintained. Such alleles remain hidden in the large buffer of four genomes, but reveal themselves upon inbreeding when the chance for homozygosity increases. This is even more manifested at the diploid level where the homozygosity level more rapidly increases upon inbreeding (Fig. 2).

This large genetic variation is also helpful for breeding as it forms a genetic reservoir of useful genes. It is a challenge to identify alleles that contribute most to plant performance. As potato has 39000 genes, the identification and usage of the 'best alleles' per locus, including interactions between them (epistasis), will gradually take place over decades of research and breeding (PGSC, 2011). Corn may serve as a good example, whereby, after a century of dedicated breeding by numerous breeders worldwide, a genetic gain of over 1% per year is still achieved (Troyer, 2006).

2.5 Crossable diploid species and tetraploids increase genetic reservoir for diploid breeding

The potato germplasm available for breeding comprises many species, including diploid species (Jansky and Peloquin, 2006). These have been used as source to introduce resistance genes into cultivated germplasm. Breeders often use diploid potato to rapidly combine favourable traits that can be introduced into the tetraploid germplasm by direct crossings, bridge crossings or via chromosome doubling (De Mainea, 1982; Chauvin et al., 2003). The diploid breeding programme at Wageningen University (Hutten, 1994) has generated donor lines that harbour the most important traits for potato breeding (Table 1). Additional diploid germplasm is available from potato gene banks and public research institutes such as University of Wisconsin-Madison, United States; Potato Germplasm Enhancement Laboratory, Japan; Gene Bank at Gatersleben, Germany and International Potato Centre, Peru.

Another source of diploid germplasm is tetraploids that can be prickle pollinated to generate diploid offspring, designated dihaploids (Uijtewaal et al., 1987a). A collection of dihaploids obtained from one tetraploid harbours the full set of genes from the tetraploid and can be exploited in a diploid potato breeding programme.

In conclusion, the large genetic variation in potato and in its wild relatives, combined with the technology to switch between ploidy levels, provides a tremendous wealth of germplasm available for diploid hybrid breeding.

2.6 Diploid potato may perform equal to tetraploids

Most important food crops such as rice, corn and soybean are diploid. Sugar beet cultivars were initially tetraploid, then triploid and since 2000, all new cultivars are diploid. In potato, it has long been assumed that tetraploids outperform diploids (Rowe, 1967; Hutten et al., 1994). Occasional observations have contradicted this assumption: Progeny of diploid potato USW4 with *S. chacoense* M6 produced large tubers and high yield (Lipman and

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0641

Table 1 The *Sli*-gene donor, designated DS and 16 diploid potato germplasm, designated D1–D16, used for hybrid breeding at Solynta. The trait abbreviations are according to Hutten (1994)

Abbreviation	Short description
DS	*Sli*-gene donor
D1	Early (maturity), long, *Y*, Qcook
D2	Early, *Y*, Qcook
D3	*R3*, *H1*, *Gpa2*, *RXadg*, *Y* (yellow flesh)
D4	Grp1, early, long (shape), *Ro1* (*H1*?)
D5	Early, long, *Y*, Qfry
D6	Long, *Y*, Qfry, *H1*, Qcook
D7	Early, long, *Y*, *H1*, Qcook, *Zep* (orange flesh)
D8	Early, *y* (white flesh)
D9	Qstarch, *Y*
D10	Wild species hybrid: *phyt avl*
D11	Wild species hybrid: *phyt rch*
D12	Round (shape), Qcook, Qfry
D13	Early, round, *Zep*, *Y*, Spectacled, Qcook, blue anthocyans
D14	Wild species hybrid: *phyt tar*
D15	Wild species BC1: early, *phyt vnt1*, round, *Y*, *H1*
D16	Early, round, *y* (white flesh)

Zamir, 2007; Jansky et al., 2014). Uijtewaal et al. (1987b) observed that heterozygous diploid potato outperformed all homozygous di- and tetraploid derivatives. These results from potato and from other crops support the expectation that diploid potato will eventually replace tetraploid potato for commercial usage.

3 The state of the art of hybrid potato breeding

In 2008, Solynta started its research by making crosses between diploid potato germplasm, obtained from a pre-breeding programme from Wageningen University (Rutten, 1994), and a homozygous accession of the wild species *S. chacoense*, carrying the dominant self-compatibility controller gene *Sli* (Hosaka and Hanneman, 1998a,b; Phumichai et al., 2005; Lindhout et al., 2011a). The F_1 plants were extremely vigorous and about half of them produced many berries upon self-pollination. This was considered a major breakthrough as these were, to our knowledge, the first vigorous, self-compatible diploid potato plants ever obtained. These F_1 plants were highly heterozygous. The first generation after selfing (designated F_2) should harbour at least 50% homozygous loci. As many of these loci might

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0642

harbour 'weak alleles', it was expected that F_2 plants would be too weak to survive. We also made BC_1 populations by backcrossing to the *S. chacoense* parent.

As expected, the F_2 and BC_1 showed weak growth and many died in the field. However, a number of plants survived and 10% of the surviving F_2 plants even proved self-compatible (Lindhout et al., 2011a). We generated the second inbred generation, designated F_3 and made crosses between self-compatible individual F_3 plants. These inbred plants were tested with SNP markers to confirm their genetic identity as real inbreds. These results indicated that breeding hybrid potato was now feasible (Lindhout et al., 2011b).

The weak plant performance, the poor tuber quality and the low yield of the inbreds was not only due to inbreeding depression, but was also caused by the wild *S. chacoense*, a species that hardly produces tubers. So, by this approach, we not only started a hybrid potato breeding system, but also the process of domestication of a new 'diploid potato' based on an interspecific cross of diploid *S. tuberosum* and the wild species *S. chacoense*.

We hypothesized that developing vigorous inbred lines is challenging as for each of the 39 000 loci the most favourable alleles should be identified and combined. Unfavourable genes with large effects on plant performance are identified easily and hence selecting increased plant performance is initially easy and fast. Undesired characters from the *S. chacoense* parent such as abundant stolons, small leaves and twisted, small and low-yielding tubers were removed in a few breeding generations.

Sli is a dominant gene (Phumichai et al., 2005; Phumichai and Hosaka, 2006). However, the successful expression of this gene requires a vigorous plant that is fertile and supports self-pollination. Often these criteria are not met. Therefore, the frequency of self-compatible plants is usually lower than expected based on a monogenic trait. Inbreeding depression is exhibited as weaker plant growth upon higher generations of inbreeding (Fig. 3). As a consequence, the self-compatibility level tends to decrease upon further selfings. The first inbred lines obtained by Solynta, containing over 95% homozygous

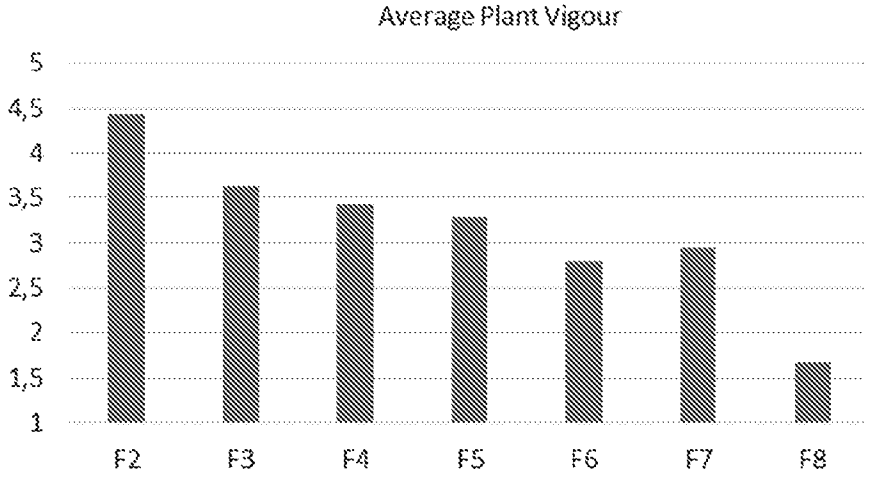


Figure 3 Inbreeding in the diploid Solynta germplasm in winter 2014–15. The Fx indicates x-1 generation of selfing after the last cross was made. Data are averaged over the complete trial consisting of over 5000 plants. The scale of plant vigour ranges from 1 = very weak via 3 = average to 5 = very strong.

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0643

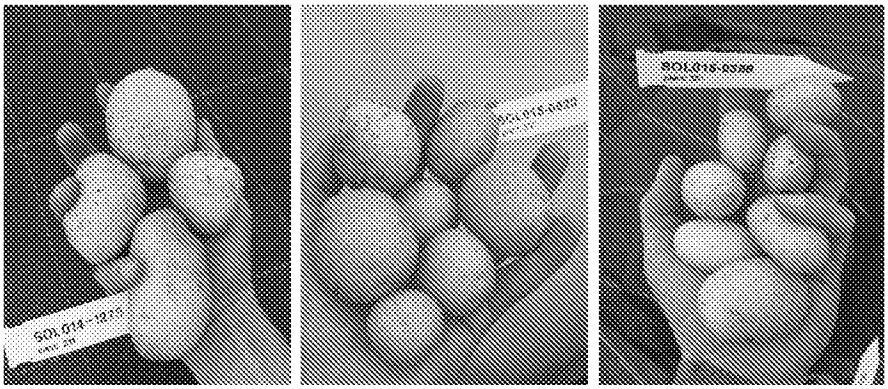


Figure 4 Examples of Solynta diploid germplasm. Plants were raised from seedlings and grown in the greenhouse in the summer season of 2014 (left panel) and 2015 (middle and right panel). They were among the best genotypes in a breeding programme comprising over 15000 plants.

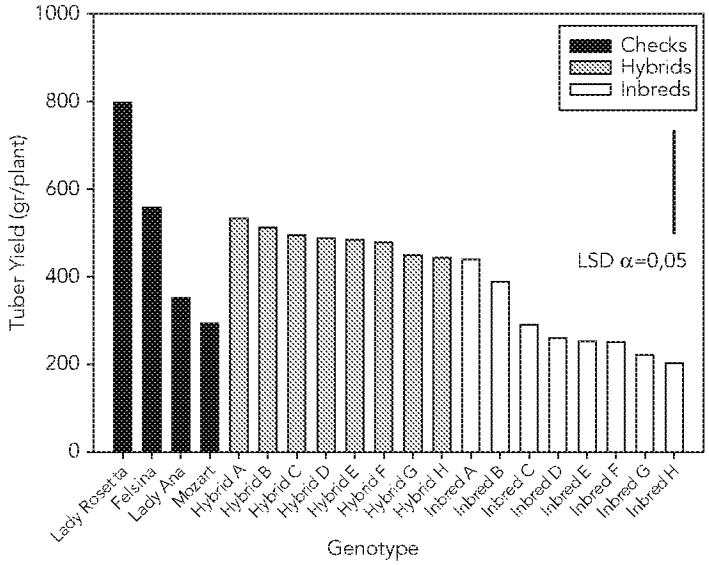


Figure 5 Hybrid performance of the first diploid experimental potato hybrids. Seed tubers were harvested from greenhouse-grown plants, raised in the winter from tubers (checks) and seedlings (hybrids and inbreds). The seed tubers were planted on 8 May 2015 in a trial field on sandy soil in Wageningen and harvested on 17 September 2015.

loci, hardly produced progenies and the seedlings were extremely weak. Therefore, new crosses were made between F_3 and F_5 inbred lines and selfing was started again from these F_1's to continue selecting parent lines that combine beneficial traits. As a result, performance of the inbreds improved over the following generations (Fig. 4).

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0644



Figure 6 Example of heterosis in diploid hybrid potato. The plants were from the same trial as in Figure 5. At the left the female F_3 parent and at the right the male F_5 parent, while the hybrid is in the middle.

In mature hybrid breeding systems, parents of the hybrids are selected based on general combining abilities (GCA), whereby molecular markers can be used to better predict the breeding value of the parents (Tobias et al., 2009). As no historic data were available on combining abilities of diploid potato parents, the selection of the first parents was based on the performance of the parents themselves.

Following the predictions of good performing inbred lines, crosses between these lines were made resulting in 45 potato hybrids which were tested in the field together with 20 inbred lines in two replicates of two plants per plot. The yields varied from 83 to 580 g/plant (Fig. 5). Thirteen hybrids scored higher than any of the inbreds, also outperforming the check variety Lady Anna, while some also showed a strong heterosis for yield (Fig. 6). The trials are being repeated in 2016 by using seed tubers raised from the field.

In addition, a new series of 216 experimental hybrids was generated in the winter-season 2014–15, mainly based on F_6 parent lines. The hybrid seeds were sown immediately after harvest and ten seedlings per hybrid were transplanted into the field in June, 2 months later than the usual seed tuber plantings. Still the yield of some hybrids was higher than 500 g/plant and the tubers were similar in size and shape to commercial seed tubers.

The results of the first experimental hybrids illustrate the potential of diploid hybrid potato varieties. As the first hybrids were randomly made without any a prior information about the combining abilities of the parents, it is expected that the next series of hybrids based on the results of these field trials will perform better and may show a stronger overlap with commercial controls.

4 Production of and commercialization of hybrid seed cultivars

The production of hybrid potato seeds is mainly done by hand pollinations. The seed yield per plant varies from hundreds to many thousands of seeds. Each successful hand pollination generates a berry with 50–150 seeds and each plant produces 5–50 berries. This

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0645

is similar to other vegetable *Solanum* crops, like tomato and pepper, where commercial seed is produced by hand pollination.

The emasculation of the flowers is a time-consuming part of the hand pollinations. Male sterility systems have been reported that make emasculations redundant and hence reduce seed production costs (Li, 2008). Alternatively, functional male sterility may be used, whereby pollen is prevented to land on the stigma of the same flower as the exerted stigma may be manually pollinated by pollen of the male parent before the own pollen may reach the stigma (Lössl et al., 2000; Abrol et al., 2012).

The transport of potato seeds over the world is very restricted. This is because the dominant breeding systems are based on the production of seed tubers and hardly any rules are in place for potato seeds. As a consequence, most countries consider potato seeds as belonging to the highest risk classes. This is remarkable as seed tubers may harbour any of over 200 species of pathogens that attack potato (Delleman et al., 2004). In contrast, only six pathogens are seed-borne (Solomon-Blackburn and Barker, 2001). These are five viruses and a viroid, which are absent in major potato-growing regions like the Netherlands. So, potato seeds are very safe and regulations will need to be adjusted accordingly.

The registration process for breeders' rights poses a similar situation: in countries which are members of the International Union for the Protection of new Varieties of Plants (UPOV), the path to commercialization of a hybrid starts with the registration process to obtain breeders' rights, which is based on seed tubers. So, protection by breeders' rights of hybrid potato seed cultivars is not yet possible. The European Union (EU) is adjusting the legislation process, but it may still take several years before this is established. Other non-UPOV countries will likely follow later.

5 Inbred lines for genetic research

Inbred lines allow the generation of mapping populations such as F_2, BC_1 and BC_2. As the parents have limited allelic variation, the signal-to-noise ratio is much higher than in studies with heterozygous tetraploid populations. Moreover, putative quantitative trait loci (QTL) can effectively be confirmed in dedicated populations that are selected to segregate for the loci under investigation, and are fixed for other regions on the genome (Wang et al., 2008; Schmalenbach and Pillen, 2009; Fu et al., 2010). In addition, new genetic populations can be generated, which are very powerful for quantitative genetic studies, like nearly isogenic lines (NILs), recombinant inbred lines (RILs) and libraries of introgression lines (Young et al., 1988; Paran et al., 1995; Jeuken and Lindhout, 2004; Finkers et al., 2007; Zhang et al., 2005; Chen et al., 2010; Viquez et al., 2014).

Genetic studies in potato have been done at the tetraploid level and at the diploid level. Tetraploids may support simple genetics like the mapping of resistance genes (Solomon-Blackburn and Barker, 2001), but quantitative studies are less reliable as the genetic noise of the numerous highly heterozygous loci is high (unexplained error). Genome-wide association studies (GWAS) at the tetraploid level will always generate hundreds of potential leads, but only a few hits may be meaningful (Li et al., 2010; D'Hoop et al., 2014). More accurate and reliable quantitative studies were done at the diploid level, initially by crossing heterozygous parents (Prasher et al., 2014) and, more recently, in a diploid F_2 population (Endelman and Jansky, 2016).

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0646

The possibility of using self-compatible, homozygous inbred lines for genetic studies offers three powerful advantages:

1 Only one allele per homozygous locus is present.
2 Only two alleles per heterozygous locus are present.
3 Backcrosses and selfings are feasible for confirmation studies.

For more than five decades, mutant studies have uncovered new alleles of important genes and have helped to confirm or determine the function of genes. Although advanced technologies like the CRISPR/Cas system (Belhaj et al., 2013) are likely to replace the methods by which mutants are made, mutants will remain powerful tools to discover unknown phenotypic traits or to study induced alleles that also have the advantage to be free of deregulation rules.

5.1 The first completely homozygous self-compatible diploid inbred line in potato

Most *Solanum* species that are crossable with cultivated potato are self-incompatible. An exception is *S. chacoense* (Hosaka and Hanneman, 1998a; Hawkes, 1990; Jansky et al., 2014) and introducing the *Sli*-gene from *S. chacoense* into cultivated diploid potato resulted in fertile self-compatible offspring (Lindhout et al., 2011a). After several rounds of inbreeding, highly homozygous self-compatible inbreds were generated. The level of homozygosity was assessed by using SNP markers to investigate the effects of inbreeding (Fig. 7). A strong correlation was observed between the overall level of homozygosity and reduced self-compatibility. By new series of crosses, selections and selfings, the agronomic

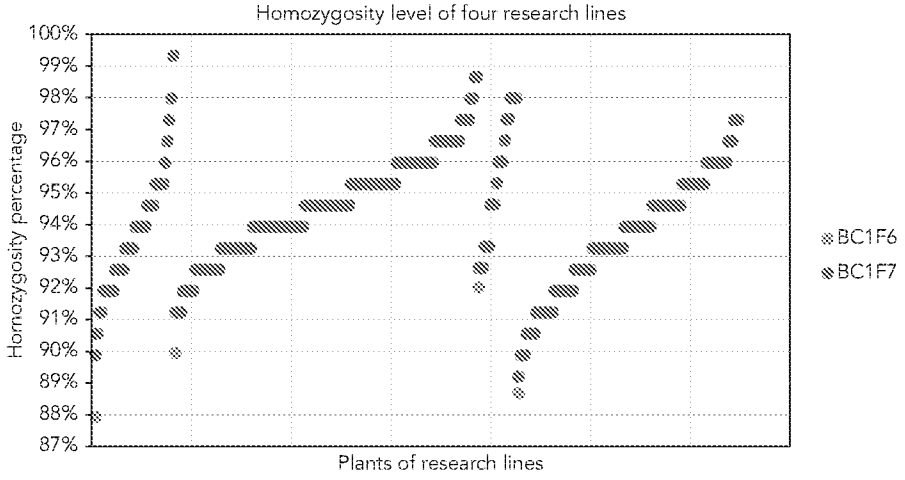


Figure 7 The increase of the level of heterozygosity in the offspring (BC_1F_7) of four BC_1F_6 plants obtained by selfing of the BC_1(D1 × F_1[DS × D1]) TQ: Please check the usage of brackets is as per style. (Table 1). The width of the bar indicates the number of plants in the progeny within the class with plants of the same homozygosity level. The calculations were based on a total of 150 markers.

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0647

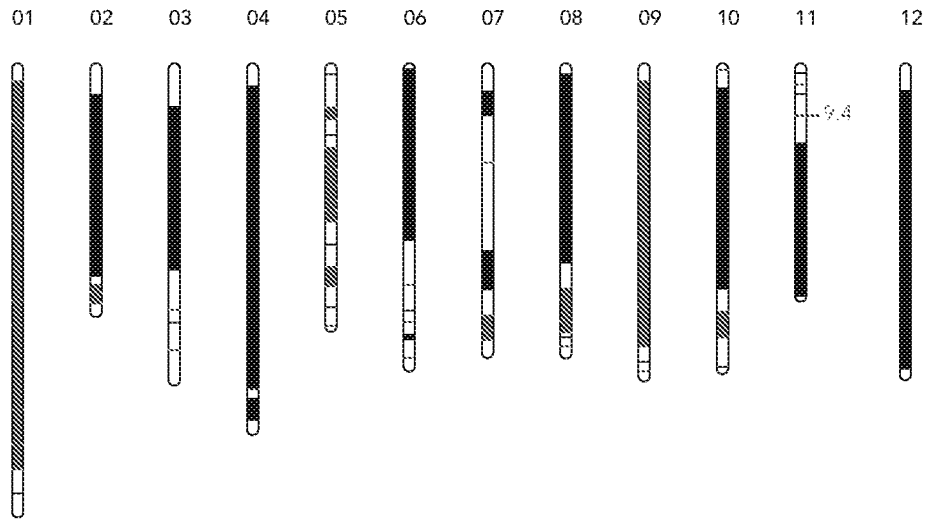


Figure 8 The first essentially homozygous self-compatible diploid potato. The position of the SNP markers is based on the published sequence (PSGC, 2011). The 12 vertical bars indicate the 12 chromosomes. Red bars indicate homozygous D1, blue bars indicate homozygous DS (Table 1). The green line on chromosome 11 indicates a heterozygous scored marker.

performance of the inbred lines continuously improved. After six generations of selfing, a homozygous self-compatible line was generated with only 1 out of 150 SNP markers still heterozygous (Fig. 8). Genotyping by sequencing techniques make it now possible to check the level of homozygosity in more detail.

5.2 Genetic studies in segregating diploid F_2 populations

Recently, Endelman and Jansky (2016) published the first results of a mapping study in an F_2 population of diploid potato. This was based on a cross between the doubled monoploid potato DM1-3 and M6, which is an S_7 inbred line derived from the self-compatible wild relative *S. chacoense*. A single F_1 plant was then self-pollinated and an F_2 population of 109 genotypes was grown, genotyped (>2200 SNPs) and phenotyped. Meijer et al. (2016) analysed an F_2 population (108 markers) based on a cross between two clones, namely DS (a homozygous diploid *S. chacoense* clone containing the self-incompatibility overcoming *Sli*-gene) and D2 (a partly heterozygous diploid *S. tuberosum* clone; see also Table 1). The results of both studies are comparable: tuber shape is associated with a region on chromosome 10, flesh colour with a region on chromosome 3 and tuber and pigment colour on chromosomes 2, 10 and 11. These QTLs were identified at the same loci as described in literature (van Eck et al., 1993, 1994). In both studies, additional QTLs were identified. Furthermore, there is an overwhelming reservoir of potential useful QTLs in the potato germplasm (Bradshaw et al., 2007) and thus also in the dihaploids that can be made. Such QTLs can now be more reliably studied at the diploid level and this will ultimately lead to the identification of the underlying genes. A limited subset of the diploid germplasm may already harbour many important traits for potato breeding (Table 1). Relevant genes

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0648

for these traits can be introgressed into vigorous and fertile diploid genotypes to develop progenies with maximum genetic fixation to minimize genetic noise. Such genotypes are unique materials for further detailed genetic studies.

5.3 Marker-assisted backcrossing

Another application for inbred lines in potato is introgression breeding. This requires knowledge of diagnostic markers for the gene of interest, preferably inside the gene, markers for the recurrent parent genome and a self-compatible homozygous backcrossing parent. There are dozens of well-studied resistance genes in potato that can be used for introgression breeding. This paves the way for a marker-assisted backcrossing (MAB) programme in potato (Frisch and Melchinger, 2005).

To this end, homozygous inbred lines are crossed with a diploid donor carrying a specific gene of interest. In two backcrosses, NILs can be generated by selection with diagnostic markers for the gene of interest and against markers in its flanking regions, combined with selection for markers well distributed over the potato genome (whole background selection). Such NILs can harbour over 98% of the recurrent genome in combination with the specific gene. Both parents of a hybrid may have an introgressed gene, resulting in a double stack hybrid. To introgress specific genes in a homozygous parental line will take 2–3 years. These MAB programmes are routinely used in other crops and are also feasible in potato (Mallick et al., 2015; Jeong et al., 2015).

6 Cropping systems based on true seeds

The production of commercial tubers in most parts of the world starts with seed tubers. These have a large reservoir of nutrients for the growing shoots, allow a rapid initial plant growth and fast leaf coverage of the soil, which is one of the most critical factors for potato yield. In contrast, potato seeds are extremely tiny, about 2500 seeds per gram. As a result, during the first period after germination the young seedlings are very vulnerable for abiotic stresses like drought, frost and heat. Field emergence has been reported between 50% and 80% with acceptable tuber yields under different experimental conditions (El-Bedewy et al., 1994; Renia, 1995). However, without a protective environment, the risk is very high that an emerging seedling will not survive, even when it is pelleted or primed.

This chapter describes alternative strategies to circumvent the exposure of week seedlings to harsh conditions.

6.1 Production of seedling tubers in greenhouse

Seedling tubers can be produced under greenhouse conditions by sowing in a medium with sufficient water supply and at optimum germination temperature of 15–20°C (Struik and Wiersema, 2012). As soon as seedlings reach 5–10 cm in length, they are transplanted in pots. The desired tuber size, the available space in the greenhouse and the length of the growing period will determine the pot size, nutrient supply, day/night temperature and light regime (for a detailed protocol see Struik and Wiersema, 2012). Densities of 80–170 plants/m^2 are common in greenhouses (Lommen, 1995; Tierno et al., 2014). There is a trade-off between number of tubers, size of tubers, planting density and time to harvest.

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0649

Depending on the exact conditions, end-product requirements and production costs, the optimal combination is chosen. Alternatively, hydroponic techniques are available, whereby the roots are grown in a dark, humid and soilless environment in two layers, one for nutrient uptake and the other layer for producing mini-tubers (Lommen, 2007). The seedling tubers are picked at regular intervals and the total yield per plant may reach dozens of tubers. The soilless culture assures clean seed tubers. The conditions and picking regime are set to have optimal numbers and tuber sizes.

6.2 Production of seedlings for commercial crop

Greenhouse-grown seedlings can also be used to start the cultivation of a commercial crop. This system is equivalent to the one used for lettuce, leek, cabbage and onion (Leskovar et al., 2014). Technically, commercial potato production from seedlings is feasible and maximizes the benefits of true seeds. When potato transplants are grown as a ware potato crop, a whole new cultivation system must be developed. Important elements are: transplanting systems, plant spacing including ridging or bedding, the use of soil coverage, weed control, irrigation and harvesting methods. Further mechanization and dedicated cultivation systems will be optimized for cropping systems that start with potato seedlings (Roy et al., 2015). In Kenya, tuber yields of 30 tonnes/ha were obtained, whereby seedling transplants were used as starting material for a commercial cultivation (Muthoni et al., 2014). This already represented 50% higher yield than average in Kenya (Wang'om and van Dijk, 2013).

6.3 Seedling tubers as starting materials for a commercial crop

Seedling tubers are equivalent to mini-tubers that are produced from tissue culture, which is routinely done to start a new multiplication round with clean basic seeds (Amin et al., 2014). They are certified as G1 material. The great advantage of seedlings are the reduced costs, compared to *in vitro* grown plants, and the flexibility to start the production whenever and wherever needed, as seeds can easily be stored and transported. As the cost to produce mini-tubers from *in vitro* grown plants is very high, in the present potato system at least three rounds of field multiplications are needed to dilute these high costs over many seed tubers. For a hybrid seed system, the cost of producing seedling tubers is much lower and hence fewer propagation rounds are needed. Therefore, seedling tubers should be multiplied only 1 year and then released to commercial farmers. Such a system also fits better to the fast introduction of new cultivars, which is typical for a hybrid breeding system.

6.4 Production of seed tubers from seedlings in field

At present, the production of seedling tubers is mostly done under tropical conditions. Seeds are sown in a simple greenhouse or in the field under plastic cover with plant densities of 80–100 plants/m^2 (Kumar, 2014; Struik and Wiersema, 2012; Fig. 9). When the seedlings have reached 5–10 cm in length, they are transplanted to the field, in ridges, at a defined plant density. Additional hilling will increase the number of seed tubers per plant (Wiersema, 1986). To decrease the risk of root damage, the complete substrate is transplanted with the seedling. Plantlets need some time to adapt to outdoor conditions before transplanting directly to the soil (Gopal, 2004). In South Asia, transplants are

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0650



Figure 9 The first seedlings of the first diploid hybrid potato hybrids in Democratic Republic of Congo.

planted on the sides of the ridges to benefit from the shade and higher soil humidity. The planting distance may be adjusted to compensate for a shorter growing season compared to seed tubers, if appropriate. The cultivation conditions are similar to traditional potato cultivation systems. In Egypt, seed tuber yields of 40–60 tonnes/ha were obtained in such a system, based on tetraploid TPS populations (El-Yazied et al., 2004).

Seedlings are more sensitive to frost and drought than seed tubers. Thus, transplanting is done in a frost-free season and with irrigation. Compared to the traditional systems of producing seed tubers, whereby the tubers are planted far before the last night with frost, the length of the growing season of transplants may be 2 months shorter. In addition, the plant development may be further delayed due to a transplanting shock and weak initial growth. Hence, tuber numbers are lower and tuber sizes are smaller compared to seed tuber grown plants, causing severe yield reductions. Plant density may be increased to compensate for these reduced yields per plants.

7 Case studies

7.1 Combatting *Phytophthora infestans*

Late blight, caused by the oomycete *Phytophthora infestans*, was responsible for the Irish famine of 1845–47 (Fry, 2008). All potato cultivars were susceptible to the disease and suffered severe yield losses that led to food shortage. Since then, breeders have selected cultivars with field resistance and from the early twentieth century onwards, have introduced specific *R*-genes, often sourced from wild relatives. However, cultivar Pentland Dell, which

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0651

carries three *R*-genes, already turned out susceptible to a new virulent race of the fungus even before its widespread use and just 4 years after its introduction (Malcomsum, 1969).

At present, the optimal way to control *P. infestans* is a combined approach of clean seed tubers, clean soils, early and preventive chemical protection and destruction of crop debris after harvest. The global annual cost of cultivation measures and yield loss is estimated at US$5 billion (Duncan, 1999).

P. infestans reproduces mainly clonally. With hundreds of billions of spores produced per hectare in an infected crop (Skelsey et al., 2010) and a mutation rate of $1:10^9$, mutations in any given gene of *P. infestans* are likely to occur in a disease-infected field. Sexual recombination, combined with the redundancy of several effectors that are recognized by *R*-genes, explains why *P. infestans* easily mutates effector genes and develops virulence (Jiang and Tyler, 2012).

As a result of the high genetic variation in potato, dozens of resistance sources have been identified and are available for breeding (Park et al., 2009). Whereas single genes are easily defeated by virulent races to *P. infestans*, combinations of *R*-genes are more effective, although the Pentland Dell case indicates that a more dynamic approach may be needed (Niks et al., 2011).

Ideally, isogenic cultivars are developed that only differ in the combination of *R*-genes. This would allow to deploy the most suitable cultivar, dependent on the epidemiology of *P. infestans*. However, the introduction of one gene from a wild related species into a tetraploid cultivar by traditional breeding already takes several decades, and to combine different *R*-genes in a breeding programme is simply too complicated.

Since 1990, many *R*-genes to *P. infestans* have been mapped and cloned (Ballvora et al., 2002; Huang et al., 2005; Park et al., 2009). These all belong to the so-called class of 'NBS/LRR genes' and have a cytoplasmic interaction with effector genes of *P. infestans*, resulting in defence responses that block the growth of the pathogen (Jones and Dangl, 2006). A genetic modification (GM) approach to develop a series of isogenic cultivars with different *R*-genes from crossable species is being pursued (Haverkort et al., 2016, Jacobsen and Schouten, 2008). These so-called cisgenic plants might fall under the highly costly and complex GM legislation, which would hamper their commercial opportunities.

The hybrid breeding system offers a clear path towards resilient resistance to *P. infestans*: *R*-genes can be stacked in a potato hybrid via marker-assisted introgression (Park et al., 2009). Two genes can be combined via the two parents in 2–3 years, and additional *R*-genes can be added within a year to generate multi-stack resistance hybrids. In this way, series of *R*-gene isogenic hybrids can be generated as a dynamic resource to select the best combination of *R*-genes to protect the crop against the prevailing races of *P. infestans*.

7.2 Hybrid potato breeding for East Africa

Hybrid potato cultivars will bring great benefits, not only to modern commercial farmers in the developed world, but they may even have a greater social impact in tropical regions where the population rely on potato as a major source of energy and nutrition (FAOstat, 2016). It is very challenging to start an initiative to develop hybrid potato for these regions (Thomas-Sharma et al., 2016; Kumara et al., 2015). When legal and physical protection of the breeding germplasm is not secured in these regions, the development of inbred lines and the hybrid crosses are done elsewhere. The implementation of hybrid potato cultivars in these regions requires considerable investments and strong cooperation of committed

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0652

Table 2 Potato area and production in six East-African countries in 2014 (FAOstat, 2016)

Country	Area (000 ha)	Production (000 tonnes)	Yield (tonnes/ha)
Burundi	24.4	181.2	7.4
Kenya	115.6	1626.0	14.1
Rwanda	166.4	2225.1	13.4
Tanzania	211.5	1761.0	8.3
Uganda	39.0	188.0	4.8
Ethiopia	67.4	921.8	13.7

AQ: We have changed 'T' to 'tonnes' in "Table 2". Please check if this is correct?

public and private partners. As an example, the implementation of hybrid potato cultivars for East Africa is briefly described below.

The highlands of East Africa are traditionally important production areas for potato because the crop is an important component of the local diet (Table 2). However, yields remain very low (Table 2). A range of traditional varieties is used from local sources (Kaguongo et al., 2008) as well as improved material from the International Centre for Potato (CIP). Seed tubers are produced by farmers and storage conditions are far from optimal (Kaguongo et al., 2008; Gildemacher et al., 2009). Janssens et al. (2013) concluded that bacterial wilt, lack of clean seed tubers and poor storage are the most prominent production constraints. Gildemacher et al. (2009) showed that only 3% of the seed tubers sold were free of viruses.

True hybrid potato seeds are devoid of contaminating pathogens and therefore offer an excellent opportunity to potato production improvement in East Africa. Such hybrids should be attuned to the needs of the farmers, who grow their crop at the typical local conditions like a short growing cycle of 90–100 days, high temperatures and tuber development under short days. The prerequisites for establishing dedicated hybrid potato breeding system for East Africa are:

- A (private) organization executing a breeding programme tailored to the needs of the region.
- Secured supply of hybrid seeds for the region.
- Regulations supporting imports and exports of seeds, seed tubers and commercial tubers.
- Formal registration system for breeders' rights protection.
- Local organizations testing new experimental hybrids.

Such breeding programme can only become sustainable if the complete downstream part of potato food chain is also well organized. This includes the following:

- Production systems of disease-free seed tubers from seedlings.
- An efficient supply system for farmers to obtain hybrid cultivars.
- Efficient farmers' cropping systems to produce high quality potato tubers for the target markets.
- Efficient logistics to transport farm produce to consumers and processors.

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0653

- A well-developed consumer market.
- A business plan over the whole potato chain, whereby all stakeholders benefit.

Solynta is already testing the first experimental hybrids in the highlands of the Albertine Rift in Ituri Province, Democratic Republic of Congo. These are experimental hybrids derived from the European hybrid breeding programme. At a local farm, established by the Lake Albert Foundation, seeds have been sown and seedlings have been transplanted into the field (Fig. 9). These hybrids are evaluated in good cooperation with local farmers and the results are shared with the Solynta breeding programme. These data are used to adjust the selection of inbred lines to the needs in East Africa and to continuously generate new experimental hybrids, which will be tested at the Congo farm again. This iterative and interactive process may already select the first dedicated hybrids for East Africa in 2–3 years.

This breeding programme is accompanied by research on cropping systems for the region. In addition, training programmes for research institutions, agronomists, local staff and interested farmers in the regions will make the farmers' communities and relevant institutions familiar with the new concepts of hybrid potato cultivars. Also market studies are needed to identify and secure stable and sustainable markets for the farmers' potato products. This market may comprise other countries in the Great Lakes Region (South Sudan, Uganda, Burundi and Rwanda).

The support for this programme by national authorities and development agencies – also in the neighbouring countries like Uganda – is required, but it will take time before the concept of hybrid potato cultivars is fully understood and accepted.

8 Conclusion

Since the first crosses in 2008, the Solynta research efforts have been focused on the development of a hybrid potato breeding system. This research has now reached the stage where hybrid potato breeding will become reality. This has recently been supported by two leading potato breeding companies in EU, KWS and HZPC, who have also expressed their conviction that hybrid potato breeding will be future main breeding system (KWS, 2016). In addition, 21 leaders in the industry and potato science in the United States have expressed their opinion on 'Reinventing potato as a diploid inbred line-based crop' with a scientific base for diploid hybrid potato breeding (Jansky et al., 2016).

We have made great advances in the development of useful homozygous inbred lines and the first field evaluations of experimental hybrids have shown the potential of hybrid cultivars to harvest heterosis.

The technologies to develop new cropping systems adapted to various climate zones and agronomic practices, which allow the production of commercial seed tubers from seedlings, are already available.

We envision that future hybrid potato varieties, similar to modern tomato hybrids, will harbour up to 15 resistance genes. In addition, the lifetime of new potato hybrid cultivars will be reduced to less than 5 years as is the experience in sugar beet, where the lifetime of new cultivars is only 2–3 years since the first diploid hybrids have been introduced into the market.

Our inbred lines will also be of great value for research purposes as they allow the development of sophisticated populations that are very helpful in genetic and genomic

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

studies as has been shown in other crops. This will give a boost in the exploration and exploitation of the genetic variation in the potato germplasm.

The self-compatible potato inbred lines will greatly stimulate quantitative research on the genetic control of complex traits such as interaction with mycorrhiza, biotic stresses, nutrient uptake, nutrition value and processing quality.

The application of hybrid potato breeding technology will not be restricted to the most advanced research groups in the public or private institutions. New players in the scientific and applied breeding field of the potato business will arise and new cooperations will be established to take full advantage of the hybrid breeding technology in science and product development.

Finally, hybrid potato breeding will require the skills of the breeder as well as the knowledge and tools of scientists. As a result, a new generation of potato breeding teams will be established combining the skills of the breeder with the in-depth knowledge of plant scientists.

9 Where to look for further information

This chapter describes the first implementation of a hybrid potato breeding strategy and the progress that is made since 2008. We direct the interested reader to the papers of Almekinders (1996, 2009) to learn about 'conventional TPS'. As a textbook on plant breeding, we suggest Brown and Cagliari (2011), while more advanced potato genetics and genomics is found in Bradeen and Kole (2011). The history and mechanism of *Phytophthora* attack is well described by Fry (2008). Finally, FAO (FAOstat, 2016) statistics provide numerous data on potato cultivation.

Leading centres of research include Wageningen University in The Netherlands, James Hutton Institute in the United Kingdom, the International Potato Centre in Peru, Wisconsin University and Michigan State University both in the United States, whereby the most recent paper of Jansky et al. (2016) can be considered a must for anybody interested in hybrid potato breeding.

10 Acknowledgements

We are grateful to Jan Leemans and Herman Fleer for critically reading and reviewing this manuscript.

11 References

Abrol, D. P. (2012). Pollination for hybrid seed production. In *Pollination Biology. Biodiversity Conservation and Agricultural Production*. Publisher Springer Netherlands, pp. 397–411.

Almekinders, C. J. M., Chujoy, E. and Thiele, G. (2009). The use of true potato seed as pro-poor technology: The efforts of an international agricultural research institute to innovating potato production. *Pot. Res.* 52, 275–93.

Almekinders, C. J. M., Chilver, A. S. and Renia, H. M. (1996). Current status of the TPS technology in the world. *Pot. Res.* 39, 289–303.

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Amin, N., Amin, A. R., Roy, T. S., Ali, M. A., Rashid, M. M., Hossain, M. M. and Hasan, N. (2014). Bulking behavior of seedling tubers derived from true potato seed as affected by its size and harvesting time. *App. Sci. Rep.* 8, 1–8.

Ballvora, A., Ercolano, M. R., Weiss, J., Meksem, K., Bormann, C. A., Oberhagemann, P., Salamini, F. and Gebhardt, C. (2002). The *R1* gene for potato resistance to late blight (*Phytophthora infestans*) belongs to the leucine zipper/NBS/LRR class of plant resistance genes. *Plant J.* 30, 361–71.

Belhaj, K., Chaparro-Garcia, A., Kamoun, S. and Nekrasov, V. (2013). Plant genome editing made easy: targeted mutagenesis in model and crop plants using the CRISPR/Cas system. *Plant Meth.* 9, 39.

Birchler, J. A., Yao, H., Chudalayandi, S., Vaiman, D. and Veitia, R. A. (2010). Heterosis. *Plant Cell* 22, 2105–12.

Bradeen, J. M. and Kole, C. (2011). *Potato Genetics: Genetics, Genomics and Breeding of Potato.* CRC Press, USA

Bradshaw, J. E., Hackett, C. A., Pande, B., Waugh, R. and Bryan, G. J. (2007). QTL mapping of yield, agronomic and quality traits in tetraploid potato (*Solanum tuberosum* subsp. *tuberosum*). *Theor. Appl. Genet.* 116, 193–211.

Brown, J. and Caligari, P. (2011). *An Introduction to Plant Breeding.* Wiley-Blackwell, USA, p. 224.

Cappadocia, M. (1990). Wild Potato (*Solanum chacoense* Bitt.). In Y. P. S. Bajaj (ed.), *Vitro Production of Haploids. Biotechnology in Agriculture and Forestry, Vol. 12 Haploids in Crop Improvement I*, pp. 514–29. Springer-Verlag, Berlin, Heidelberg.

Chani, E., Veilleux, R. E. and Boluarte-Medina, T. (2000). Improved androgenesis of interspecific potato and efficiency of SSR markers to identify homozygous regenerants. *Plant Cell Tissue Organ Cult.* 60, 101–12.

Charlesworth, D. and Willis, J. H. (2009). The genetics of inbreeding depression. *Genetics* 10, 783–96.

Chauvin, J. E., Souchet, C., Dantec, J. P. and Ellisseche, D. (2003). Chromosome doubling of 2x *Solanum* species by oryzalin: method development and comparison with spontaneous chromosome doubling *in vitro*. *Plant Cell Tissue Organ Cult.* 73, 65–73.

Chen, X., Niks, R. E., Hedley, P. E., Morris, J., Druka, A., Marcel, T. C., Vels, A. and Wauh, R. (2010). Differential gene expression in nearly isogenic lines with QTL for partial resistance to *Puccinia hordei* in barley. *Genomics* 11, 629.

Crow, J. F. (1998). 90 Years ago: The beginning of hybrid maize. *Genetics* 148, 923–8.

CSAC (2005). Initial sequence of the chimpanzee genome and comparison with the human genome. *Nature* 437, 69–87.

D'hoop, B. B., Keizer, P. L. C., João Paulo, M., Visser, R. G. F., Van Eeuwijk, F. A. and Van Eck, H. J. (2014). Identification of agronomically important QTL in tetraploid potato cultivars using a marker-trait association analysis. *Theor. Appl. Genet.* 127, 731–48.

De Jong, H. and Rowe, P. R. (1971). Inbreeding in cultivated diploid potatoes. *Pot. Res.* 14, 74–83

De Mainea, M. J. (1982). An evaluation of the use of dihaploids and unreduced gametes in breeding for quantitative resistance to potato pathogens. *J. Agric. Sci.* 99, 79–83.

Delleman, J., Mulder, A. and Turkensteen, L. J. (2004). *Potato Diseases: Diseases, Pests and Defects.* Potatoworld and NIVAP, The Hague, the Netherlands.

Douches, D. S., Maas, D. J., Astrzebski, K. and Chase, R. W. (1996). Assessment of potato breeding progress in the USA over the last century. *Crop Sci.* 36, 1544–52.

Duncan, J. M. (1999). *Phytophthora*-an abiding threat to our crops. *Microbiol. Today* 26, 114–16.

Duvick, D. N. (2005). The contribution of breeding to yield advances in maize (Zea mays L.). *Adv. Agron.* 86, 83–145.

El-Bedewy, R., Crissman, C. and Cortbaoui, R. (1994). Progress report. Egypt's seed system based on true potato seed. *CIP Circular* 20, 5–8.

El-Yazied, A., Elminiawy, S. E., Hamoud, N. K. and El-Kheima, S. (2004). Seed tuber production of some hybrids using true potato seed. Mansoura University. *J. Agric. Sci.* 32, 1329–41.

Endelman, J. B. and Jansky, S. H. (2016). Genetic mapping with an inbred line-derived F_2 population in potato. *Theor. Appl. Genet.* 1–9

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0656

FAO, IFAD and WFP. (2015). The State of Food Insecurity in the World 2015. Meeting the 2015 international hunger targets: taking stock of uneven progress. Rome, FAO.

FAOstat. (2016). http://faostat3.fao.org/home/E

Finkers, R., Van Heusden, A. W., Meijer-Dekens, F., Van Kan, J. A. L. and Lindhout, P. (2007). The construction of a *Solanum habrochaites* LYC4 introgression population and the identification of QTLs for resistance to *Botrytis cinerea*. *Theor. Appl. Genet.* 114, 1071–80.

Frisch, M. and Melchinger, A. E. (2005). Selection theory for marker-assisted backcrossing. *Genetics* 170, 909–17.

Fry, W. (2008). Plant diseases that changed the world *Phytophthora infestans*: the plant (and *R* gene) destroyer. *Molec. Pl. Path.* 9, 385–402.

Gildemacher, P. R., Demo, P., Barker, I., Kaguongo, W., Woldegiorgis, GT., Wagoire, W. W., Wakahiu, M., Leeuwis, C. and Struik, P. C. (2009). A Description of seed potato systems in Kenya, Uganda and Ethiopia. *Am. J. Pot. Res.* 86, 373–82.

Gopal, J. (2004). True potato seed: Breeding for hardiness. In A. Haneafi (ed.), Sixth Triennial Congress of the African Potato Association. *Proc. APA Congr.*, 5–10 April, Agadir, Morocco, pp. 39–57.

Gopal, J. (2014). Heterosis breeding in potato. *Agric. Res.* 3, 204–17.

Haldane, J. (1930). Theoretical genetics of autopolyploids. *J. Genet.* 22, 359–72.

Haverkort, A. J., Boonekamp, P. M., Hutten, R., Jacobsen, E., Lotz, L. A. P., Kessel, G. J. T., Vossen, J. H. and Visser, R. G. F. (2016). Durable late blight resistance in potato through dynamic varieties obtained by cisgenesis: Scientific and societal advances in the DuRPh project. *Pot. Res.*, 59, 35–66.

Hawkes, J. G. (1990). *The Potato: Evolution, Biodiversity, and Genetic Resources*. Belhaven Press, London.

Hosaka, K. and Hanneman, R. E. (1998a). Genetics of self-compatibility in a self-incompatible wild diploid potato species *Solanum chacoense*. 1. Detection of an *S* locus inhibitor (*Sli*) gene. *Euphytica* 99, 191–7.

Hosaka, K. and Hanneman, R. E. (1998b). Genetics of self-compatibility in a self-incompatible wild diploid potato species *Solanum chacoense*. 2. Localization of an *S* locus inhibitor (*Sli*) gene on the potato genome using DNA markers. *Euphytica* 103, 265–71.

Hua, J., Xing, Y., Wu, W., Xu, C., Sun, X., Yu, S. and Zhang, Q. (2003). Single-locus heterotic effects and dominance by dominance interactions can adequately explain the genetic basis of heterosis in an elite rice hybrid. *Proc. Natl. Acad. Sci. USA* 100, 2574–9.

Huang, S., Van der Vossen, E. A., Kuang, H., Vleeshouwers, V. G., Zhang, N., Borm, T. J., Van Eck, H. J., Baker, B., Jacobsen, E. and Visser, R. G. (2005). Comparative genomics enabled the isolation of the *R3a* late blight resistance gene in potato. *Plant J.* 42, 251–61.

Hutten, R. C. B. (1994). Basic Aspects of Potato Breeding Via the Diploid Level. PhD Thesis, Wageningen University, p. 93.

Hutten, R. C. B., Schippers, M. G. M., Hermsen, J. G. Th. and Jacobsen, E. (1994). Comparative performance of diploid and tetraploid progenies from 2x.2x crosses in potato. *Euphytica* 81, 187–92.

Jacobsen, E. and Schouten, H. J. (2008). Cisgenesis, a new tool for traditional plant breeding, should be exempted from the regulation on genetically modified organisms in a step by step approach. *Pot. Res.* 51, 75.

Jansky, S. H. and Peloquin, S. J. (2006). Advantages of wild diploid *Solanum* species over cultivated diploid relatives in potato breeding programs. *Genet. Res. Crop Evol.* 53, 669–74.

Jansky, S. H., Charkowski, A. O., Douches, D. S., Gusmini, G., Richael, C., Bethke, P. C., Spooner, D. M., Novy, R. G., De Jong, H., De Jong, W. S., Bamberg, J. B., Thompson, A. L., Bizimungu, B., Holm, D. G., Brown, C. R., Haynes, K. G., Sathuvalli, V. R., Veilleux, R. E., Miller Jr., J. C., Bradeen, J. M. and Jiang, J. M. (2016). Reinventing potato as a diploid inbred line-based crop. *Crop Sci.* 56, 1–11.

Jansky, S. H., Chung, Y. S. and Kittipadakul, P. (2014). M6: A diploid potato inbred line for use in breeding and genetics research. *J. Plant Registr.* 8, 195–9.

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0657

Janssens, S. R. M., Wiersema, S. G., Goos, H. and Wiersema, W. (2013). The value chain for seed and ware potatoes in Kenya; Opportunities for development LEI. Memorandum 13-080, p. 57.

Jeong, H-S., Jang, S., Han, K., Kwon, J-K. and Kang, B-C. (2015). Marker-assisted backcross breeding for development of pepper varieties (*Capsicum annuum*) containing capsinoids. *Molec. Breeding* 35, 226.

Jeuken, M. J. W. and Lindhout, P. (2004). The development of lettuce backcross inbred lines (BILs) for exploitation of the *Lactuca saligna* (wild lettuce) germplasm. *Theor. Appl. Genet.* 109, 394–401.

Jiang, R. H. Y. and Tyler, B. M. (2012). Mechanisms and evolution of virulence in oomycetes. *Ann. Rev. Phytop.* 50, 295–318.

Jones, J. D. G and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–9.

Kaguongo, W., Gildemacher, P., Demo, P., Wagoire, W., Kinyae, P., Andrade, J., Forbes, G., Fuglie, K., and Thiele, G. (2008). Farmer practices and adoption of improved potato varieties in Kenya and Uganda. International Potato Center (CIP), Lima, Peru. Social Sciences Working Paper 2008–5. 85 p.

Kumar, V. (2014). True potato seed technology – Prospects and problems. In N. K. Pandey, D. K. Singh and R. Kumar (eds), *Current Trends in Quality Potato Production, Processing and Marketing*, pp. 175–82. Central Potato Research Institute, India.

Kumara, N. S., Govindakrishnan, P. M., Swarooparani, D. N., Nitin, Ch. Surabhi, J. and Aggarwal, P. K. (2015). Assessment of impact of climate change on potato and potential adaptation gains in the Indo-Gangetic Plains of India. *Intern. J. Pl. Prod.* 9 (1), 151–70.

KWS press release (2016). http://www.kws.com/aw/KWS/company-info/Products/Potatoes/News-Articles/~hjqx/KWS-to-fully-focus-on-hybrid-potato-bree/

Leskovar, I. D., Crosby, M. K., Palma, A. M. and Edelstein, M. (2014). Vegetable crops: Linking production, breeding and marketing. In R. G. Dixon and E. D. Aldous (eds), *Horticulture: Plants for people and places, Volume 1: Production Horticulture*, pp. 75–96. Springer Netherlands, Dordrecht.

Li, L., Paulo, M.-J., Van Eeuwijk, F., and Gebhardt, C. (2010). Statistical epistasis between candidate gene alleles for complex tuber traits in an association mapping population of tetraploid potato. *Theor. Appl. Genet.* 121, 1303–10.

Li, X.-Q. (2008). Male sterility systems for hybrid seed production in Brassica crops. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources* 3, 1–14.

Lindhout, P., Meijer, D., Schotte, T., Hutten, R. C. B., Visser, R. G. F. and Van Eck, H. J. (2011a). Towards F1 hybrid seed potato breeding. *Pot. Res.* 54, 301–12.

Lindhout, W. H., Schotte, T. P., Visser, R. G. F., Van Eck, H. J. and Hutten, R. C. B. (2011b). Hybrid seed potato breeding. European Patent Office nr EP 2514303 A1

Lippman, Z. B. and Zamir, D. (2007). Heterosis: revisiting the magic. *Trends Genet.* 23, 60–6.

Lommen, W. J. M. (1995). Basic studies on the production and performance of potato minitubers. PhD Thesis, Wageningen Agricultural University, Wageningen, The Netherlands, p. 181.

Lommen, W. J. M. (2007). The canon of potato science: Hydroponics. *Pot. Res.* 50, 315–18.

Lössl, A., Götz, M., Braun, A. and Wenzel, G. (2000). Molecular markers for cytoplasm in potato: Male sterility and contribution of different plastid-mitochondrial configurations to starch production. *Euphytica* 116, 221–30.

Malcolmson, J. F. (1969). Races of *Phytophthora infestans* occurring in Great Britain. *Trans. Br. Mycol. Soc.* 53, 417–23.

Mallick, N., Vinod, Sharma, J. B., Tomar, R. S., Sivasamy, M. and Prabhu, K. V. (2015). Marker-assisted backcross breeding to combine multiple rust resistance in wheat. *Plant Breed.* 134, 172–7.

Meijer, D. A., Abdullah, S., Rothengatter, R., Van Eck, H. J., Visser, R. G. F., Lindhout, P. and Van Heusden, S. (2016). An F_2 QTL mapping study in a cross between *S. tuberosum* and *S. chacoense*. *Theor. Appl. Genet.* (submitted).

Muthoni, J., Shimelis, H., Melis, R. and Kinyua, Z. M. (2014). Response of potato genotypes to bacterial wilt caused by *Ralstonia solanacearum* (Smith) (Yabuuchi et al.) in the tropical highlands. *Am. J. Pot. Res.* 91, 215–32.

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0658

Niks, R. E., Parlevliet, J. E., Lindhout, P. and Bai, Y. (2011). *Breeding Crops with Resistance to Diseases and Pests*. Wageningen Academic Publishers, p. 200.

Paran, I., Goldman, I., Tanksley, S. D. and Zamir, D. (1995). Recombinant inbred lines for genetic mapping in tomato. *Theor. Appl. Genet.* 90, 542–8.

Park, T. H., Vleeshouwers, V. G. A. A., Jacobsen, E., Van der Vossen, E. and Visser, R. G. F. (2009). Molecular breeding for resistance to *Phytophthora infestans* (Mont.) de Bary in potato (*Solanum tuberosum* L.): a perspective of cisgenesis. *Plant Breed.* 128, 109–17.

PGSC (2011). Genome sequence and analysis of the tuber crop potato. *Nature* 475, 189–94.

Phumichai, C. and Hosaka, K. (2006). Cryptic improvement for fertility by continuous selfing of diploid potatoes using *Sli* gene. *Euphytica* 149, 251–8.

Phumichai, C., Mori, M., Kobayashi, A., Kamijima, O. and Hosaka, K. (2005). Toward the development of highly homozygous diploid potato lines using the self-compatibility controlling *Sli* gene. *Genome* 48, 977–84.

Prashar, A., Hornyik, C., Young, V., McLean. K., Kumar Sharma, S., Dale, M. F. B. and Bryan, G. J. (2014). Construction of a dense SNP map of a highly heterozygous diploid potato population and QTL analysis of tuber shape and eye depth. *Theor. Appl. Genet.* 127, 2159–71.

Renia, H. (1995). True seed is a commercial reality in USA. *Pot. Rev.* 5, 48–51.

Rijk, B., van Ittersum, M. and Withagen, J. (2013). Genetic progress in Dutch crop yields. *Field Crops Res.* 149, 262–8.

Rowe, P. R. (1967). Performance and variability of diploid and tetraploid potato families. *Am. Pot. J.* 44, 263–71.

Roy, T. S., Baque, M. A., Chakraborty, R., Haque, M. N. and Suter, P. (2015). Yield and economic return of seedling tuber derived from True Potato Seed as influenced by tuber size and plant spacing. *Univ. J. Agric. Res.* 3, 23–30.

Schmalenbach, I. and Pillen, K. (2009). Detection and verification of malting quality QTLs using wild barley introgression lines. *Theor. Appl. Genet.* 118, 1411–27.

Skelsey, P., Rossing, W. A. H., Kessel, G. J. T. and Van der Werf, W. (2010). Invasion of *Phytophthora infestans* at the landscape level: How do spatial scale and weather modulate the consequences of spatial heterogeneity in host resistance? *Phytopath.* 100, 1146–61.

Solomon-Blackburn, R. M. and Barker, H. (2001). A review of host major-gene resistance to potato viruses X, Y, A and V in potato: genes, genetics and mapped locations. *Heredity* 86, 8–16.

Struik, P. C. and Wiersema, S. (2012). *Seed Potato Technology*. Wageningen Academic Publishers, p. 383.

Su, C. F., Lu, W. G., Zhao, T. J. and Gai, J. Y. (2009). Verification and fine-mapping of QTLs conferring days to flowering in soybean using residual heterozygous lines. *Chin. Sci. Bull.* 6, 499–508.

Thomas-Sharma, S., Abdurahman, A., Ali, S., Andrade-Piedra, J. L., Bao, S., Charkowski, A. O., Crook, D., Kadian, M., Kromann, P., Struik, P. C., Torrance, L., Garrett, K. A. and Forbes, G. A. (2016). Seed degeneration in potato: the need for an integrated seed health strategy to mitigate the problem in developing countries. *Plant Path.* 65, 3–16.

Tierno, R., Carrasco, A., Ritter, E. and Ruiz de Galarreta, J. I. (2014). Differential growth response and minituber production of three potato cultivars under aeroponics and greenhouse bed culture. *Amer. J. Pot. Res.* 91, 346–53.

Tobias, A., Schrag, T. A., Möhring, J., Melchinger, A. E., Kusterer, B., Dhillon, B. S., Piepho, H-P. and Frisch, M. (2009). Prediction of hybrid performance in maize using molecular markers and joint analyses of hybrids and parental inbreds. *Theor. Appl. Genet.* 120, 451–61.

Troyer, A. F. (2006). Adaptedness and heterosis in corn and mule hybrids. *Crop Sci.* 46, 528–43.

Uijtewaal, B. A., Huigen, D. J. and Hermsen, J. G. Th. (1987a). Production of potato monohaploids (2n = x = 12) through prickle pollination. *Theor. Appl. Genet.* 73, 751–8.

Uijtewaal, B. A., Jacobsen, E. and Hermsen, J. G. Th (1987b). Morphology and vigour of monohaploid potato clones, their corresponding homozygous diploids and tetraploids and their heterozygous diploid parent. *Euphytica* 36, 745–53.

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0659

Uitdewilligen, J. G. A. M. L., Wolters, A. M. A, D'hoop, B. B., Borm, T. J. A., Visser, R. G. F. and Van Eck, H. J. (2013). A next-generation sequencing method for genotyping-by-sequencing of highly heterozygous autotetraploid potato. *PLoS ONE* 8(5), e62355.

Van Breukelen, E. W. M., Ramanna, M. S. and Hermsen, J. G. Th. (1977). Pathenogenetic monohaploids (2n = 2x = 12 from *Solanum tuberosum* L. and *S. verrucosum* Schlechtd. and the production of homozygous potato diploids. *Euphytica* 26, 263–71.

Van Eck, H. J., Jacobs, J. M. E., van den Berg, P. M. M. M., Stiekema, W. J. and Jacobsen, E. (1994), The inheritance of anthocyanin pigmentation in potato (*Solanum tuberosum* L.) and mapping of tuber skin colour loci using RFLPs. *Heredity* 73, 410–21.

Van Eck, H. J., Jacobs, J. M. E., van Dijk, J., Stiekema, W. J. and Jacobsen, E. (1993) Identification and mapping of three flower colour loci of potato (*S. tuberosum* L.) by RFLP analysis. *Theor. Appl. Genet.* 86, 295–300.

Viquez-Zamora, M., Caro, M., Finkers, R., Tikunov, Y., Bovy, A., Visser, R. G. F., Bai, Y. and Van Heusden, S. (2014). Mapping in the era of sequencing: high density genotyping and its application for mapping TYLCV resistance in *Solanum pimpinellifolium*. *BMC Genomics* 15, 1152.

Visser, R. G. F., Bachem, C. W. B., Borm, T., de Boer, J., Van Eck, H. J., Finkers, R., Van der Linden, G., Maliepaard, C. A., Uitdewilligen, J. G. A. M. L., Voorrips, R., Vos, P. and Wolters, A. M. A. (2014). Possibilities and challenges of the potato genome sequence. *Pot. Res.* 57, 327–30.

Vos, P. G., Uitdewilligen, J. G. A. M. L, Voorrips, R. E., Visser, R. G. F. and Van Eck, H. J. (2015). Development and analysis of a 20K SNP array for potato (*Solanum tuberosum*): an insight into the breeding history. *Theor. Appl. Genet.* 128, 2387–401.

Wang, C. M., Lo, L. C, Feng, F., Zhu, Z. Y. and Yue, G. H. (2008). Identification and verification of QTL associated with growth traits in two genetic backgrounds of Barramundi (*Lates calcarifer*). *Animal Genet. Vol.* 39, 34–9.

Wang'om, W. G. and van Dijk, M. P. (2013). Low potato yields in Kenya: do conventional input innovations account for the yields disparity? *Agric. Food Sec.* 2, 14.

Wiersema, S. G. (1986). A method of producing seed tubers from true potato seed. *Pot. Res.* 29, 225–37.

Young, N. D., Zamir, D., Ganal, M. W. and Tanksley, S. D. (1988). Use of isogenic lines and simultaneous probing to identify DNA markers tightly linked to the *tm-2a* gene in tomato. *Genetics* 120, 579–85.

Zhang, Y-M., Mao, Y., Xie, C., Smith, H., Luo, L. and Xu, S (2005). Mapping Quantitative Trait Loci using naturally occurring genetic variance among commercial inbred lines of maize (*Zea mays* L.). *Genetics* 169, 2267–75.

© Burleigh Dodds Science Publishing Limited, 2016. All rights reserved.

Aardevo Exhibit 1010
Page 0660

Potato Research
https://doi.org/10.1007/s11540-019-09444-x

Contribution and Stability of Yield Components of Diploid Hybrid Potato

Julia Stockem[1] · Michiel de Vries[1] · Edwin van Nieuwenhuizen[1] · Pim Lindhout[1] · Paul C. Struik[2]

Received: 31 January 2019 / Accepted: 31 October 2019/
© European Association for Potato Research 2020

Abstract

Recently, a hybrid breeding system was developed for diploid potato. We compared performance of diploid hybrids with commercially available tetraploid cultivars. Therefore, seedling tubers were produced from true hybrid seeds in field conditions. In the subsequent year, diploid hybrids grown from seedling tubers showed a yield potential comparable with commercial tetraploid cultivars: the highest yielding diploid hybrids showed a yield comparable with the lower yielding tetraploid cultivars. Yields of hybrids and commercial tetraploid cultivars were broken down into different yield components and the interactions with growing conditions were quantified. The stability of yield and other relevant traits in different growing conditions was similar between hybrids and commercial cultivars. The contribution of the different yield components to total yield over different environments was compared between diploid hybrids and tetraploid cultivars. In diploid hybrids as well as tetraploid cultivars, more tubers per stem resulted in the highest yield gain, while an increase in tuber size resulted in a relatively smaller increase of total yield.

Keywords GxE interaction · Hybrid breeding · Solanum tuberosum · True seed

Introduction

Potato (*Solanum tuberosum* L. and its tuber bearing crossable species) is the third food crop after rice and wheat (Zaheer and Akhtar 2016). A large proportion of the

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11540-019-09444-x) contains supplementary material, which is available to authorized users.

✉ Julia Stockem
julia.stockem@solynta.com

[1] Solynta, Dreijenlaan 2, 6703 HA Wageningen, The Netherlands

[2] Centre for Crop Systems Analysis, Wageningen University, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands

Aardevo Exhibit 1010
Page 0661

production is used for other purposes such as seed, industrial processing and feed (Haverkort and Struik 2015). Despite its importance, yield increase by genetic gain has been limited for the last century, especially in cultivars for consumption (Douches et al. 1996; Piepho et al. 2014). The most important factor limiting genetic gain is the lack of an efficient breeding system. An advanced system is hybrid breeding based on crosses between two homozygous inbred lines (Lindhout et al. 2011). By choosing contrasting parents, yield contributing alleles can be combined (Lindhout et al. 2011). The segregation of potato traits after several generations of inbreeding was described by Meijer et al. (2018). Until now, the absence of a self-compatibility system hampered the creation of homozygous inbred lines in potato. Since the discovery of the *Sli*-gene (Hosaka and Hanneman 1998), it has become possible to develop a diploid hybrid breeding system (Lindhout et al. 2018). The discovery not only creates opportunities for commercial breeding programmes, it also greatly facilitates genetic research by using advanced segregating populations such as introgression libraries and backcross populations (Jeuken and Lindhout 2004; Endelman and Jansky 2016; Prinzenberg et al. 2018). Diploid germplasm allows high throughput phenotyping and efficient QTL detection, accelerating the breeding process (Meijer et al. 2018; Prinzenberg et al. 2018; Su et al. 2019).

With the development of this hybrid breeding system, which is new for potato, using diploid hybrids instead of tetraploid clones, a system of testing new cultivars needs to be developed. Commonly in breeding systems, new cultivars are grown at several locations from tubers and their performance is benchmarked against a set of already existing commercial tetraploid cultivars (Tiemens-Hulscher et al. 2013). To determine relevant genetic gain, performance of diploid hybrids needs to be benchmarked against tetraploid cultivars commonly used by farmers. Assessing the genotype by environment interactions in farmers' fields is an important aspect of acceptance of new (types of) cultivars.

Besides the use of new genetic material in diploid breeding, the starting material is also different from the traditional breeding germplasm. In tetraploid breeding, a cross is made only once and the true seeds derived from this cross are grown to seedling tubers (Tiemens-Hulscher et al. 2013). After that, genotypes are clonally propagated, which means that starting material of tetraploid cultivars always consists of seed tubers. Hybrid breeding in potato results in true (botanical) seeds. Plant development is highly affected by the type of starting material (true seed or seed tuber) and by its physiological, physical, genetic and phytosanitary quality. Size and physiological age of seed tubers have large effects on ware crop yield (Arsenault et al. 2001; Struik and Wiersema 2001). Therefore, seedling tubers need to be produced from diploid hybrids to evaluate the performance of hybrids in comparison to existing benchmark cultivars.

Although for most field crops, dry matter yield is the most important economic output, for potato fresh tuber yield, tuber size distribution, dry matter content and tuber shape are main output determinants. These traits are important criteria whether a cultivar is suitable for fry or chips production (Tiemens-Hulscher et al. 2013). Dry matter percentage should range between 22 and 25% for chips (crisps) and 21 and 23% for fries. Tubers that are processed into chips should be round, while tuber shape of frying cultivars should be oblong for optimal processing (Tiemens-Hulscher et al. 2013). Potato yield can be dissected into different components and be expressed as the product of tuber weight, tubers per stem and stems per unit area (Haverkort et al.

Aardevo Exhibit 1010
Page 0662

1990; Lynch et al. 1995; Struik and Wiersema 2001; Asghari-Zakaria et al. 2007). Potato production is highly affected by the environment (Haverkort 1990; Kooman et al. 1996). To investigate the specific development aspects of diploid hybrids in different environments, the contribution of these components that make up potato yield was investigated.

In this paper, we show the first results of such a hybrid breeding programme focusing on yield and yield components. As testing environments, we have chosen farmers' fields in The Netherlands, Belgium and France, representative for the north-western European potato cultivation environment. This region is known for its high technological level of potato cultivation and its conducive climate, resulting in high yields.

The objectives of this study were to (1) evaluate the performance of diploid hybrids in comparison to existing tetraploid cultivars, (2) to compare the environmental effect on yield and yield components between diploid hybrids and tetraploid cultivars and (3) to evaluate the contribution of different yield components to total yield.

Materials and Methods

Genetic Material

Diploid heterozygous *Solanum tuberosum* accessions were crossed with the self-compatibility donor *S. chacoense* (Hosaka and Hanneman 1998). Inbred lines of diploid potato were developed in a dedicated diploid breeding programme as described by Lindhout et al. (2011, 2018). The inbred parents were crossed after 5 to 7 generations of self-pollinations to produce the test hybrids. These inbreds should theoretically be more than 95% homozygous, but this was not tested (Lindhout et al. 2011). In winter 2015–2016, crosses between parent lines were made manually to produce diploid hybrid seeds and 572 hybrids were grown in one location in a first year trial to select the most uniform hybrids. This resulted in a panel of 65 diploid hybrids that was used in the present study. These hybrids were selected based on a wide variation in yield and yield components to represent the genetic variation among these hybrids and to allow a detailed analysis.

Nineteen commercial tetraploid cultivars were used as controls for the different potato product types, representing the frying, chip and fresh consumption markets: Annabelle, Lady Cristil, Lady Claire, Pirol, Innovator, Lady Rosetta, Agata, Arsenal, Hermes, Brooke, Nicola, Bintje, Markies, Russet Burbank, Fontane, Spunta, Mozart, Agria and Milva. The main differences between these cultivars are dry matter content, shape and tuber size. The diploid hybrids were not yet differentiated for the different market segments. These control cultivars are commonly grown in the regions where the trials took place. Hence, these controls are a realistic representation of farmers' practice.

Seed tubers of commercial cultivars were classified in class A in the NAK (Nederlandse Algemene Keuringsdienst voor zaaizaad en pootgoed van landbouwgewassen, The Dutch General Inspection Service) classification system (NAK 2018). Seed tubers were produced and stored in the conditions optimal for the specific cultivar for a realistic representation of the cultivar's potential, so storage conditions were not identical for all cultivars. Diploid hybrids were all stored at 4 °C as optimal conditions for the specific hybrids were yet

Aardevo Exhibit 1010
Page 0663

unknown. Seed tubers of equal size and sprout distribution were selected for the trial. To assess the suitability of the seedling tubers for a ware crop trial, sprouting was scored after 6 months of storage (data not shown) (Tiemens-Hulscher et al. 2013). Generally, seed tubers should be planted when dormancy has been broken (Struik and Wiersema 2001). Seedling tubers with a sprouting score between 3 and 7 were used in the trial; sprouting of seed tubers of tetraploid cultivars was comparable with the sprouting score of 5 of the diploid hybrids.

Seedling Tuber Production

Field Trial Description

Seedling tuber production from true diploid hybrid seeds was performed on a heavy marine clay in Wolphaartsdijk [NL], in 2016. The botanical seeds of the hybrids were extracted from the berries and sown into 104 plug trays (2.5 × 2.5 × 3.8 cm) filled with soil (Horticoop) in a greenhouse compartment in the first week of April 2016. Germination after 2 weeks was between 54 and 98%. Six weeks after sowing the seedlings were mechanically transplanted into the open field, into ridges of 75 cm width and a plant distance of 30 cm within the row. The crop was treated as a standard seed-tuber crop, according to farmers' practices. Irrigation was applied at 1 and 3 days after planting to prevent the seedlings from drying out. Pests and diseases were chemically controlled. Both mechanical weeding and hand-weeding were applied twice. After 90 growing days in the field, the haulm was chemically killed. Three weeks after haulm killing, the seed tubers of all diploid hybrids were lifted by a harvesting machine. The seed tubers were stored in a climate-controlled storage room at 4 °C from October 1st onwards until the end of February.

Design and Measurement

Seedling tuber production was done in a non-replicated design with a plot size between 90 and 488 plants, depending on hybrid seed availability. Seedlings were transplanted semi-mechanically with an adjusted 4-row cabbage planter with a planting distance of 30 cm between plants. After harvest, seedling tubers were sorted by size and yield was determined for tubers > 28 mm. Phytosanitary control on quarantine diseases was routinely carried out by the national inspection service (NAK at Emmeloord, NL). To assess the quality of the seedling tubers, sprouting was scored in February, at the preparation of the yield trials according to a 1–9 scale, with 9 being completely dormant and 1 abundantly sprouting (Tiemens-Hulscher et al. 2013).

Yield Trials

Field Trial Description

In 2017, yield trials were conducted using the seedling tubers as starting material that were produced in 2016. Certified seed tubers (class A) of the tetraploid cultivars were obtained to serve as control cultivars in the trials. The field trials were conducted at five different sites (Warmeriville [FR], Berthem [BE], Hilvarenbeek [NL], Est [NL],

Aardevo Exhibit 1010
Page 0664

Table 1 Location, soil characteristics, crop management system, planting and haulm killing dates of the five experimental sites

Site	Longitude (°E)	Latitude (°N)	% clay	% org C	pH	Ridge/bed system	Planting date	Haulm killing date
Warmeriville [FR]	4.219	49.345	13	4.2	7.6	90 cm ridge	19 Apr. 2017	29 Aug. 2017
Hilvarenbeek [NL]	5.086	51.483	2	3.1	6.8	150 cm beds	21 Apr. 2017	6 Oct. 2017
Est [NL]	5.329	51.845	33	3.7	6.1	75 cm ridge	14 Apr. 2017	15 Sept. 2017
Berthem [BE]	4.633	50.871	13	2.2	6.4	75 cm ridge	25 Apr. 2017	2 Oct. 2017
Giethoorn [NL]	6.054	52.671	1	7.4	5.4	75 cm ridge	24 Apr. 2017	5 Oct. 2017

Aardevo Exhibit 1010
Page 0665

Giethoorn [NL]). These sites differed in soil type, climate and crop management (Table 1). Two of the sites were typical sandy soils, one a loamy soil, one a light clay and one a heavy clay soil, covering the full range of physical soil types on which potatoes are grown in NW Europe. At all sites, the trial was laid out within a farmers' field, hence crop management was according to farmers' practice. Typically, the tubers were planted in ridges, spaced at 75 cm, except for the experiment in Warmeriville, where the distance between ridges was 90 cm (Table 1). Spacing between seed(ling) tubers in all locations was 25 cm. In Hilvarenbeek, the farmer had adopted a bed system, which was better adapted to his conditions. Due to the fine sand at this location, ridges would be prone to erosion.

Design and Measurements

At preparation of the field trials, the weight of the seed(ling) tubers for each plot was recorded. Trials were planted mechanically in a randomized complete block design with three repetitions and 20 plants per plot, divided over two rows. Between each plot, two border plants of the cultivar Miss Blush were planted to obtain an equal border effect over all plots. The bi-colored cultivar Miss Blush was chosen to avoid mixture at harvest, as test cultivars and diploid hybrids showed uniform skin color.

The whole experiment was bordered by two ridges of Miss Blush at the sides and one full plot of Miss Blush at the beginning and end of each ridge. During the growing season, stem number was counted per plot. After harvest, tuber number and size were measured automatically using a 3D camera (RMA Techniek, 's Heer Arendskerke, Netherlands). Total tuber weight was determined per plot. A subsample of approximately 5 kg was used to determine dry matter content (see below for formulas).

Data Analysis

Data of the yield trials were statistically analyzed using R (R Core Team 2017). A component analysis was done on total yield (Formula 1) to determine how these components contribute to total yield in each cultivar. Yield and yield components were adjusted for spatial field trends with Spatial Analysis of Field Trials with Splines (SpATS) (Rodríguez-Álvarez et al. 2018). In this model, differences in plant development due to environmental variation within the field are distinguished from differences in the genetic compositions between the cultivars. In SpATS, variation in the field is

Table 2 Analysis of variance table, calculated using the AMMI analysis (additive main effects and multiplicative interaction models)

Factor	*df*	Sum of squares	*F* value	*p* value
Location	4	160537	239.0	< 0.001***
Replicate	10	1679	1.8	0.05
Genotype	83	448198	59.0	< 0.001***
Genotype × location (GxE)	332	104915	3.5	< 0.001***
Residuals	789	72212		

Aardevo Exhibit 1010
Page 0666

analyzed in two directions (rows and columns of the trial) using P-splines; effects of spatial variation are described by a standard mixed model (Rodríguez-Álvarez et al. 2018). By adjusting the data using SpATS, effects of environmental variation within trial fields were reduced, as the focus of this analysis was the genetic differences and the differences between trial fields. To meet the assumptions of normality of the SpATS model, data were log transformed, means and standard errors were back-transformed to linear data for graphical presentation. To examine to what extent the different yield components contribute to total yield, the variation in each yield component was compared with variation in total yield by linear regression. The contribution was defined as the change in yield by changing one unit of the yield component. This was determined by the slope of a linear model, which was fitted through the data of all sites. Genotype by environment interaction was calculated with the AMMI analysis (Additive Main effects and Multiplicative Interaction models), using the Agricolae package in R (Crossa 1990). Results of the AMMI analysis are presented in Fig. 2 and in Table 2. For the AMMI analysis, the raw data were used; the rest of the presented data of yield, stems/m^2, tubers/stem and tuber size were adjusted for spatial effects within the field using SpATS. Under water weight was used to determine dry matter content (Formula 3, 4) (Tiemens-Hulscher et al. 2013). Tuber shape was determined using Formula 5.

$$yield\ (g/m^2) = tubers/stem \times stems/m^2 \times gram/tuber \tag{1}$$

$$y_{ij} = Block + \boldsymbol{row}_{r(j)} + \boldsymbol{col}_{c(j)} + f(row, col) + \boldsymbol{g}_i + \boldsymbol{e}_{ij} \tag{2}$$

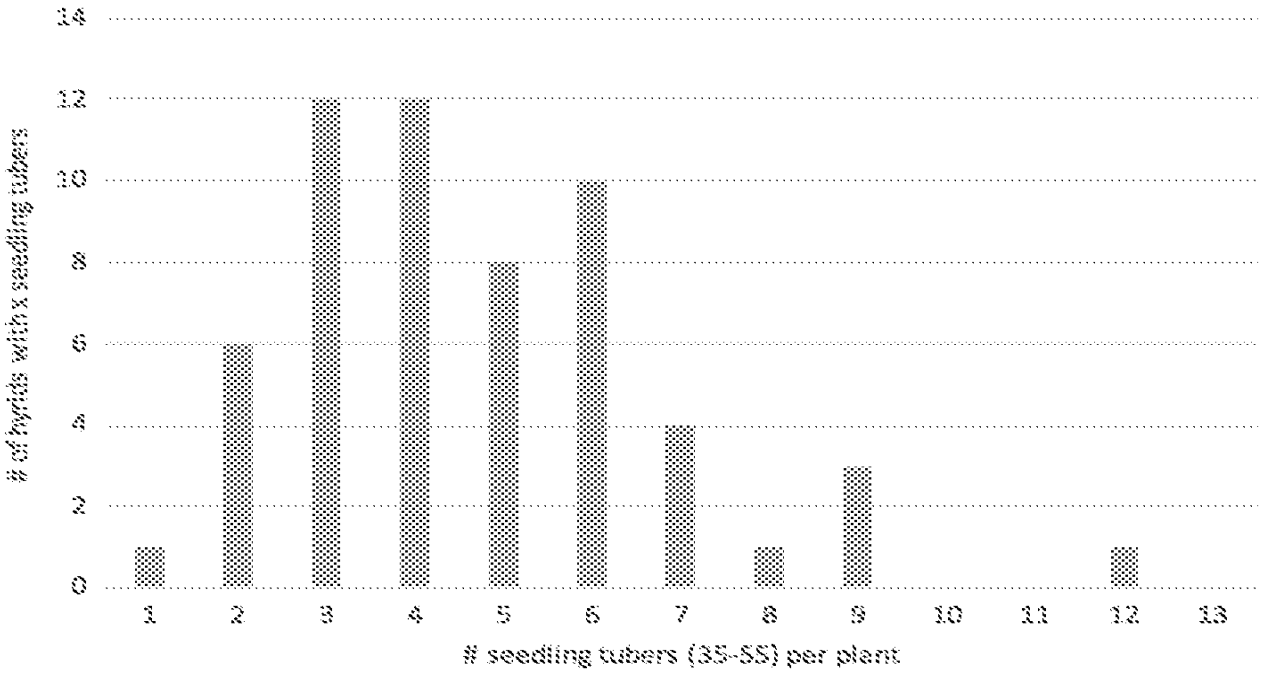


Fig. 1 Frequencies of number of seedling tubers (35–55 mm) produced per plant

Aardevo Exhibit 1010
Page 0667

$$Under\ water\ weight = \frac{5050}{weight\ above\ water} \times weight\ under\ water \quad (3)$$

$$Dry\ matter\ content\ (\%) = under\ water\ weight \times 4.90713 \times 10^{-2} + 2.054 \quad (4)$$

$$Tuber\ shape = 1/\frac{tuber\ length}{\sqrt{(\pi \times (0.5 \times tuber\ width)) \times (0.5 \times tuber\ height)}} \quad (5)$$

Results

Seedling Tuber Production from Seedlings

In contrast to regular potato seed tuber productions, seedling tubers were produced from diploid hybrid seedlings. After transplanting to the field, seedlings grew rapidly and hardly any plant died. For most hybrids, the canopy was more or less closed at the end of June. After harvest of the seedling-tuber production, the number of seedling tubers was assessed (Fig. 1). Although diploid hybrids were not selected for yielding capacity, a good seedling tuber production was achieved.

Yield Trials

Seed tubers produced from true hybrid seeds in 2016 were used to conduct multi-location yield trials in 2017. In these trials, the performance of diploid hybrids grown from tubers was compared to the performance of tetraploid cultivars in different environments. Diploid hybrids that were used in this experiment were the first test hybrids derived from a hybrid breeding system based on homozygous inbred lines (Lindhout et al. 2011). It is known that the correlation between yield of single greenhouse-grown plants and larger blocks in the field of the same cultivar is low. Therefore, traits for high yield were not considered as selection criteria during the selection of parent lines for these hybrids.

Yield

Among the different sites, significant differences ($p < 0.001$) in yield were found (Table 2). Highest yielding site was Hilvarenbeek with on average 54 Mg/ha, while lowest average yield was measured in Berthem with 27 Mg/ha. Average yield for the

Aardevo Exhibit 1010
Page 0668



Fig. 2 Biplot of yield and PC1 of the AMMI analysis (Additive Main effects and Multiplicative Interaction models)

Netherlands and France was 43 Mg/ha, in Belgium 50 Mg/ha (Eurostat), so differences in production between sites were due to local environmental and field management differences.

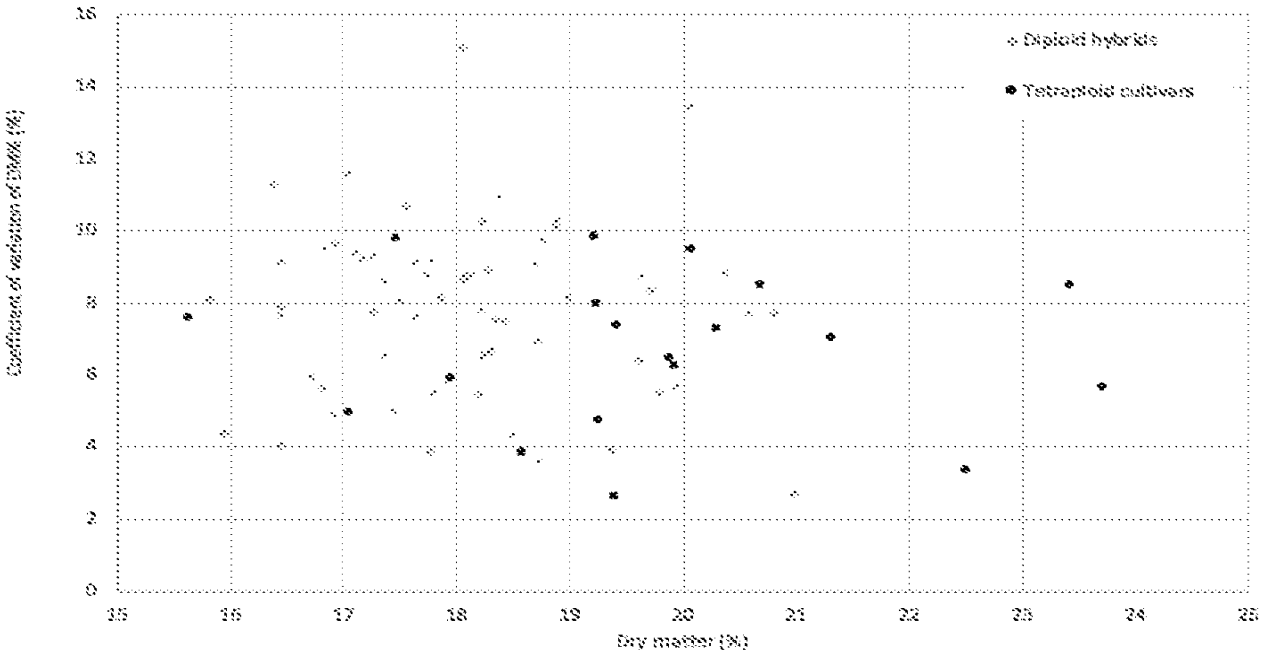


Fig. 3 Average dry matter (DM) percentage and coefficient of variation (CV) of DM% of diploid hybrids and tetraploid cultivars

Aardevo Exhibit 1010
Page 0669

Fresh tuber yield differed significantly among cultivars ($p < 0.001$). Diploid hybrids yielded on average between 16 and 52 Mg/ha. For the tetraploid cultivars, average yield was between 52 and 101 Mg/ha. Performance of cultivars among the different sites varied; indeed, a significant interaction (GxE, $p < 0.001$) in yield between cultivar and site was found (Table 2).

The genotype × environment interaction of the hybrids and cultivars was analyzed using the AMMI analysis. The AMMI parameters can be used to make a biplot in which the interaction effects of genotype and environment are shown (Gauch and Zobel 1997). In the biplot (Fig. 2), 62.9% of the GxE interaction is explained in the principal component axis PC1. Also, the principal component axis PC2 explained a significant part of the G × E interaction (23.6%); the rest of the PC axes were not significant. On average, the yield of diploid hybrids was lower than the yield of tetraploid cultivars; however, the highest yielding hybrids were similar to the lowest yielding tetraploid cultivars. For these higher yielding hybrids, the interaction with the environment was also similar to several tetraploid cultivars (Fig. 2). The large distance between the trial locations in Fig. 2 shows that they differed in environmental effect, except for Warmeriville and Berthem that were very close to each other (Fig. 2).

Dry Matter

Dry matter percentage (DM%) was measured in each genotype on all fields separately. A wide genetic variation for DM% was observed, as well as variation in DM% among the different sites. Diploid hybrids showed a broad range of DM%, between 15.8 and 21% (Fig. 3). This was in range with most of the tetraploid cultivars, except for three

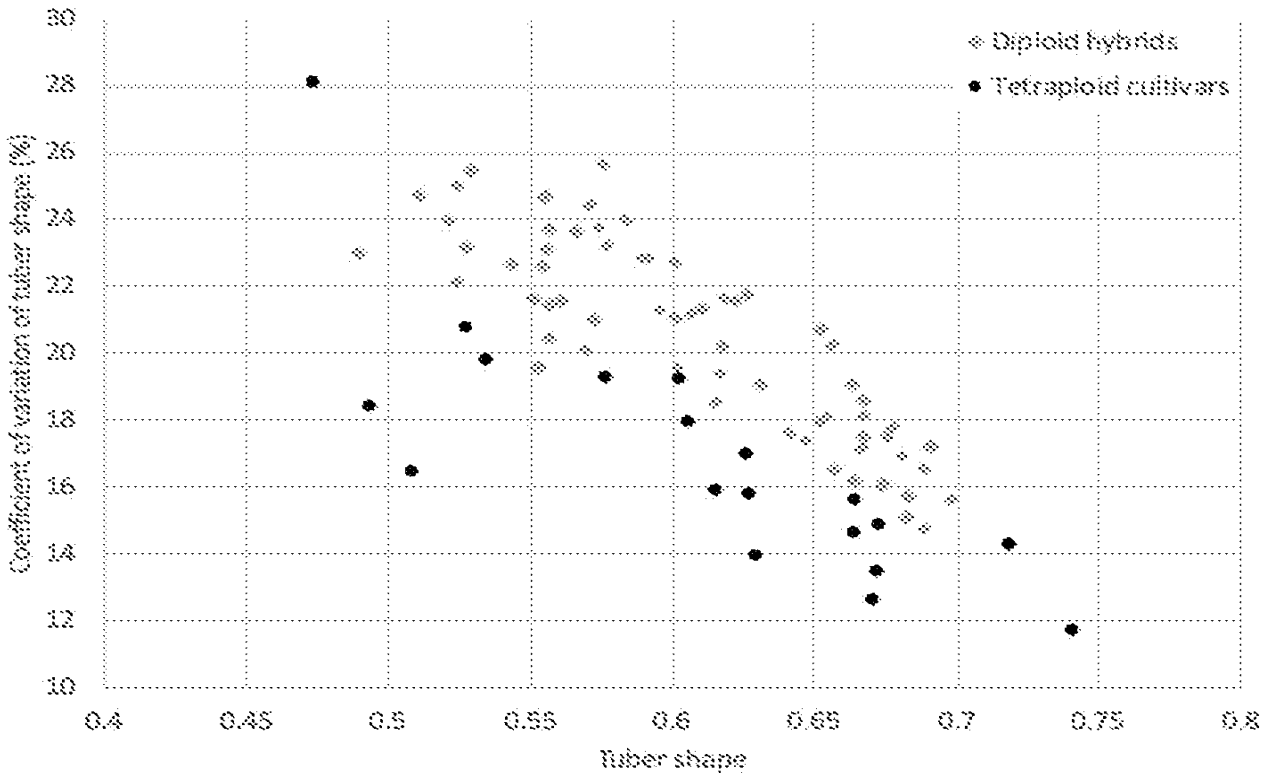


Fig. 4 Average tuber shape and coefficient of variation (CV) of tuber shape of diploid hybrids and tetraploid cultivars. Tuber shape is a dimensionless number between 1 and 0, with 1 representing a perfect sphere

Aardevo Exhibit 1010
Page 0670

chip cultivars with a DM% of more than 22.5% (Brooke, Pirol and Lady Rosetta). The rest of the chip and frying cultivars had a DM% between 19.2 and 21.3%, 12 of the diploid hybrids also had a DM% within this range. The rest of the hybrids had a DM% comparable with the tetraploid table cultivars that produced a DM% between 19.2 and 15.6. So in general, in this test panel, the diploid hybrids had similar DM% to the tetraploid cultivars.

To compare the extent of the environmental effect on genotypes, the coefficient of variation (CV) of DM% over the sites was calculated for each diploid hybrid and tetraploid cultivar (Fig. 3). Variation of DM% between fields in most diploid hybrids was in the same range as the variation of DM% in tetraploid cultivars. The most stable diploid hybrid (Hybrid 1) had a CV of 2.7%, which was similar to the CV of the tetraploid cultivar Innovator, while the DM% in hybrid 1 was even higher than in Innovator (21% and 19.4%, respectively). So, dry matter percentage and stability in different environments were as good as, and sometimes even better in hybrids than in tetraploid cultivars.

Tuber Shape

Tuber shape is an important trait for cultivars in the chip and the frying industry, because tubers need to be suitable for the processing machines (Tiemens-Hulscher et al. 2013). Tuber shape is here expressed by Formula 4, in which the length, width and height of individual tubers are taken into account. The result is a dimensionless number between 1 and 0, with 1 representing a perfect sphere. Without selection for specific tuber shape in the diploid hybrids during the breeding process, the same range from round to long was found in hybrids as in tetraploid cultivars (Fig. 4). The variation for

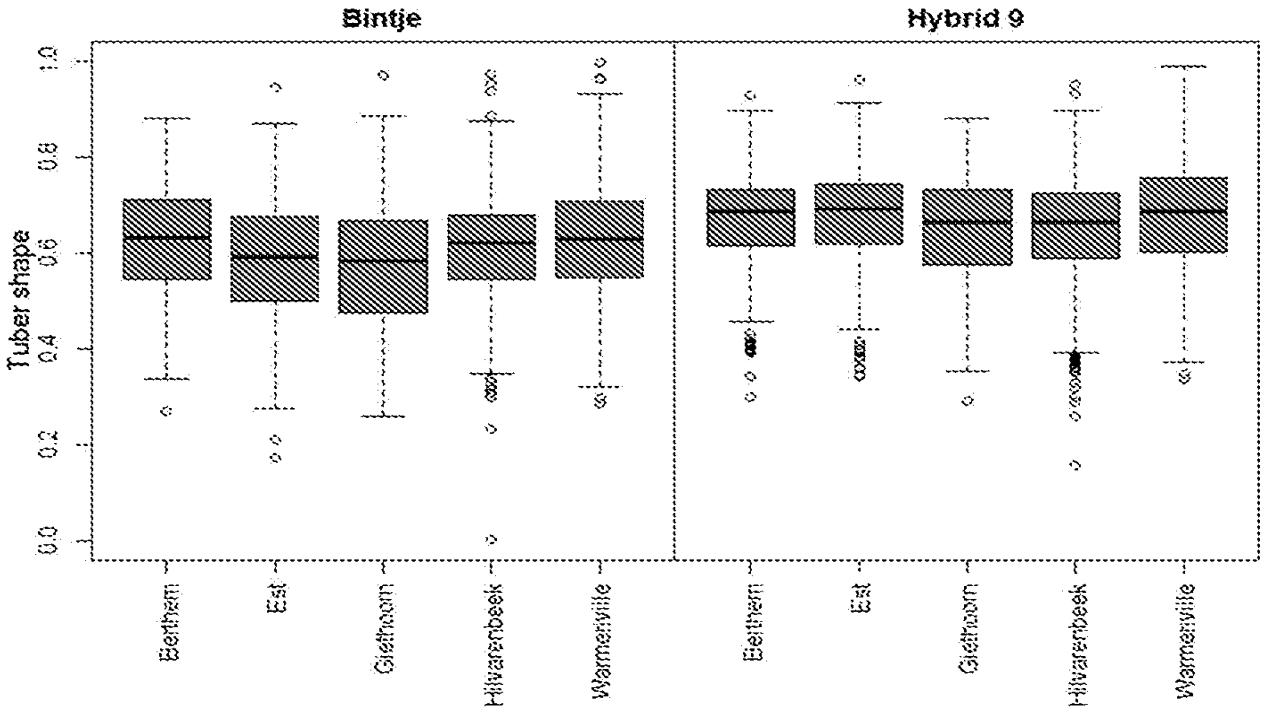


Fig. 5 Boxplots of shape of individual tubers of tetraploid cultivar Bintje (n = 3484) and diploid hybrid 9 (n = 2782) grown at five different locations in 2017

Aardevo Exhibit 1010
Page 0671

long tubers for fries represented by frying cultivars like Innovator and Russet Burbank was similar to the variation of several hybrids, as well as a round shape represented by several chip cultivars like Hermes and Lady Rosetta.

The uniformity of the shape is important for mechanical processing. Therefore, the coefficient of variation of the shape data of the individual tubers was calculated for each hybrid and cultivar (Fig. 4). Most diploid hybrids had a higher CV than the tetraploid cultivars. On average, the CV for the hybrids was 20%, and for the tetraploid cultivars, it was 17%; however, the most stable hybrids showed overlap with the least stable tetraploid cultivars. To gain insight into the effects of the different environments on the distribution of shape, we show boxplots of two cultivars (Fig. 5). Both Hybrid 9 and Bintje had a stable shape across the different environments. The differences in mean shape and variation between the five locations were smaller for Hybrid 9 than for cultivar Bintje.

Yield Components

Potato yield was analyzed as the product of different plant components that can contribute to yield. Individual tuber weight, number of tubers per stem and number of stems per surface unit were chosen as relevant yield components to compare the growth of diploid hybrids and tetraploid cultivars. These yield components were compared between tuber grown diploid hybrids and commercial tetraploid cultivars that grew in yield trials on five different locations.

Generally, the yield components differed between the tetraploid cultivars and the diploid hybrids. The mass of the individual tubers of diploid hybrids was lower than that of the tetraploid controls (71 vs. 177 g on average). The hybrids had on average a higher number of stems (25.6 stems/m^2) than the tetraploids (14.9 stems/m^2). The number of tubers per stem was higher in the tetraploids, compared to the diploids (2.9 and 1.8, respectively). This resulted in approximately equal number of tubers per unit area (45.2 and 43.5 tubers/m^2 for diploids and tetraploids, respectively) and all hybrids performed with the number of tubers/m^2 within the range of the tetraploid cultivars.

When comparing yield components of individual tetraploid cultivars and diploid hybrids, the differences were small. A positive correlation was found between yield and tubers per stem ($p < 0.001$). Indeed, the higher yielding hybrids had a relatively high number of tubers per stem. Hybrid 9 for example produced 2.3 tubers per stem on average, which was nearly as much as Annabelle (2.6) and more than Innovator (1.9). Tuber size also was positively correlated with yield ($p < 0.001$); also for the diploid hybrids, the larger tubers resulted in higher yield, even though all hybrid tubers were relatively small.

The cultivars with higher yield also had a relatively high number of tubers per m^2. For example, Bintje showed a high number of tubers/m^2 (60.3) and the highest yield (7.2 kg/m^2) across all cultivars, while Hybrid 1 had the lowest yield (1.6 kg/m^2) and a low number of tubers m^{-2} (31.2). In higher yielding hybrids, the number of tubers/m^2 was also high, with both Hybrid 9 and tetraploid cultivar Bintje having the largest number of tubers/m^2.

Aardevo Exhibit 1010
Page 0672

Table 3 Average values of adjusted yield and adjusted yield components of a selection of representative hybrids and tetraploid cultivars and adjusted yield, adjusted yield components and effect of yield components on total yield per cultivar (regression coefficient). A table with the values of all hybrids and cultivars can be found in the Table S1. Average values of yield components and yield were calculated using the full dataset of diploid hybrids and tetraploid cultivars

		Yield		Yield components			Contribution yield components to yield		
Cultivar, hybrid	Seed(ling) tuber weight (kg/plot)	kg/m^2	Tubers/m^2	Stems/m^2	Tubers/stem	g/tuber	Contribution per stem [g/m^2/(stems/m^2)]	Contribution tuber size [g/m^2/(g/tuber)]	Contribution tuber number [g/m^2/(tubers/stem)]
Hybrid 1	0.72	1.6	31.2	29.9	1.0	48	84	104	3161
Hybrid 2	0.91	2.8	31.1	17.9	1.7	89	− 20	90	1822
Hybrid 3	0.71	2.9	51.9	34.1	1.5	56	− 174	145	2020
Hybrid 4	1.00	3.3	36.1	19.6	1.8	89	160	62	2124
Hybrid 5	0.82	3.8	54.7	31.3	1.7	71	− 2	119	2033
Hybrid 6	0.97	3.8	43.7	22.4	2.0	91	96	1	426
Hybrid 7	0.76	3.8	52.0	19.8	2.6	68	867	179	2642
Hybrid 8	0.83	4.8	56.0	25.5	2.2	85	127	126	2305
Hybrid 9	0.73	5.2	60.6	26.4	2.3	93	− 111	− 8	1617
Annabelle	1.14	5.2	44.6	17.4	2.6	117	165	5	2123
Innovator	1.44	6.4	29.8	16.0	1.9	222	322	− 41	3387
Hermes	0.45	6.9	43.1	11.6	3.7	159	956	− 101	2153
Bintje	0.51	7.2	60.3	16.0	3.8	125	257	− 42	1164
Site									
Berthem	–	3.0	38.6	27.6	1.4	93	–	–	–
Warmeriville	–	3.1	37.4	20.9	1.8	99	–	–	–
Est	–	3.3	36.6	18.5	2.0	98	–	–	–
Giethoorn	–	5.3	60.8	25.3	2.4	90	–	–	–
Hilvarenbeek	–	5.5	63.7	23.7	2.7	96	–	–	–

Aardevo Exhibit 1010
Page 0673

Contribution of Yield Components to Yield

Variation in yield was found across sites as well as between cultivars. The variation in yield was due to the difference in responses of individual genotypes to the environments. Although there are trade-offs and interdependencies between yield components, they explain differences in yield between cultivars and environments.

The number of tubers per stem showed the largest variation across sites (CV = 24.8%); between Berthem and Hilvarenbeek, the difference was almost a factor two. For stems/m^2, the variation was much lower (CV = 15.3%) and the weight of the tubers was rather stable (CV = 3.7%). In conclusion, the yield component that was affected most by the environment was the number of tubers per stem.

To compare the extent to which different yield components were affected by the environment between cultivars, the contribution of individual yield components to total yield over the different sites was determined by calculating the slope between total yield and yield component across sites (Table 3 and Table S1). It estimated the yield gain for a single yield component for each cultivar, with a positive value indicating a contribution to higher yield when the specific yield component increases, and a negative value indicating a loss in total yield when increasing a specific yield component.

To compare the relative effects of yield components on yield between the tetraploid cultivars and the diploid hybrids, correlations between yield components and yield were calculated. Every pair of average yield and yield component value of each location was used in the regression analysis, data are shown in the last three columns of Table 3. It compared the difference in contribution to yield of yield components. Number of tubers per stem explained 80% of the variation in yield in tetraploids and 90% in the diploid hybrids. The relative effect of number of stems per m^2 on yield was much smaller: 18% in tetraploid cultivars and 5% in hybrids. Tuber size affected yield least in tetraploids and hybrids (2% and 5%, respectively). So, on average, hybrids and tetraploid commercial cultivars shared the most important contributing factor to yield.

A higher number of tubers per stem contributed positively to total yield in all cultivars (Table 3). For diploid hybrids in the selection of Table 3, the contribution was between 426 and 3161 g yield increase per m^2 for each extra tuber per stem. In the tetraploids, this was between 1164 and 3387 g/m^2 yield increase/(tuber/stem). More stems per m^2 led in some hybrids to a decrease in total yield while in others, total yield increased with more stems per m^2 (contribution between − 174 and 867 g/m^2 yield increase/(stems/m^2) in Table 3). In the tetraploid cultivars, higher stem numbers were positively correlated with yield with contributions between 165 and 956 yield g/m^2 yield increase/(stems/m^2). There was a large variation in the contribution of larger tubers to total yield between diploid hybrids as well as in tetraploid cultivars, with values between − 8 and 175 g/m^2 yield increase/(g/tuber) for hybrids and − 101 and 5 g/m^2 yield increase/(g/tuber) for tetraploid cultivars. Overall, the range of yield contribution by the different yield components was overlapped between diploid hybrids and tetraploid cultivars.

Variation was found between the different cultivars in total yield as well as in the contribution of the separate yield components to yield. To examine whether there is an optimal value for each yield component for high yield, the gain in yield with an increase of the yield component was compared with the average value of that yield component for each cultivar. Although yield gain and increase in yield component are not completely independent, it provides insight into whether there are optimal yield component values.

Aardevo Exhibit 1010
Page 0674

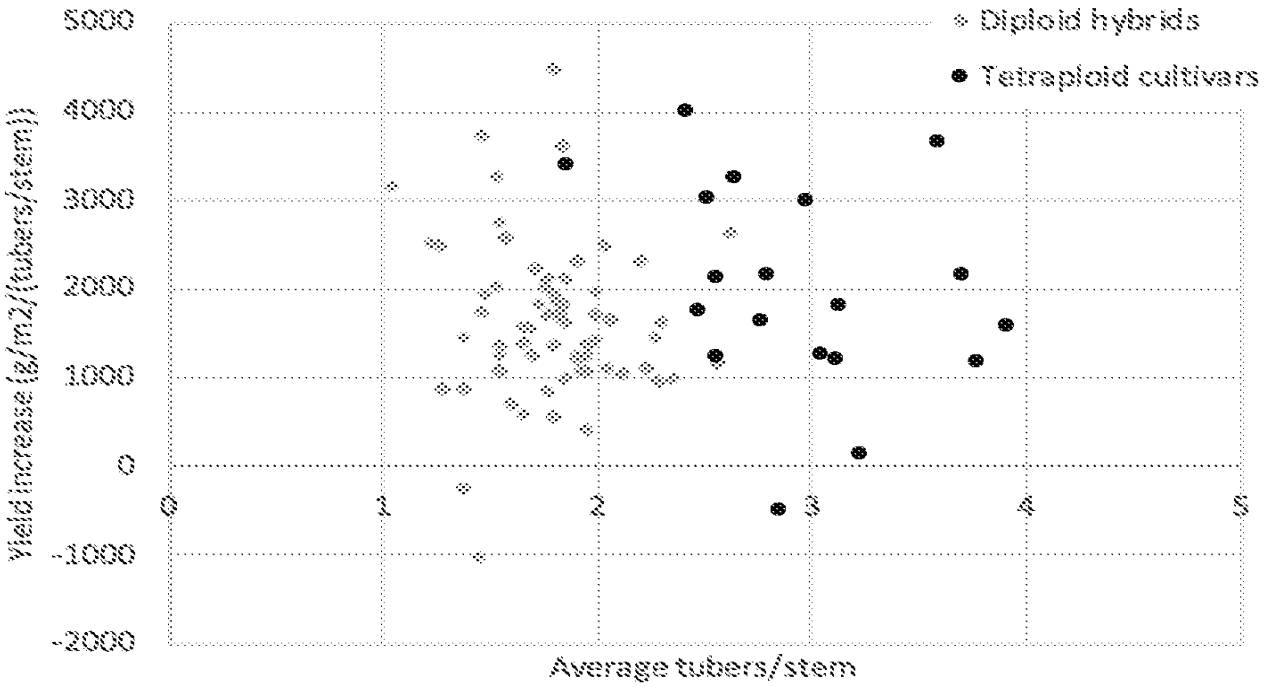


Fig. 6 Contribution of more tubers per stem to total yield, expressed in yield increase per extra tuber/m^2, compared to average number of tubers per stem over five sites (Berthem, Giethoorn, Hilvarenbeek, Est, Warmeriville)

Genotypic variation was found for the contribution of yield components to total yield. The effect of differences in yield components between genotypes on the correlation between yield component and yield was examined (Figs 6, 7 and 8). Most genotypes showed a positive correlation between yield and tubers/stem when the

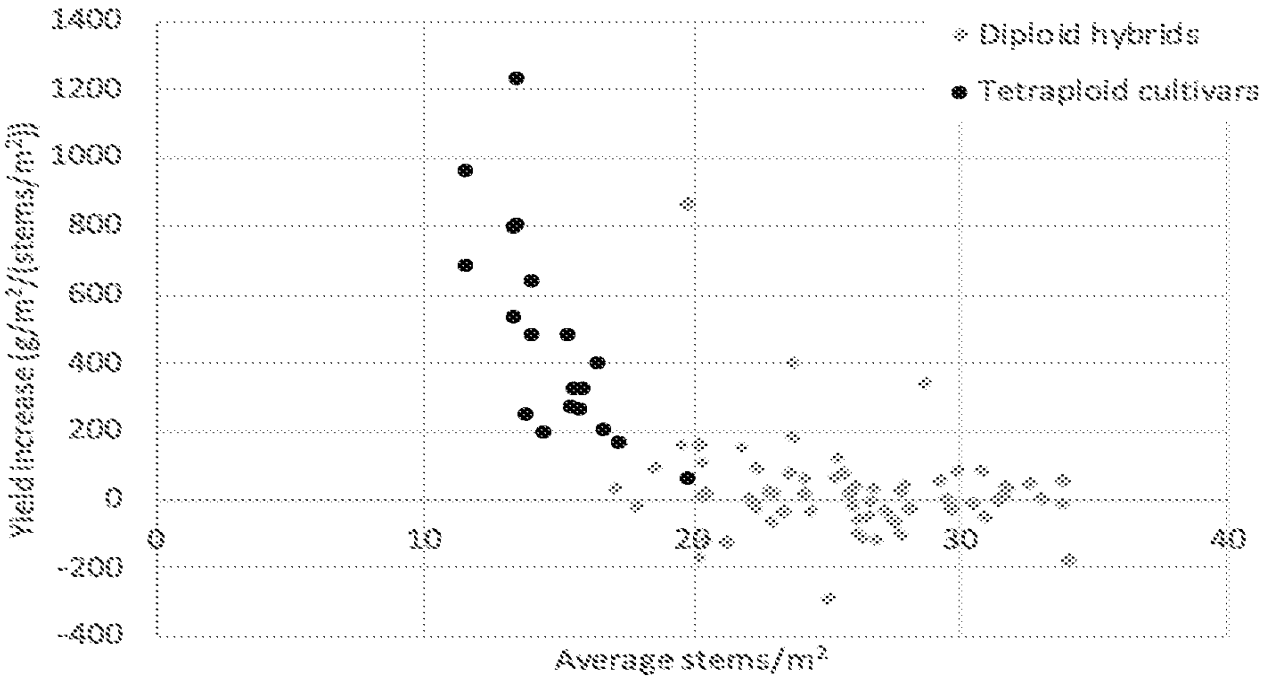


Fig. 7 Contribution of more stems per m^2 to total yield, expressed in total yield increase per extra stem/m^2, compared to average number of stems/m^2 over five sites (Berthem, Giethoorn, Hilvarenbeek, Est, Warmeriville)

Aardevo Exhibit 1010
Page 0675

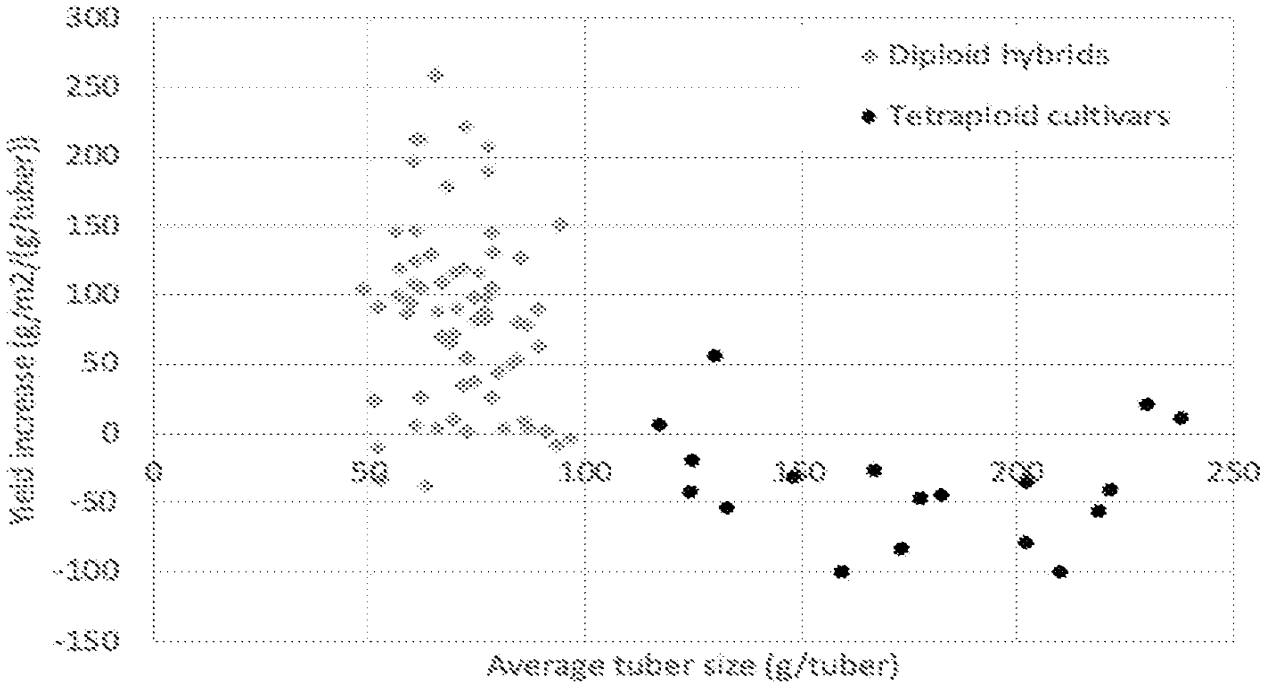


Fig. 8 Contribution of larger tubers to total yield, expressed in total yield increase per extra g tuber (tuber size), compared to average tuber size over five sites (Berthem, Giethoorn, Hilvarenbeek, Est, Warmeriville)

average number of tubers/stem was between 1.0 and 3.8, so for this dataset, more tubers per stem contributed positively to total yield in the whole range.

In contrast to the number of tubers/stem, larger tubers also affected yield negatively in some cultivars (Fig. 8). A negative relation was found between contribution of larger tubers to total yield and average tuber size of a genotype (Fig. 8). Until a tuber size of about 90 g, the production of larger tubers resulted in a higher total yield. When a genotype already produced large tubers on average, larger tubers decreased total yield. So, with increasing average tuber size of a genotype contribution of even larger tubers to total yield decreased.

Discussion

In this research, we have presented results of replicated field trials across multiple locations of diploid hybrids in comparison with tetraploid cultivars. We have analyzed the yield components to explain differences in yield level and stability.

Hybrid Seedling-Tuber Production

To perform yield trials in which diploid hybrids could be benchmarked against tetraploid commercial cultivars, we first produced seedling tubers from diploid hybrid seeds. This was done to decrease the difference in starting material, as plant performance is largely affected by the starting material (Arsenault et al. 2001; Struik and Wiersema 2001). The breeding process of the test hybrids that were used in this trial was done exclusively in the greenhouse, so no selection for yield was applied.

Aardevo Exhibit 1010
Page 0676

Seedling tubers that were produced were used in the subsequent year for yield trials. The physiological age of seed tubers is one of the main determinants for seed tuber quality (Struik and Wiersema 2001) and it is affected by many genetic and environmental factors such as management of the seed tuber crop, storage conditions, seed tuber size and the state of dormancy (Struik 2007). Before the seed tubers were selected for the ware crop trial, sprouting was scored. The results showed that dormancy was broken in all tubers; however, there was some variation probably due to different genetic background of the diploid hybrids.

To minimise the experimental error, caused by differences in the physiological conditions of the tubers, seed tubers were stored under optimal conditions and equally sprouting tubers were selected for the yield trial. If present, the largest unexplained error would occur in the diploid hybrids, due to differences in physiological conditions as the tubers of the different hybrids were not stored under optimal conditions for each hybrid. In addition, some genetic variation within the hybrids may still occur as the parents were not 100% homozygous. However, the within-plot variation of the hybrids was not higher than this variation within commercial cultivars (data not shown). So, this supports the conclusion that the differences between the hybrids and the commercial cultivars were due to genetic differences.

Yield Trials

Yield trials were conducted to examine yield potential of diploid hybrids, the GxE interaction and to compare yield components between diploid hybrids and tetraploid cultivars. The trials were conducted at five sites in France, Belgium and Netherlands (3 sites) from which insight into the variation due to genetics, the different environments and their interactions was obtained.

Performance of Diploid Hybrids and Tetraploid Cultivars

For a long time, the assumption that tetraploid potatoes are inherently higher yielding than diploid potatoes was based in a large part on the untested hypothesis that having four allelic variants per locus contributes to yield superiority (Bani-Aameur et al. 1991; Jong and Tai 1991; Werner and Peloquin 1991; Buso et al. 1999). When grown in a farmer-managed ware crop, we have shown that two of the diploid hybrids and cultivar Annabelle show similar average yields with 48, 52 and 52 Mg/ha, respectively, thereby proving the yield potential of diploid hybrids. This was suprising; as diploid hybrids were never selected for high yielding under field conditions, they were not expected to keep up with yield of tetraploid commercial cultivars in which selection for high yield has been performed for hundreds of years.

These results are supported by trials of Hutten (1994) and Jansky et al. (2016) in which they demonstrated yield potential of diploid potato.

Next to fresh tuber yield, other important criteria that determine the economic value of a cultivar were assessed. The diploid hybrids that were used in this trial had a range for DM% as well as for tuber shape that overlapped with the tetraploid cultivars for chips and fries (Hermes and Innovator). As heritability of tuber shape is high (Bisognin et al. 2012; Lindqvist-Kreuze et al. 2015; Meijer et al. 2018), it is a trait that can be selected for efficiently.

Aardevo Exhibit 1010
Page 0677

Environmental Effect on Plant Performance

Insight into the stability of desired traits across different environments is important to be able to select for desired trait properties efficiently in the breeding process. These diploid hybrids were a set of first test hybrids, developed while the breeding programme was still immature. Therefore, more variation was expected in the hybrids than in the tetraploid cultivars, which were clones. This expected variation was investigated for yield as well as a number of relevant traits.

Yield of diploid hybrids and tetraploid cultivars was affected by the environment. In line with the results found in this study, potato tuber yield is known to be highly influenced by different environments (Haverkort 1990). Several approaches of measuring yield stability are possible (Lin et al. 1986). In this study, the AMMI analysis was used as a useful method to distinguish the effects of genotype, environment and their interaction (Gauch et al. 2008). The first principal component axis PC1 explained 62.9% of the GxE interaction and is therefore used to discuss the interaction. Although we cannot attribute differences in performance between hybrids or tetraploid commercial cultivars to specific environmental conditions, we were able to determine that yields of both diploid hybrids and tetraploid cultivars were affected by GxE. The magnitude on the PC1 axis was comparable between hybrids and cultivars; however, the scores of the tetraploid cultivars generally were higher. A higher score of a cultivar in a specific environment indicates a better performance in that environment, while genotypes that score close to zero perform more constant across environments (Gauch and Zobel 1997). Even though the hybrids generally had lower yields and performed less stable across the environments than cultivars, there were also hybrids that perform similar to tetraploid cultivars in both yield and stability in this set of unselected test-hybrids.

Dry matter content of both diploid hybrids and tetraploid cultivars was affected similarly by the environment. When stability of a cultivar for DM% is high, efficient selection for specific dry matter percentages is possible (Wang et al. 2017). Although there was still genetic variability within the diploid hybrids and the hybrids were never tested in the field before, overall stability of the performance was similar to clonally propagated tetraploid cultivars. This genetic variability may have led to the slightly higher CV of shape in diploid hybrids compared to tetraploid cultivars; however, it was small in effect, which is in line with earlier reports (Yildirim and Celal 1985; Love et al. 1997).

The variation of yield components in the different environments was compared between the diploid hybrids and the tetraploid cultivars. The relation of the three different yield components was similar for tetraploids and diploids: for both, the number of tubers that was produced per stem was affected most by the environment, followed by number of stems per m^2 and finally tuber size.

No difference was found in the average effect of environment on yield components between hybrids and tetraploid cultivars. This comparison shows that diploid hybrid cultivars express similar variation to various environments as commercial tetraploid cultivars despite the genetic variation that is still present in these first test hybrids, and the difference in starting material (seedling tubers compared to normal seed tubers). Moreover, it shows that yield and dry matter are highly affected by the environment, so multiple environments are needed for selection, while tuber shape is more stable across different environments.

Aardevo Exhibit 1010
Page 0678

Contribution of Yield Components to Total Yield

The variation of yield components in different environments was compared to the difference in total yield between those environments to evaluate the contribution of each yield component to the total yield. An increase in number of tubers produced per stem showed the strongest positive effect on yield in diploid hybrids as well as in tetraploid cultivars. For tetraploid cultivars, a similar effect of number of tubers per plant was found by Asghari-Zakaria et al. (2007). In contrast to the effect of the number of stems, the effect of tuber size on yield differed between tetraploid cultivars and hybrids. The commercial tetraploid cultivars already produced relatively large tubers and larger tubers did not result in more yield or affected yield even negatively. This finding is in contrast to the results of Asghari-Zakaria et al. (2007) who showed a positive correlation between tuber size and yield. However, as both studies contained only 1 year of field trials, different findings could be explained by year effects. The results suggest that there is an optimum in tuber size above which larger tuber size has a neutral or negative effect on total yield.

The distribution of yield components was different between diploid hybrid cultivars and tetraploid cultivars. Diploids had smaller tubers and fewer tubers per stem, but more stems/m^2 than tetraploids. The large number of stems/m^2 in diploid hybrids even affected total yield negatively, while in all tetraploid cultivars, an increase of stem density had a positive effect on yield. Firman and Allen (2007) showed that increasing stem density up to 10 stems/m^2 resulted in a higher total yield, but higher densities did not affect total yield, and in our study, we found an optimum of 18 stems/m^2. With larger stem densities, the tuber size distribution can be steered: higher density results in more smaller tubers (Firman and Allen 2007). Indeed, in this experiment, a negative correlation was found between stem density and tuber size.

As most commercial tetraploids cultivars produced larger tubers than diploid hybrids, the effect of increased tuber weight was larger in hybrids than in tetraploid cultivars. The estimated optimal tuber size was 100 g/tuber and larger tubers did not contribute to higher total yield or even resulted in lower yield. A larger number of tubers per stem resulted in a higher total yield in most diploid hybrids and tetraploid commercial cultivars. This comparison shows the similarity of distribution of yield components contributing to yield in diploid hybrids and tetraploid cultivars. In addition, it gives insight in which yield components need most focus in the diploid hybrid cultivars to improve yield potential.

Implications for Breeding

The analysis of yield components was performed for different environments and the variation of specific plant properties in different environments was evaluated. With this information, we determined traits that need to be tested in several environments to make reliable selections, for example yield, dry matter and tuber number were affected by the environment. Specific yield components that can be used as selection criteria in a breeding program were identified. In this trial, optimal configurations of yield components for the tested environments were found: a tuber size of approximately 100 g/tuber and a stem density of about 18 stems/m^2, with as many tubers as possible as more tubers always increased total yield. These configurations can be used as breeding

Aardevo Exhibit 1010
Page 0679

targets. Moreover, we were able to identify more stable hybrids over more variable ones, and hence we can start selecting for trait stability.

Conclusion

In this study, we compared the performance of diploid test-hybrid cultivars to tetraploid commercial ones in field trials at representative locations in the NW-European potato-growing area. In these trials, some diploid hybrids have shown a yield potential comparable to the tetraploid cultivars. For major economically relevant traits such as dry matter percentage, tuber number/m^2 and tuber size, the diploid hybrids only slightly underperformed compared to the tetraploid cultivars. For yield, the best hybrids performed as good as the lower yielding tetraploid cultivars, the diploid hybrid cultivars having never been selected under field conditions. Stability of these traits across different locations was remarkably similar to the that of tetraploid cultivars. Moreover, the stability was different for the measured traits. For making selections in breeding, this information helps to determine the number of environments that is needed to test a certain trait. There was a large overlap in performance of the diploid hybrids and tetraploid cultivars for all the yield components analyzed (stems per m^2, tubers per stem and tuber size). In both tetraploid cultivars and diploid hybrids, the number of tubers per stem affected the total yield most, followed by stems/m^2. Tuber size had the smallest effect on total yield. With the variation for these traits present in diploids, desired properties can be combined in a cultivar through hybrid breeding which is competitive with the current commercial tetraploid cultivars.

References

Arsenault WJ, LeBlanc DA, Tai GCC, Boswall P (2001) Effects of nitrogen application and seedpiece spacing on yield and tuber size distribution in eight potato cultivars. Am J Potato Res 78(4):301–309

Asghari-Zakaria D, Fathi M, Hasan-Panah D (2007) Sequential Path Analysis of Yield Components in Potato. Potato Res. 49(4):273–279

Bani-Aameur F, Lauer FI, Veilleux RE, Hilali A (1991) Genomic Composition of 4x–2x Potato Hybrids: Influence of Solanum Chacoense. Genome 34(3):413–420

Bisognin DA, Rigão MA, Lopes SJ, Storck L (2012) Heritability and Correlation among Potato Tuber Traits. Crop Breed. Appl. Biotechnol. 12(3):215–219

Buso JA, Boiteux LS, Peloquin SJ (1999) Multitrait Selection System Using Populations with a Small Number of Interploid (4x-2x) Hybrid Seedlings in Potato: Degree of High-Parent Heterosis for Yield and Frequency of Clones Combining Quantitative Agronomic Traits. Theor Appl Genet 99(1–2):81–91

Crossa J (1990) Statistical analysis of multilocation trials. Adv. Agron. 44:55–85

Douches DS, Maas D, Jastrzebski K, Chase RW (1996) Assessment of potato breeding progress in the USA over the last century. Crop Sci 26(6):1544–1552

Endelman JB, Jansky SH (2016) Genetic Mapping with an Inbred Line-Derived F2 Population in Potato. Theor Appl Genet 129(5):935–943

Firman DM, Allen EJ (2007) Potato Biology and Biotechnology: Advances and Perspectives. Chapter 33: Agronomic practices. Elsevier, Amsterdam. 719–38

Aardevo Exhibit 1010
Page 0680

Gauch H, Piepho H, Annicchiarico P (2008) Statistical Analysis of Yield Trials by AMMI and GGE: Further Considerations. Crop Sci 48. https://doi.org/10.2135/cropsci2007.09.0513

Gauch HG, Zobel R (1997) Identifying mega-environment and targeting genotypes. Crop Sci 37:381–385

Jong H, Tai GCC (1991) Evaluation of Potato Hybrids Obtained from Tetraploid-Diploid Crosses I. Parent-Offspring Relationships. Plant Breeding 107(3):177–182

Haverkort AJ (1990) Ecology of Potato Cropping Systems in Relation to Latitude and Altitude. Agr. Syst. 32(3):251–272

Haverkort AJ, Struik PC (2015) Yield Levels of Potato Crops: Recent Achievements and Future Prospects. Field Crop. Res. 182:76–85

Haverkort AJ, van de Waart M, Bodlaender KBA (1990) Interrelationships of the Number of Initial Sprouts, Stems, Stolons and Tubers per Potato Plant. Potato Res 33(2):269–274

Hosaka K, Hanneman RE (1998) Genetics of self-compatibility in a self-compatible wild diploid potato species Solanum chacoense. 2. Localization of an S- locus inhibitor (Sli) gene on the potato genome using DNA markers. Euphytica 103:265–271

Hutten RCB (1994) Basic Aspects of Potato Breeding via the Diploid Level. Wageningen

Jansky SH, Charkowski AO, Douches DS, Gusmini G, Richael C, Bethke PC, Spooner DM, Novy RG, De Jong H, De Jong WS, Bamberg JB, Tomphson AL, Bizimungu B, Holm DG, Brown CR, Haynes KG, Sathuvalli VR, Veilleux RE, Miller C, Bradeen JM, Jiang J (2016) Reinventing Potato as a Diploid Inbred Line-Based Crop. Crop Sci 56(4):1412–1422

Jeuken MJW, Lindhout P (2004) The Development of Lettuce Backcross Inbred Lines (BILs) for Exploitation of the Lactuca Saligna (Wild Lettuce) Germplasm. Theor Appl Genet 109(2):394–401

Kooman PL, Fahem M, Tegera P, Haverkort AJ (1996) Effects of Climate on Different Potato Genotypes 2. Dry Matter Allocation and Duration of the Growth Cycle. Eur J Agron 5(3–4):207–217

Lin CS, Binns MR, Lefkovitch LP (1986) Stability Analysis: Where Do We Stand? Crop Sci 26:894–900

Lindhout P, de Vries M, ter Maat M, Su Y, Viquez-Zamora M, van Heusden S (2018) Hybrid Potato Breeding for Improved Varieties. Achieving Sustainable Cultivation of Potatoes 1(1):1–24

Lindhout P, Meijer D, Schotte T, Hutten RCB, Visser RGF, van Eck HJ (2011) Towards F 1 Hybrid Seed Potato Breeding. Potato Res 54(4):301–312

Lindqvist-Kreuze H, Khan A, Salas E, Meiyalaghan S, Thomson S, Gomez R, Bonierbale M (2015) Tuber Shape and Eye Depth Variation in a Diploid Family of Andean Potatoes. BMC Geneti 16(1):1–10

Love SL, Werner BK, Pavek JJ (1997) Selection for Individual Traits in the Early Generations of a Potato Breeding Program Dedicated to Producing Cultivars with Tubers Having Long Shape and Russet Skin. Am Potato J 74(3):199–213

Lynch DR, Foroud N, Kozub GC, Fames BC (1995) The Effect of Moisture Stress at Three Growth Stages on the Yield, Components of Yield and Processing Quality of Eight Potato Varieties. Am Potato J 72:375–385

Meijer D, Viquez-Zamora M, van Eck HJ, Hutten RCB, Su Y, Rothengatter R, Visser RGF, Lindhout WH, van Heusden AW (2018) QTL Mapping in Diploid Potato by Using Selfed Progenies of the Cross S. Tuberosum × S. Chacoense. Euphytica 214(7):121

NAK (2018) Inspection of seed potatoes; https://www.nak.nl/wp-content/uploads/2018/10/Inspection-of-seed-potatoes-DIS-2018-definitief.pdf

Piepho HP, Laidig F, Drobek T, Meyer U (2014) Dissecting Genetic and Non-genetic Sources of Long-term Yield Trend in German Official Variety Trials. Theor Appl Genet 127(5):1009–1018

Prinzenberg AE, Víquez-Zamora M, Harbinson J, Lindhout P, van Heusden S (2018) Chlorophyll Fluorescence Imaging Reveals Genetic Variation and Loci for a Photosynthetic Trait in Diploid Potato. Physiol Plant 164(2):163–175

R Core Team (2017) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. https://www.R-project.org/

Rodríguez-Álvarez MX, Boer MP, van Eeuwijk FA, Eilers PHC (2018) Correcting for Spatial Heterogeneity in Plant Breeding Experiments with P-Splines. Spat Stat 23:52–71

Struik PC (2007) The Canon of Potato Science: 40. Physiological Age of Seed Tubers. Potato Res 50:375–377

Struik PC, Wiersema SG (2001) Seed Potato Technology. Wageningen Academic Publishers, The Netherlands

Su Y, Viquez-Zamora M; den Uil D; Simnige J; Kruyt H; Vossen J; Lindhout P; van Heusden S (2019) Introgression of Genes for Resistance against Phytophthora infestans in Diploid Potato. American Journal of Potato Research

Aardevo Exhibit 1010
Page 0681

Tiemens-Hulscher M, Delleman J, Eising J, Lammerts van Bueren ET (2013) Potato Breeding. Drukkerij De Swart, Den Haag

Wang Y, Snodgrass LB, Bethke PC, Bussan AJ, Holm DG, Novy RG, Pavek MJ, Porter GA, Rosen CJ, Sathuvalli V, Thompson AL, Thornton MT, Endelman JB (2017) Reliability of Measurement and Genotype × Environment Interaction for Potato Specific Gravity. Crop Sci 57:1–7

Werner JE, Peloquin SJ (1991) Yield and Tuber Characteristics of 4x Progeny from 2x x 2x Crosses. Potato Res 34(3):261–267

Yildirim MB, Celal F (1985) Genotype x environment interactions in potato. Am Potato J62(7):371–375

Zaheer K, Akhtar MH (2016) Potato Production, Usage, and Nutrition---A Review. Crit Rev Food Sci Nutr 56(5):711–721. https://doi.org/10.1080/10408398.2012.724479

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Aardevo Exhibit 1010
Page 0682

Electronic Patent Application Fee Transmittal

Application Number:	16541071
Filing Date:	14-Aug-2019
Title of Invention:	Hybrid Seed Potato Breeding
First Named Inventor/Applicant Name:	Willem Hendrik Lindhout
Filer:	Sheila Walker/Annette Armstrong
Attorney Docket Number:	VONL:004USC1

Filed as Small Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 2 months with $0 paid	2252	1	300	300
Miscellaneous:				
SUBMISSION- INFORMATION DISCLOSURE STMT	2806	1	120	120
		Total in USD ($)		**420**

Electronic Acknowledgement Receipt

EFS ID:	39360306
Application Number:	16541071
International Application Number:	
Confirmation Number:	4348
Title of Invention:	Hybrid Seed Potato Breeding
First Named Inventor/Applicant Name:	Willem Hendrik Lindhout
Customer Number:	125771
Filer:	Sheila Walker
Filer Authorized By:	
Attorney Docket Number:	VONL:004USC1
Receipt Date:	05-MAY-2020
Filing Date:	14-AUG-2019
Time Stamp:	19:25:58
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$420
RAM confirmation Number	E202055J26325401
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		VONL_004USC1_Response.pdf	8537793 97c8d7be0aefaa4480748e614f8fe29fabae2a5a	yes	45

Multipart Description/PDF files in .zip description		
Document Description	**Start**	**End**
Amendment/Req. Reconsideration-After Non-Final Reject	1	1
Specification	2	10
Claims	11	14
Applicant summary of interview with examiner	15	15
Applicant Arguments/Remarks Made in an Amendment	16	25
Affidavit-traversing rejectns or objectns rule 132	26	45

Warnings:

Information:

2	Extension of Time	VONL_004USC1_EOT.pdf	94917 9b53f12fd3ba3984e6699a5b06361768ac776578	no	1

Warnings:

Information:

3		VONL_004USC1_IDS.pdf	81908 895caef679063d5a73aa1a5880715466648c4b8e	yes	5

Multipart Description/PDF files in .zip description		
Document Description	**Start**	**End**
Transmittal Letter	1	2

	Information Disclosure Statement (IDS) Form (SB08)		3	5	
Warnings:					
Information:					
4	Non Patent Literature	De_Vries_2016pto.pdf	1948093 8c7a11b0cc5bf5b45c5b2ac6a331211b7b3f3b36	no	25
Warnings:					
Information:					
5	Non Patent Literature	Jansky_2015.pdf	1823893 f18f6ddbd9166f4411dbb2d4fcc6d18442ded692	no	3
Warnings:					
Information:					
6	Non Patent Literature	Jansky_2018pto.pdf	305590 cda617cae0a61fb6d4e6097b1e6a98bb3962fc7f	no	47
Warnings:					
Information:					
7	Non Patent Literature	Lindhout_2017pto.pdf	1948093 282f5a2a9a97a0a26ec6075b796e7bc9685dc362	no	25
Warnings:					
Information:					
8	Non Patent Literature	Stockem_2020.pdf	1226342 36f7c39e00eca6e75e04312e193ee82a49296b84	no	22
Warnings:					
Information:					
9	Fee Worksheet (SB06)	fee-info.pdf	32676 6ad122516be9b147c6458fdc8942e4f3e941b9c4	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	15999305		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 16/541,071	Filing Date 08/14/2019	☐ To be Mailed

ENTITY: ☐ LARGE ☑ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $50 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $230 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT	05/05/2020	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 10	Minus	** 20	= 0		x $50 =	0
	Independent (37 CFR 1.16(h))	* 2	Minus	*** 3	= 0		x $230 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	LIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/WILLIAM N PHILLIPS/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Willem Hendrik Lindhout *et al.*	Group Art Unit: 1663
Serial No.: 16/541,071	Examiner: Russell T. Boggs
Filed: August 14, 2019	Atty. Dkt. No.: VONL:004USC1
For: Hybrid Seed Potato Breeding	Confirmation No.: 4348

REQUEST TO TREAT APPLICATION AS SPECIAL PURSUANT TO MPEP § 707.02 AND § 708.01(I)

Mail Stop Amendment
Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

Applicant hereby requests that the current application be treated as special and examined on an expedited basis accordingly. MPEP § 707.02 states that any application that has been pending five or more years should be carefully studied by the supervisory patent examiner and every effort should be made to terminate its prosecution and, in order to accomplish this result, "the application ***is to be considered 'special' by the examiner***." (emphasis added). Pendency under this provision is measured to include prior related U.S. applications. MPEP § 708.01(I).

The current case is a continuation of United States Application No. 13/504,156, filed July 10, 2012, and also claims priority to other U.S. applications filed more than five years prior to the current date. Thus, the total pendency of the present application in accordance with MPEP § 708.01(I) is more than five years. This application should therefore be treated as special pursuant to MPEP § 707.02 and such action is thus respectfully requested.

Aardevo Exhibit 1010
Page 0690

No fees are believed due in connection with the filing of the instant paper. However, should any such fees become due under 37 C.F.R. §§ 1.16 to 1.21 for any reason relating to the instant request, the Commissioner is authorized to deduct said fees from Dentons US LLP's Deposit Account No. 19-3140/VONL:004USC1.

DENTONS US LLP
2000 McKinney Avenue, Suite 1900
Dallas, TX 75201-1858
214-259-0931
214-259-0910 (fax)

Respectfully submitted,

/Robert E. Hanson/

Robert E. Hanson
Reg. No. 42,628
Attorney for Applicant

Date: June 30, 2020

Electronic Acknowledgement Receipt

EFS ID:	39865583
Application Number:	16541071
International Application Number:	
Confirmation Number:	4348
Title of Invention:	Hybrid Seed Potato Breeding
First Named Inventor/Applicant Name:	Willem Hendrik Lindhout
Customer Number:	125771
Filer:	Robert E. Hanson
Filer Authorized By:	
Attorney Docket Number:	VONL:004USC1
Receipt Date:	30-JUN-2020
Filing Date:	14-AUG-2019
Time Stamp:	23:35:06
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Petition for review by the Technology Center SPRE	VONL_004USC1_Treat_App_Special_Req.pdf	25927 ec8c6d46ede8a69397ba954eebd323dbd3839cfb	no	2

Warnings:

Information:	
Total Files Size (in bytes):	25927

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

United States Patent and Trademark Office

Office of the Chief Financial Officer

Document Code:WFEE

User :66270

Sale Accounting Date:07/10/2020

Sale Item Reference Number	Effective Date
16541071	06/30/2020

Document Number	Fee Code	Fee Code Description	Amount Paid	Payment Method
I202070A42141289	2464	PETITION FEE-37CFR 1.17(H) (GROUP II)	$70.00	Deposit Account

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/541,071	08/14/2019	Willem Hendrik Lindhout	VONL:004USC1	4348

125771 7590 07/17/2020

DENTONS US LLP
P.O. Box 061080
Chicago, IL 60606

EXAMINER	
BOGGS, RUSSELL T	

ART UNIT	PAPER NUMBER
1663	

NOTIFICATION DATE	DELIVERY MODE
07/17/2020	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

patents.us@dentons.com

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

DENTONS US LLP
P.O. Box 061080
Chicago IL 60606

In re Application of	:
Lindhout et al.	:
Serial No.: 16/541,071	: Decision on Petition
Filed: August 14, 2019	:
Attorney Docket No: VONL:004USC1	:

This letter is in response to the petition filed under MPEP §§ 707.02 and 708.01 filed on June 30, 2020 to treat as "special" for expedited prosecution.

BACKGROUND

Relevant parts of the prosecution history are summarized below.

- The instant application filed on August 14, 2019 is a continuation of US Application No. 13/504,156, filed July 10, 2012.

- On June 30, 2020, applicants submitted a petition requesting that the instant application be treated as "special" since the instant application has been pending for a total of five years or more taken into consideration the pendency of the 13/504,156 parent application.

    - It is noted that the Petition provides authorization to charge any necessary petition fee to Deposit Account No. 19-3140/VONL: 004USC1.

RELEVANT PROVISIONS

- **MPEP § 707.02 Applications Up for Third Action and 5-Year Applications**

… Any application that has been pending five years or more should be carefully studied by the supervisory patent examiner and every effort should be made to terminate its prosecution. In order to accomplish this result, the application is to be considered *"special"* by the examiner.

- **. MPEP § 708.01 List of Special Cases (in relevant part)**

37 C.F.R. 1.102 Advancement of examination.

…. (b) Applications wherein the inventions are deemed of peculiar importance to some branch of the public service and the head of some department of the Government requests immediate action for that reason, may be advanced for examination.

(c) A petition to make an application special may be filed without a fee if the basis for the petition is:

(1) The applicant's age or health; or

(2) That the invention will materially:

(i) Enhance the quality of the environment;

(ii) Contribute to the development or conservation of energy resources; or

(iii) Contribute to countering terrorism.

(d) A petition to make an application special on grounds other than those referred to in paragraph (c) of this section must be accompanied by the fee set forth in § 1.17(h).

The following is a list of special cases (those which are advanced out of turn for examination): …

(l) *Applications pending more than 5 years, including those which, by relation to a prior United States application*, have an effective pendency of more than 5 years. See MPEP § 707.02

DISCUSSION

The petition has been carefully considered.

The instant application has been pending greater than five years in light of the effective filing date of the parent application.

The petition fee set forth in § 1.17(h) has been charged to Deposit Account No. 19-3140/VONL: 004USC1, as authorized.

DECISION

The petition is **GRANTED.**

Should there be any questions about this decision please contact Bennett Celsa, by letter addressed to Director, TC 1600, at the address listed above, or by telephone at 571-272-0807 or by facsimile sent to the general Office facsimile number, 571-273-8300.

/GARY JONES/
Director, Technology Center 1600

Technology Center Petition Count Sheet

Application No.	Applicant(s)	
16/541,071	Lindhout et al.	

Petition Code	Petition Type
☐ 601	Petition to Make Special, Age or Health (37 CFR 1.102, MPEP § 708.02)
☐ 604	Petition to Make Special, Accelerated Examination (37 CFR 1.102, MPEP § 708.02)
☐ 607	Petition to Make Special, For Reasons not Provided in 601-606 and 639-640 (37 CFR 1.102, MPEP § 708.02)
☐ 608	Petition to Reopen After a Board Decision (37 CFR 1.198)
☐ 609	Petition for Review of Final Restriction Requirement (37 CFR 1.144)
☐ 610	Petition Invoking Authority of Commissioner under 37 CFR 1.181 Not Specified in Codes 611-617
☐ 611	Petition Relating to Prematureness of Final Rejection (37 CFR 1.181, MPEP § 706.07)
☐ 612	Petition Relating to Refusal to Enter an Amendment (37 CFR 1.181 and 1.127, MPEP § 714.09)
☐ 613	Petition Relating to Withdraw of holding of Abandonment (37 CFR 1.137, MPEP § 711.03)
☐ 614	Petition Relating to Withdraw of holding of Abandonment (37 CFR 1.137, MPEP § 7.14.19)
☐ 615	Petition Relating to Formal Sufficiency / Propriety of Affidavits (37 CFR 1.131, 1.132, 1.608, MPEP § 608.04(C)
☐ 617	Petition Relating to Refusal to Enter an Amendment (37 CFR 1.312)
☐ 620	Petition for Extension of Time (37 CFR 1.136 (b))
☐ 622	Petition Concerning Appeal Application before Transfer of Jurisdiction to Board of Appeals (MPEP § 1206)
☐ 623	Petition for Second or Subsequent Suspension of Action (37 CFR 1.103, MPEP § 709)
☐ 634	Petition to Correct Inventorship in a Patent not in Interference (37 CFR 1.324, MPEP § 1481)
☐ 635	Petition to Correct Inventorship in a Patent Application (37 CFR 1.48)
☐ 636	Petition to Change Inventorship in a Patent (37 CFR 1.324)
☐ 637	Petition to Withdraw from Issue, before Payment of an Issue Fee (37 CFR 1.313(a))
☐ 639	Petition for Make Special Prospective Manufacture (37 CFR 1.102, MPEP § 708.02)
☐ 646	Petition for Make Special Accelerated Examination
☐ 690	Petition Related Color Drawings of Photographs
☑ 699	Petition for Matters Before Technology Center

Information Required for OPESS Processing

☑ GRANTED ☐ GRANTED-IN-PART ☐ DENIED ☐ DISMISSED

☑ Mail Decision and Enter Decision in Petition Database/PALM
☐ Copy Decision, Send to Scanning
☐ Fees Need to be Charged
☐ _____

To: patents.us@dentons.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 125771

Jul 18, 2020 06:50:55 AM

Dear PAIR Customer:

DENTONS US LLP
P.O. Box 061080
Chicago, IL 60606
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 125771 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

Disclaimer:
The list of documents shown below is provided as a courtesy and is not part of the official file wrapper. The content of the images shown in PAIR is the official record.

Application	Document	Mailroom Date	Attorney Docket No.
16541071	PETDEC	07/17/2020	VONL:004USC1

To view your correspondence online or update your email addresses, please visit us anytime at https://sportal.uspto.gov/secure/myportal/privatepair.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16541071
Filing Date	2019-08-14
First Named Inventor	Willem Hendrik Lindhout
Art Unit	1663
Examiner Name	Russell T. Boggs
Attorney Docket Number	VONL:004USC1

U.S.PATENTS

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1					

If you wish to add additional U.S. Patent citation information please click the Add button.

U.S.PATENT APPLICATION PUBLICATIONS

Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1					

If you wish to add additional U.S. Published Application citation information please click the Add button.

FOREIGN PATENT DOCUMENTS

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1							☐

If you wish to add additional Foreign Patent Document citation information please click the Add button

NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	16541071
	Filing Date	2019-08-14
	First Named Inventor	Willem Hendrik Lindhout
	Art Unit	1663
	Examiner Name	Russell T. Boggs
	Attorney Docket Number	VONL:004USC1

	1	Response to Non-Final Office Action regarding U.S. Application No. 14/112,747 (Atty. Dkt. No. VONL:006US), dated July 16, 2020.	☐

If you wish to add additional non-patent literature document citation information please click the Add button

EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16541071
Filing Date	2019-08-14
First Named Inventor	Willem Hendrik Lindhout
Art Unit	1663
Examiner Name	Russell T. Boggs
Attorney Docket Number	VONL:004USC1

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☒ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☒ See attached certification statement.

☐ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☐ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Amy Dunstan/	Date (YYYY-MM-DD)	2020-08-20
Name/Print	Amy Dunstan	Registration Number	61069

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Willem Hendrik Lindhout *et al.*	Group Art Unit: 1663
Serial No.: 14/112,747	Examiner: Russell T. Boggs
Filed: December 23, 2013	Atty. Dkt. No.: VONL:006US
For: HYBRID SEED POTATO BREEDING	Confirmation No.: 5919

RESPONSE TO NON-FINAL OFFICE ACTION DATED JANUARY 16, 2020

Mail Stop Amendment
Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

This paper is submitted in response to the non-final Office Action dated January 16, 2020, for which the date for response is July 16, 2020, in view of the enclosed Petition for Extension of Time and fees. No additional fees are believed due in connection with the filing of the instant paper. However, should any such fees become due under 37 C.F.R. §§ 1.16 to 1.21 for any reason relating to the instant response, the Commissioner is authorized to deduct said fees from Dentons US LLP's Deposit Account No. 19-3140/VONL:006US.

Amendments to the Claims begin on page 2.

Telephonic Interview Summary begins on page 6.

Remarks begin on page 7.

AMENDMENTS TO THE CLAIMS

Please amend the claims as follows:

1. (Currently Amended) A diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line comprising plants having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant, wherein said self-compatibility originates from *Solanum chacoense* and is referred to as S-locus inhibitor (*Sli*) gene, wherein a copy of said gene[[as]] is present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, representative seeds of said lines having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively, wherein said line in addition to said tuber yield comprises at least one agronomically desirable trait or gene, wherein said at least one agronomically desirable trait or gene provides resistance to *Phytophthora infestans* infections.

2. (Currently Amended) A hybrid potato seed resulting from a cross between two essentially homozygous parental potato breeding lines, wherein at least one of the lines is ~~which is a plant of~~ the line according to claim 1.

3. (Previously Presented) A hybrid potato plant grown from the seed of claim 2.

4. (Previously Presented) A tetraploid plant produced by doubling the genome of a plant of the line of claim 1.

5. (Currently Amended) A method for producing a diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line comprising plants having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant, comprising:

(a) providing a first potato plant, which first potato plant is a plant of a first diploid, self-compatible and fertile potato line, wherein said self-compatibility originates from *Solanum chacoense* and is referred to as S-locus inhibitor (*Sli*) gene, wherein a copy of said gene[[as]] is present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, representative seeds of said lines having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively;

Aardevo Exhibit 1010
Page 0705

(b) ~~providing~~cross-pollinating said first potato plant with a second potato plant to produce a plurality of hybrid offspring seeds, wherein said second potato plant is a plant of a diploid or tetraploid *Solanum tuberosum* potato line which may have any level of homozygosity, said line of said second potato plant comprising plants having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant;

(c) ~~cross pollinating said first and second potato plants to provide~~ seeds ~~and collecting said seeds to thereby provide an offspring generation in the form of a hybrid offspring seed;~~

~~(d)~~ growing a plurality of hybrid offspring potato plants from said plurality of hybrid offspring seeds ~~a population of hybrid offspring potato plant and;~~

(d) selecting ~~from said population~~at least a first hybrid offspring plant[[s]], wherein said plant is ~~being~~ fertile and produces~~producing~~ tubers at a yield expressed in grams of fresh weight of at least 200 grams per plant;~~, and~~

(e) ~~selfing~~crossing said selected hybrid offspring potato plant with itself or a plant of the potato line of step (a)~~or backcrossing said hybrid offspring potato plant to a potato plant of said line of said first potato plant,~~ for between 1 to 8 generations~~selfings or backcrosses,~~ to ~~thereby provide~~produce a diploid, self-compatible and essentially homozygous *Solanum tuberosum* potato breeding line comprising plants having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant;

(f) said method further comprising the step of introducing into a plant of said diploid, self-compatible and essentially homozygous potato breeding line obtained in step (e) at least one trait or gene provides resistance to *Phytophthora infestans* infections.

6. (Previously presented) The method of claim 5, wherein said first potato plant is a plant of potato line NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, representative seeds of said line having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively.

7. (Previously presented) A diploid potato breeding line obtained by the method of claim 5.

8. (Previously Presented) A method for producing a uniform hybrid potato seed, comprising:

Aardevo Exhibit 1010
Page 0706

(a) providing a first potato plant of the line according to claim 1,

(b) providing a second potato plant, wherein said second potato plant is a plant of a diploid, fertile, self-compatible and essentially homozygous potato line, and

(c) cross pollinating said first and second potato plant and allowing one of either said first or second potato plant to produce berries with seeds and collecting said seeds from said berries to provide an offspring generation in the form of a hybrid offspring seed.

9. (Original) A seed produced by the method of claim 8.

10. (Original) A hybrid potato plant, or part thereof, produced by growing the seed of claim 9.

11. (Previously Presented) A pollen or an ovule of the plant of claim 10.

12. (Original) A tuber from the plant of claim 10.

13-17. (Cancelled)

18. (Previously Presented) A set of at least 2 intercrossable diploid, fertile, self-compatible and essentially homozygous potato lines according to claim 1.

19. (Previously presented) An elite diploid potato breeding line obtained by the method of claim 5.

20.-21. (Cancelled)

22. (Previously Presented) The diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line according to claim 1, wherein said potato line is a progeny line of potato line NCIMB 41663, NCIMB 41664, NCIMB 41665, or NCIMB 41765, representative seeds of said NCIMB lines having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively.

23. (Cancelled)

24. (Previously Presented) The method according to claim 5, wherein the at least one trait or gene that is introduced into the plant of said diploid, self-compatible and essentially homozygous potato breeding line obtained in step (e) is selected from *S. venturii* genes *Rpi-vnt1.1*, *Rpi-vnt1.2,* and *Rpi-vnt1.3* on chromosome 9.

Aardevo Exhibit 1010
Page 0707

25. (Previously Presented) The method according to claim 5, wherein the at least one trait or gene that is introduced into the plant of said diploid, self-compatible and essentially homozygous potato breeding line obtained in step (e) is introduced by transgenic means.

26. (Previously Presented) The diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line of claim 1, wherein at least 25% of the homozygous genomic loci in a plant of said line are from one or more of NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, and NCIMB accession number 41765.

Aardevo Exhibit 1010
Page 0708

TELEPHONIC INTERVIEW SUMMARY

On April 6, 2020, Applicants' undersigned representatives, Robert E. Hanson, Reg. No. 42,628, and Sheila Walker, Reg. No. 71,087, spoke telephonically with Examiner Boggs regarding the outstanding rejections in the present Office Action. The Examiner indicated that amendments to claim 1 and 5 to recite that a copy of the *Sli* gene is present in the recited deposits would address the indefiniteness rejection over the claims. The Examiner also indicated that the portions of the written description and enablement rejections that are similar to those raised in U.S. Application No. 16/541,071 can be addressed through the submission of the same Declaration of Dr. Willem Hendrik Lindhout under 37 C.F.R. § 1.132.

With regard to the indefiniteness, written description, and enablement rejections that relate to the limitation "the trait or gene provides resistance to *Phytophthora infestans* infections" in claims 1, 5, and 20, the Examiner indicated that additional evidence that potato germplasm is "known and readily available" for each of the *Phytophthora infestans* resistance genes listed in Table 10 of the specification would be needed to address the rejections.

Applicants thank Examiner Boggs for the time taken to discuss the case.

Aardevo Exhibit 1010
Page 0709

REMARKS

A. Status of the Claims

Claims 1-12, 18-20 and 22-26 are pending in the application. Claims 1-12, 18-20 and 22-26 are rejected. Claims 1, 2, and 5 are amended herein. Support for the amendments can be found, at least, in the Specification and claims as filed.

B. Claim Objections

The Action objects to claim 5 for containing informalities. Applicants note that claim 5 is amended herein. The objection is thus believed to be moot and withdrawal thereof respectfully requested.

C. Specification Objection

The Action objects o Table 10 in the specification as improperly incorporating essential material. Specifically, the Examiner asserts that the meaning and scope of claims 20 and 23 can only be understood in view of the listed non-patent references in Table 10 and that the incorporation of these references in the specification is improper. Applicants respectfully traverse.

Applicants note that the genes in Table 10 for *Phytophthora* resistance (*Rpi* genes) are available from at least three sources: (i) from plants of commercial potato varieties; (ii) from plants held in publicly accessible culture collections and genebanks (seedbanks); and (iii) from plants studied by academic research groups. At Wageningen University and Research (WUR) in the Netherlands, for instance, many of the potato cultivars and wild *Solanum* accessions studied are obtained from an-in-house collection and from the Center of Genetic Resources the Netherlands (CGN), Wageningen, The Netherlands (http://www.cgn.wur.nl/uk). The genebank for plant genetic resources, maintained by CGN, holds a collection of over 1700 potato varieties

Aardevo Exhibit 1010
Page 0710

and wild accessions. A simple search in the CGN database provides a number of accessions of potato species *Solanum bulbocastanum* that are late blight resistant, for instance, CGN18311, CGN18310, CGN17690, CGN22367, CGN17692, CGN17693, and CGN17688 (https://cgngenis.wur.nl/). Propagation material of these accessions can be ordered from CGN and is thus readily available to the public.

Huang 2005, listed in Table 10 of the specification, discloses that *Solanum demissum* accession CGN17810 contains *Rpi* genes R1, R2, R3, R4, R7, and R8. This accession is publicly available from the CGN.

In a recent publication by Rakosy-Tican *et al.*, 2020 (**EXHIBIT A**) the *Rpi* genes and their location is provided for five commercial potato cultivars ('Baltica', 'Delikat', 'Quarta', 'Romanze', and 'Sarpo Mira') and *S. bulbocastanum* accession GLKS31741 (blb41) (Groß Lüsewitz Potato Collections (GLKS) of the IPK Genebank, Leibniz-Institute of Plant Genetics and Crop Plant Research, Germany). In particular in Tables 1-3 of that publication and page 3 identify GLKS31741 as a source. The commercial potato variety 'Sarpo Mira,' which may *inter alia* be obtained from Danespo A/S, Give, Denmark (https://www.danespo.com/english/varieties/varieties/fresh-market/sarpo-mira) comprises *Phytophthora infestans* resistance genes *R3a* and *R3b*.

Foster *et al.* 2009, listed in Table 10 of the specification, discloses *S. venturi* accession CGN18108 contains *Rpi-vnt1.1*, *S. venturi* accession CGN18279 contains *Rpi-vnt1.2*, and *S. venturi* accession CGN17998 contains *Rpi-vnt1.3*. All of these accessions are publicly available from the CGN.

Lokossou 2010 (**EXHIBIT B**, p. 51, Table 1a and p. 52, Table 1b) discloses that the *Rpi-blb1* gene is present in *S. bulbocastanum* accessions PI275194, PI275195, CGN17689,

CGN21306, CGN17692, and CGN17690 and that *Rpi-blb2* is present in *S. bulbocastanum* CGN17692, 960631, PI 275195, PI498011, and PI498223. European Patent publication EP1334979A1 also discloses *Phytophthora infestans* resistance in CGN17692 (which also carries the designation BGRC 8005) is based on the *Rpi-blb1* gene. All of these accessions are publicly available from the CGN or at the U.S. National Plant Germplasm System (USDA-GRIN/NPGS).

Oosumi *et al.* 2009, listed in Table 10 of the specification, discloses *Rpi-bt1* as found in plants of *S. bulbocastanum* that were obtained from the Inter-Regional Potato Introduction Station (NRSP-6), Sturgeon Bay, Wisconsin.

Lokossou 2009, listed in Table 10 of the specification, discloses that the R2-*like Rpi* gene is present in genotype AM3778-16 and that the *Rpi-abpt* gene is present in clone 707TG11-1, and refers to for these clones to Park 2005a and Park 2005b, respectively. Park 2005a and Park 2005b, both of which are listed in Table 10 of the specification, disclose that both clones are produced at the Laboratory of Plant Breeding, Department of Plant Sciences, Wageningen University, Wageningen, The Netherlands. Although not a facility for regular ordering, such university collections provide for public availability of the plant material.

Malcolmson 1966, listed in Table 10 of the specification, teaches the presence of *Rpi* genes *R5* and *R6* in *S. demissum* accessions 3053-18 and XD2-21, respectively. *S. demissum* accessions 3053-18 and XD2-21 are obtainable from the in vitro collection at Science and Advice for Scottish Agriculture (SASA), Edinburgh, UK.

Trognitz 2007 (**EXHIBIT C**, Table 1) discloses *R9*, *R10*, and *R11* of *S. demissum* are present in cultivars CIP800994, CIP800995, and CIP800996, which are available from the potato collection of the International Potato Center (CIP), Lima, Peru.

Aardevo Exhibit 1010
Page 0712

The foregoing demonstrates that the references in Table 10 provide evidence of availability of the *Phytophthora* resistance genes to the public and thus one of skill in the art would be able to obtain accessions containing the genes based on the disclosure of the references in the Table. In view of this, the objection is thus believed to be moot and withdrawal thereof respectfully requested.

D. Rejection of Claims Under 35 U.S.C. §112, Second Paragraph

The Action rejects claims 1-12, 18-20 and 22-26 under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the applicant regards as the invention.

(1) Claims 1 and 5 are rejected as indefinite on the basis that the claims as amended now encompass a genus of equivalents to the *Sli* gene, but the specification teaches only one embodiment. Applicants respectfully traverse but note that claims 1 and 5 are amended herein consistent with the Telephonic Interview Summary above.

(2) Claims 1, 5, and 20 are rejected on the basis that the limitation "the trait or gene provides resistance to *Phytophthora infestans* infections" is insufficiently defined in the specification. Claims 20 and 23 are rejected as indefinite for allegedly including terms that lack a general art-accepted meaning, in view of the asserted improper incorporation of references listed in Table 10. Applicants respectfully traverse.

Applicants note that the genes in Table 10 for *Phytophthora* resistance (*Rpi* genes) are available from at least three sources: (i) from plants of commercial potato varieties; (ii) from plants held in publicly accessible culture collections and genebanks (seedbanks); and (iii) from plants studied by academic research groups. At Wageningen University and Research (WUR) in the Netherlands, for instance, many of the potato cultivars and wild *Solanum* accessions studied

Aardevo Exhibit 1010
Page 0713

are obtained from an-in-house collection and from the Center of Genetic Resources the Netherlands (CGN), Wageningen, The Netherlands (http://www.cgn.wur.nl/uk). The genebank for plant genetic resources, maintained by CGN, holds a collection of over 1700 potato varieties and wild accessions. A simple search in the CGN database provides a number of accessions of potato species *Solanum bulbocastanum* that are late blight resistant, for instance, CGN18311, CGN18310, CGN17690, CGN22367, CGN17692, CGN17693, and CGN17688 (https://cgngenis.wur.nl/). Propagation material of these accessions can be ordered from CGN and is thus readily available to the public.

Huang 2005, listed in Table 10 of the specification, discloses that *Solanum demissum* accession CGN17810 contains *Rpi* genes R1, R2, R3, R4, R7, and R8. This accession is publicly available from the CGN.

In a recent publication by Rakosy-Tican *et al.*, 2020 (**EXHIBIT A**) the *Rpi* genes and their location is provided for five commercial potato cultivars ('Baltica', 'Delikat', 'Quarta', 'Romanze', and 'Sarpo Mira') and *S. bulbocastanum* accession GLKS31741 (blb41) (Groß Lüsewitz Potato Collections (GLKS) of the IPK Genebank, Leibniz-Institute of Plant Genetics and Crop Plant Research, Germany). In particular in Tables 1-3 of that publication and page 3 identify GLKS31741 as a source. The commercial potato variety 'Sarpo Mira,' which may *inter alia* be obtained from Danespo A/S, Give, Denmark (https://www.danespo.com/english/varieties/varieties/fresh-market/sarpo-mira) comprises *Phytophthora infestans* resistance genes *R3a* and *R3b*.

Foster *et al.* 2009, listed in Table 10 of the specification, discloses *S. venturi* accession CGN18108 contains *Rpi-vnt1.1*, *S. venturi* accession CGN18279 contains *Rpi-vnt1.2*, and *S.*

venturi accession CGN17998 contains *Rpi-vnt1.3*. All of these accessions are publicly available from the CGN.

Lokossou 2010 (**EXHIBIT B**, p. 51, Table 1a and p. 52, Table 1b) discloses that the *Rpi-blb1* gene is present in *S. bulbocastanum* accessions PI275194, PI275195, CGN17689, CGN21306, CGN17692, and CGN17690 and that *Rpi-blb2* is present in *S. bulbocastanum* CGN17692, 960631, PI 275195, PI498011, and PI498223. European Patent publication EP1334979A1 also discloses *Phytophthora infestans* resistance in CGN17692 (which also carries the designation BGRC 8005) is based on the *Rpi-blb1* gene. All of these accessions are publicly available from the CGN or at the U.S. National Plant Germplasm System (USDA-GRIN/NPGS).

Oosumi *et al.* 2009, listed in Table 10 of the specification, discloses *Rpi-bt1* as found in plants of *S. bulbocastanum* that were obtained from the Inter-Regional Potato Introduction Station (NRSP-6), Sturgeon Bay, Wisconsin.

Lokossou 2009, listed in Table 10 of the specification, discloses that the R2-*like Rpi* gene is present in genotype AM3778-16 and that the *Rpi-abpt* gene is present in clone 707TG11-1, and refers to for these clones to Park 2005a and Park 2005b, respectively. Park 2005a and Park 2005b, both of which are listed in Table 10 of the specification, disclose that both clones are produced at the Laboratory of Plant Breeding, Department of Plant Sciences, Wageningen University, Wageningen, The Netherlands. Although not a facility for regular ordering, such university collections provide for public availability of the plant material.

Malcolmson 1966, listed in Table 10 of the specification, teaches the presence of *Rpi* genes *R5* and *R6* in *S. demissum* accessions 3053-18 and XD2-21, respectively. *S. demissum*

Aardevo Exhibit 1010
Page 0715

accessions 3053-18 and XD2-21 are obtainable from the in vitro collection at Science and Advice for Scottish Agriculture (SASA), Edinburgh, UK.

Trognitz 2007 (**EXHIBIT C**, Table 1) discloses *R9*, *R10*, and *R11* of *S. demissum* are present in cultivars CIP800994, CIP800995, and CIP800996, which are available from the potato collection of the International Potato Center (CIP), Lima, Peru.

The foregoing demonstrates that the references in Table 10 provide evidence of availability of the *Phytophthora* resistance genes to the public and thus one of skill in the art would be able to obtain accessions containing the genes based on the disclosure of the references in the Table. In view of this, the rejection thus believed to be moot and withdrawal thereof respectfully requested.

E. Rejection of Claims Under 35 U.S.C. §112, First Paragraph - Written Description

The Action rejects claims 1-12, 18-20, and 22-26 under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement. The Action asserts that the Specification provides written description for deposited lines AGVD1, AGVD2, AGVD3 and AGVD17 but fails to describe working examples of the broad genus of any other *Solanum* lines, which if combined with the *Sli* gene, that fall within the scope of the claims. The Action further asserts that one of skill in the art would not believe Applicants to be in possession of the genus as broadly claimed, especially in view of the discussion of the art by Applicants and the letter dated 11 December 2012 of Dr. Jonathan D G Jones. Applicants respectfully traverse.

Submitted herewith is the Declaration of Dr. Willem Hendrik Lindhout under 37 C.F.R. § 1.132. At paragraphs 10-12 of the Declaration, Dr. Lindhout provides the breeding histories for potato lines D1, D2, and D3, which are the diploid donor lines used to produce deposited lines AGVD1, AGVD2, AGVD3 and AGVD17. At paragraphs 13-15 of the Declaration, Dr.

Aardevo Exhibit 1010
Page 0716

Lindhout discusses the wide genetic diversity of potato lines D1, D2, and D3. At paragraphs 16-17 of the Declaration, Dr. Lindhout describes a study performed to determine the genetic variation between lines D1, D2, and D3, as well as the genetic variation between these lines and a random set of 22 commercial tetraploid *Solanum tuberosum* varieties and explains that the results show that D1, D2, and D3 represent a random sample of *Solanum tuberosum*.

In paragraphs 21-23 of the Declaration, Dr. Lindhout discusses the letter of 11 December 2012 of Dr. Jones cited in the Action. At paragraph 22 of the Declaration, Dr. Lindhout provides an excerpt from a response letter from Dr. Jones that further explains the statements he made in the 11 December 2012 letter. In the response letter, which is provided in the Declaration as **Exhibit A**, Dr. Jones states:

> "I herewith inform you [...] that I never made a statement to that effect, and that my words are misinterpreted. What I stated is that we attempted to test the *Sli* gene in *S. phureja*, but were not successful, and that we found that, after only a few rounds of selfing with the original material from Hosaka, inbreeding depression was already so extensive that we terminated the program, because we were unable to propagate the material. We drew no specific conclusions at the time, other than that we considered that it might have been due to the poor germplasm we worked with. However, I was well aware at the time of my letter that this was not the case, as I was convinced from personal communications, and your scientific publication in Potato Research (2011) Vol. 54:301–312, that we should have persisted inbreeding and selecting plants with seed carrying berries.
>
> I considered that your method was widely applicable to potato, and you had certainly shown that it was widely applicable to *S. tuberosum*."

Furthermore, at paragraph 34 of the Declaration, Dr. Lindhout explains that the present inventors were able to develop the experimental test-hybrids into a potato hybrid breeding system and provides publications demonstrating its use. A person of ordinary skill in the field of

Aardevo Exhibit 1010
Page 0717

plant biotechnology would be able to generate similar results using the teaching in the application with only routine experimentation. The results further demonstrate that one of skill in the art as of the filing date of the patent application would have been able to, using routine experimentation, use any diploid potato donor lines to generate a plant of a potato line that is diploid, fertile, self-compatible, produces at least 200 grams fresh weight of tubers per plant, and at least 50% of the genomic loci in said plant line are homozygous, wherein said self-compatibility in said line is conferred by the S-locus inhibitor (*Sli*) gene from *Solanum chacoense*, and wherein a copy of said gene is present in potato lines AGVD1, AGVD2, AGVD3, and AGVD17. Thus, the invention is not limited to a potato lines of a specific genotype, such as deposited lines AGVD1, AGVD2, AGVD3 and AGVD17, and the lines described in the specification are commensurate with the full scope of the claimed invention. Applicants were therefore in possession of the invention as claimed. In view of the foregoing, the rejection is believed to be moot and withdrawal thereof is respectfully requested.

F. Rejection of Claims Under 35 U.S.C. §112, First Paragraph - Enablement

The Action rejects claims 1-12, 18-20, and 22-26 under §112, first paragraph, as failing to comply with the enablement requirement. Specifically, the Action asserts that the Specification describes four deposited lines however the claimed genus is much broader. The Action further asserts that extensive guidance would be required to enable the invention as broadly claimed, given that success in practicing the claimed invention appears to be unpredictable and dependent on the genotype of the potato lines used, however such guidance is not provided in the application. Applicants respectfully traverse.

Submitted herewith is the Declaration of Dr. Willem Hendrik Lindhout under 37 C.F.R. § 1.132. At paragraphs 13-15 of the Declaration, Dr. Lindhout discusses the wide genetic diversity

Aardevo Exhibit 1010
Page 0718

of potato lines D1, D2, and D3, which are the diploid donor lines used to produce deposited lines AGVD1, AGVD2, AGVD3 and AGVD17. At paragraphs 16-17 of the Declaration, Dr. Lindhout describes a study performed to determine the genetic variation between lines D1, D2, and D3, as well as the genetic variation between these lines and a random set of 22 commercial tetraploid *Solanum tuberosum* varieties and explains that the results show that D1, D2, and D3 represent a random sample of *Solanum tuberosum*.

With regard to the assertion made in the Action that success in practicing the claimed invention appears to be unpredictable and dependent on the genotype of the potato lines used, Dr. Lindhout addresses this in paragraphs 21-23 of the Declaration. At paragraph 22 of the Declaration, Dr. Lindhout provides an excerpt from a response letter from Dr. Jonathan D G Jones that further explains statements made in the original letter of 11 December 2012 of Dr. Jones cited in the Action. In the response letter, which is provided in the Declaration as **Exhibit A**, Dr. Jones states:

> "I herewith inform you [...] that I never made a statement to that effect, and that my words are misinterpreted. What I stated is that we attempted to test the *Sli* gene in *S. phureja*, but were not successful, and that we found that, after only a few rounds of selfing with the original material from Hosaka, inbreeding depression was already so extensive that we terminated the program, because we were unable to propagate the material. We drew no specific conclusions at the time, other than that we considered that it might have been due to the poor germplasm we worked with. However, I was well aware at the time of my letter that this was not the case, as I was convinced from personal communications, and your scientific publication in Potato Research (2011) Vol. 54:301–312, that we should have persisted inbreeding and selecting plants with seed carrying berries.
>
> I considered that your method was widely applicable to potato, and you had certainly shown that it was widely applicable to *S. tuberosum*."

Aardevo Exhibit 1010
Page 0719

Dr. Lindhout states in paragraph 23 of the Declaration that the claimed methods can be successfully used in species other than *Solanum tuberosum*.

Applicants further note that the entire scope of the claimed subject matter is fully enabled because all that is required to satisfy the enablement requirement is that the specification teaches one reasonably skilled in the art how to make and use the claimed invention without undue experimentation. In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). Some experimentation and routine screening will not preclude enablement, particularly when a reasonable amount of guidance with respect to the direction in which the experimentation should proceed is provided. *In re Wands*, 858 F.2d at 737.

Thus, the invention is not limited to a potato lines of a specific genotype, such as deposited lines AGVD1, AGVD2, AGVD3 and AGVD17. Thus, one reasonably skilled in the art viewing the application as filed and coupled with information known in the art, is enabled to make and use the full scope of the claims. As such, the rejection is believed to be moot and withdrawal thereof is respectfully requested.

G. Double Patenting

The Action rejects claims 1-12, 18-19, 22, 25-26 as allegedly being unpatentable over claims 35-48 of U.S. Application No. 16/541,071 and over claims 1-19 of U.S. Patent No. 10,524,436. Applicants respectfully traverse but note that a Terminal Disclaimer will be submitted upon indication of otherwise allowable subject matter in order to expedite prosecution.

Aardevo Exhibit 1010
Page 0720

H. Conclusion

In light of the foregoing, Applicants submit that the case is in condition for allowance, and an early indication to that effect is earnestly solicited. The Examiner is invited to contact the undersigned at (214) 259-1890 with any questions, comments or suggestions relating to the referenced patent application.

Respectfully submitted,

/Sheila Walker/

Sheila Walker
Reg. No. 71,087
Agent for Applicants

Dentons US LLP
2000 McKinney Avenue, Suite 1900
Dallas, Texas 75201
(214) 259-1890

Date: July 16, 2020

Aardevo Exhibit 1010
Page 0721

EXHIBIT A

frontiers in Plant Science

ORIGINAL RESEARCH
published: 03 June 2020
doi: 10.3389/fpls.2020.00699

Introgression of Two Broad-Spectrum Late Blight Resistance Genes, *Rpi-Blb1* and *Rpi-Blb3*, From *Solanum bulbocastanum* Dun Plus Race-Specific *R* Genes Into Potato Pre-breeding Lines

Elena Rakosy-Tican[1], Ramona Thieme[2]*, Janine König[3], Marion Nachtigall[2], Thilo Hammann[2], Tunde-Eva Denes[1,4], Klaudia Kruppa[5] and Márta Molnár-Láng[5]*

[1] Plant Genetic Engineering Group, Department of Molecular Biology and Biotechnology, Babeş-Bolyai University, Cluj-Napoca, Romania, [2] Federal Research Centre for Cultivated Plants, Institute for Breeding Research on Agricultural Crops, Julius Kühn-Institut, Quedlinburg, Germany, [3] Federal Research Centre for Cultivated Plants, Institute for Breeding Research on Horticultural Crops, Julius Kühn-Institut, Quedlinburg, Germany, [4] Biological Research Centre, Jibou, Romania, [5] Centre for Agricultural Research, Hungarian Academy of Sciences, Martonvásár, Hungary

There is a wealth of resistance genes in the Mexican wild relative of cultivated *Solanum*, but very few of these species are sexually compatible with cultivated *Solanum tuberosum*. The most devastating disease of potato is late blight caused by the oomycete *Phytophthora infestans* (*Pi*). The wild hexaploid species *S. demissum*, which it is able to cross with potato, was used to transfer eleven race-specific genes by introgressive hybridization that were subsequently widely used in potato breeding. However, there are now more virulent races of *Pi* that can overcome all of these genes. The most sustainable strategy for protecting potatoes from late blight is to pyramid or stack broad-spectrum resistance genes into the cultivars. Recently four broad-spectrum genes (*Rpi*) conferring resistance to *Pi* were identified and cloned from the sexually incompatible species *S. bulbocastanum*: *Rpi-blb1* (RB), *Rpi-blb2*, *Rpi-blb3*, and *Rpi-bt1*. For this research, a resistant *S. bulbocastanum* accession was selected carrying the genes *Rpi-blb1* and *Rpi-blb3* together with race-specific *R3a* and *R3b* genes. This accession was previously used to produce a large number of somatic hybrids (SHs) with five commercial potato cultivars using protoplast electrofusion. In this study, three SHs with cv. 'Delikat' were selected and backcross generations (i.e., BC_1 and BC_2) were obtained using cvs. 'Baltica', 'Quarta', 'Romanze', and 'Sarpo Mira'. Their assessment using gene-specific markers demonstrates that these genes are present in the SHs and their BC progenies. We identified plants carrying all four genes that were resistant to foliage blight in greenhouse and field trials. Functionality of the genes was shown by using agro-infiltration with the effectors of corresponding *Avr* genes. For a number of hybrids and BC clones yield and tuber number were not significantly different from that

OPEN ACCESS

Edited by:
Mariola Plazas, Polytechnic University of Valencia, Spain

Reviewed by:
Hannele Lindqvist-Kreuze, International Potato Center, Peru
Weixing Shan, Northwest A&F University, China
Iga Tomczynska, Oregon State University, United States

***Correspondence:**
Elena Rakosy-Tican
elena.rakosy@ubbcluj.ro;
rakosy.elena@gmail.com
Ramona Thieme
ramona.thieme@julius-kuehn.de

Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *27 September 2019*
Accepted: *04 May 2020*
Published: *03 June 2020*

Citation:
Rakosy-Tican E, Thieme R, König J, Nachtigall M, Hammann T, Denes T-E, Kruppa K and Molnár-Láng M (2020) Introgression of Two Broad-Spectrum Late Blight Resistance Genes, Rpi-Blb1 and Rpi-Blb3, From Solanum bulbocastanum Dun Plus Race-Specific R Genes Into Potato Pre-breeding Lines. Front. Plant Sci. 11:699. doi: 10.3389/fpls.2020.00699

Aardevo Exhibit 1010
Page 0723

of the parent cultivar 'Delikat' in field trials. The evaluation of agronomic traits of selected BC_2 clones and of their processing qualities revealed valuable material for breeding late blight durable resistant potato. We show that the combination of somatic hybridization with the additional use of gene specific markers and corresponding *Avr* effectors is an efficient approach for the successful identification and introgression of late blight resistance genes into the potato gene pool.

Keywords: effectors, detached leaf assay, introgression breeding, late blight field resistance, gene specific markers, processing qualities, tuber yield

INTRODUCTION

Late blight caused by *Phytophthora infestans* (Mont.) de Bary (*Pi*) is one of the most damaging diseases of potatoes as it does not only affect the above ground parts of the plants but also the tubers (Colton et al., 2006). Although the *Pi* genome has been sequenced (Haas et al., 2009), a deep understanding of the complex interaction and co-evolution of this pathogen and its host is lacking (Kamoun and Smart, 2005; Haverkort et al., 2009; Aguilera-Galvez et al., 2018). However, its potential in causing an epidemic coupled with its great adaptability led to many outbreaks and destructions of potato harvests during the course of human history (Vreugdenhil, 2007; Chakrabarti et al., 2017). Even today, the cost of control and lost production are estimated six billion euros per year, with half of this cost incurred in Europe (Haverkort et al., 2016). Current disease control relies on ten up to 16 fungicide applications per season (Hanson et al., 2007; Haverkort et al., 2009). Fungicides are not only costly but they pollute the environment and affect human health (Paro et al., 2012). Moreover, late blight resistance introduced into potato cultivars by classical breeding, based on eleven race-specific resistance genes from *S. demissum* was quickly overcome by new races of *Pi*.

Solanum bulbocastanum (*blb*), a wild diploid species of Mexican potato ($2n = 2x = 24$), is highly resistant to all known races of *Pi*, even when exposed to high concentrations of its spores. This species is a typical example of a 1EBN (endosperm balance number) species and hence cannot be conventionally crossed with cultivated potato (Johnston et al., 1980). To date, four of its Nucleotide Binding Sites–Leucine Rich Repeats (NBS-LRR) resistance genes have been identified and cloned: *Rpi-blb1* (Van der Vossen et al., 2003), also known as *RB* and *Rpi-bt1*–both loci located on chromosome VIII (Song et al., 2003; Oosumi et al., 2009), *Rpi-blb2*–on chromosome VI (Van der Vossen et al., 2005) and *Rpi-blb3* on chromosome IV (Lokossou et al., 2009). Nevertheless, it is likely there are other factors involved in its late blight resistance (Hein et al., 2009; Lokosson et al., 2010; Rietman, 2011). Horizontal or durable resistance is thought to be polygenic and hence a quantitative trait, with complex pathogen and host interaction mechanisms that are still poorly understood (Colton et al., 2006). For instance, it was suggested that the pathogenesis related protein StPRp27 contributes to a race-nonspecific resistance against *Pi* by inhibiting the development of the disease and could potentially be used in breeding for durable resistance to late blight (Shi et al., 2012).

Many of the *Rpi* genes are located in meta-QTLs, like *Rpi-blb2* on chromosome VI and *Rpi-blb3* on chromosome IV (Danan et al., 2011). The *Rpi* genes from *S. bulbocastanum* are considered to be broad spectrum resistance genes, as already shown for *Rpi-blb1* (Song et al., 2003), *Rpi-blb2* (Van der Vossen et al., 2005; Orbegozo et al., 2016), and *Rpi-blb3* (Zhu et al., 2012). The race-specific genes used in this study, originally identified in *S. demissum,* are *R3a* and *R3b,* which are members of the *R3* complex locus on chromosome XI. The *R2* gene recognizes the same effector as *Rpi-blb3*, coded by *Avr2* gene (Aguilera-Galvez et al., 2018). Huang et al. (2004) showed that the *R3* locus harbours two functionally distinct genes: *R3a* and *R3b*. The *R3a* gene is classified in the fast evolving class I (Huang et al., 2005). The *R3* locus in potato is an example of natural stacking of resistance genes. It contains the two closely linked *R3a* and *R3b* genes, which are 0.4 cM apart, with distinctly different resistances to particular types of *Pi*, *R3a* recognizes *Avr3a*, while *R3b* despite its sequence being similar to that of *R3a*, recognizes another effector encoded by the *Avr3b* gene (Li et al., 2011).

It has been shown that by combining broad-spectrum resistance genes by means of genetic engineering (cisgenesis), two or more gene stacks can confer durable late blight resistance in the field (Zhu et al., 2012; Haesaert et al., 2015). Unfortunately, genetic engineering, which uses cloned resistance genes from wild species of potato, is still not considered as non-genetically modified organism (GMO) and its use is restricted in Europe (directive 2001/18/EC), and globally by skeptical consumers. Despite considerable progress in the genetic analysis of quantitative resistance to late blight, under long day conditions, based on molecular markers (Gebhardt and Valkonen, 2001; Simko, 2002), breeders have made little progress in breeding resistant cultivars using marker-assisted selection (MAS). The major drawbacks are the tetrasomic inheritance of potato and the strong linkage between foliage resistance and late maturity (Bradshaw et al., 2004). On the other hand, for more than 100 years there was little progress in obtaining resistant cultivars by introducing single genes from *S. demissum* and conventional breeding using bridging species is very time consuming. This was the case for the commercial potato varieties 'Bionica' and 'Toluca' (Haverkort et al., 2009) in which the single broad-spectrum resistance gene *Rpi-blb2* was incorporated. It took more than 46 years of conventional breeding in Netherlands that started at the end of the fifties and used the late blight resistant *blb* as the source of the resistance and *S. acaule* as the bridging species (Hermsen and Ramanna, 1973).

Aardevo Exhibit 1010
Page 0724

Finding methods of deploying major resistance genes to *Pi* remains an important goal of potato breeding. Currently breeders isolate variants of *R* genes and deploy them in pyramids or stacks. It is expected that this will result in broad-spectrum recognition of *Pi* isolates and might provide a more durable resistance in the field (Jo et al., 2016). Recent screening of germplasm has revealed *Rpi* genes in wild species of *Solanum*, particularly broad-spectrum resistance genes in *blb* species (Wang et al., 2008, 2013; Hein et al., 2009). The current challenge is to select, judiciously combine and deploy sets of different resistance genes that confer durable late blight resistance on modern potato cultivars.

Previously, a large number of somatic hybrids (SHs) between potato cultivars and *S. bulbocastanum* were regenerated and analyzed for hybridity, ploidy and fertility (Rakosy-Tican et al., 2015). The goal of this study was to transfer broad-spectrum resistance genes *Rpi-blb1* and *Rpi-blb3* from *S. bulbocastanum* together with race-specific *R* genes (*R3a* and *R3b*), into the potato gene pool by somatic hybridization. A functional profiling of resistance genes by effectoromics, phenotypic expression of resistance in detached leaf assay and quantitative resistance assessment in the field were used to evaluate the resistance of the somatic hybrids (SHs) and derived back-cross progenies (BC_1 and BC_2). We tested the hypothesis if the four resistance genes introgressed by somatic hybridization present in the back-cross progenies confer resistance that can be confirmed in a greenhouse and field trials. Thus, the pre-breeding lines produced will be a valuable material for breeding durable late blight resistance potato cultivars.

MATERIALS AND METHODS

Plant Material

The morphology and nuclear genetic constitution of the SHs and backcross (BC) progenies, BC_1 and BC_2, between five commercial potato cultivars ('Baltica', 'Delikat', 'Quarta', 'Romanze', and 'Sarpo Mira') and *S. bulbocastanum* accession GLKS–31741 (*blb*41) (Groß Lüsewitz Potato Collections (GLKS) of the IPK Genebank, Leibniz-Institute of Plant Genetics and Crop Plant Research, Germany) have been analyzed (Rakosy-Tican et al., 2015). Only fertile SHs were chosen for further resistance assays. These included three hybrids with potato cv. 'Delikat' carrying all four resistance genes and their derived BC clones (see **Tables 1–3**).

Assessment of Resistance Genes

Screening for Rpi-blb Resistance Genes

Fusion parents, SHs and their BC progenies were screened for the resistance genes *Rpi-blb1* using specific markers (Wang et al., 2008), *Rpi-blb3* (Lokossou et al., 2009; Zhu et al., 2012), *R3a* (Huang et al., 2005), and *R3b* (Rietman, 2011). Amplifications were carried out in a 20 µL reaction mixture containing 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM primer, approximately 10 ng template DNA and 0.5 Unit InviTaq DNA Polymerase (Invitek). PCR was conducted using a standard set of conditions on a MJ Thermal cycler (PTC 200): initial denaturizing at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, $T_{annealing}$ for 45 s, 72°C for 45 s and a final extension step of 72°C for 5 min. Amplification products were size-separated on a 1.5% agarose gel stained with ethidium bromide and visualized using a UV transilluminator. The expected sizes of amplified fragments were 821 bp for *Rpi-blb1*–marker Blb1 F/R (Wang et al., 2008), and 757 bp for *Rpi-blb3*–marker Blb3 F/R (Lokossou et al., 2009). For the race-specific genes previously characterized in *S. demissum*, the sizes of amplified fragments were 982 bp for *R3a* (Huang et al., 2005) and 378 bp for *R3b* (Rietman, 2011).

Agro-Infiltration

Agro-infiltration was done using a culture of the *A. tumefaciens* strain AGL1 + pVirg transformed with a pK7WG2 plasmid containing the avirulence factors for: *Rpi-blb1* (*Avrblb1*), *Rpi-blb3* (*Avr2* - recognized also by *R2*), *R3a* and *R3b* (*Avr3a* and *Avr3b*), obtained from the University of Wageningen (V. Vleeshouwers). The PITG codes are: *Avr2* = PITG_22870; *Avr3a* = PITG_14371; *Avr3b* = PITG_18215; and *Avrblb1* = PITG_21388.

Cultivation and infiltration was performed as described by Du et al. (2014). The macroscopic response was scored 4 days after infiltration (dpi), when the dead cells (hypersensitive reaction, HR) were visible. As a negative control, the pK7WG2 empty vector was used (Vleeshouwers et al., 2008, 2011), which had the advantage that the cells killed by the infiltration (background necrosis) can be detected and hence, were not scored as a false positive. The parents were used as positive controls. The agro-infiltration and evaluation was done on three leaflets of one plant and the final appreciation of HR and 0 was based on three different plants of the same genotype.

Assessment of Ploidy and Genome Composition in SHs and Derived BCs

Determination of Ploidy Level by Classical Cytological Methods

Flow cytometry

The first pair of leaves of *in vitro* plants that were 8 weeks old were chopped with a razor blade in 800 µL of LB buffer, which was then filtered through a 25 µm mesh. Subsequently, 12.5 µL of buffer containing 1 µg/mg propidium iodide (PI) were added (Doležel et al., 2007). The nuclear DNA content was determined using a Becton Dickinson FacScan flow cytometer and the data analyzed using CellQuest software.

Determination of the ploidy level using cytological methods

Root tips of 1–2 cm in length were harvested from young plants grown *in vitro* on MS medium (Murashige and Skoog, 1962) containing 1 mg L^{-1} NAA, fixed in ethanol/acetic acid (3:1) and stored in 70% ethanol at 4°C. The root tips were washed twice in distilled water (10 min each), digested with a 1% Cellulase Onozuka R-10, 0.4% Cytohelicase and 0.4% Pectolyase in citrate buffer at pH = 4.8 (30 min) and squashed in 45% acetic acid. After the cover slips were removed in liquid nitrogen, slides were dehydrated by placing them sequentially in 70, 90, and finally 100% ethanol. Chromosomes were stained using 4′,6–diamidine-2′-phenylindolehydrochloride (DAPI) and washed with 2xSSC and distilled water. Probes were mounted in Vectashield (Vectashield, Vector Laboratories) to preserve the

Aardevo Exhibit 1010
Page 0725

TABLE 1 | Assessment of the somatic hybrid (SH) 82/4, between potato cv. 'Delikat' and *Solanum bulbocastanum* (*blb*41) and derived back-crossed progenies BC_1 and BC_2 (the cultivar used for crossing is indicated), for the presence of the resistance genes as revealed by gene specific markers, functional profiling of *Avr* genes by agro-infiltration, resistance to late blight in a detached leaf assay (DLA) and in the field (Δ-rAUDPC); standard varieties 'Adretta' (susceptible) and 'Sarpo Mira' (resistant to late blight) were used; the year of the field assays is given as a subscript to each value of Δ-rAUDPC; from each genotype three (*two) plants and three leaflets were infiltrated, hypersensitive reaction is indicated by HR and lack of reaction by 0: the numbers in brackets indicate the number of repetitions with the same reaction.

Parent/ SH/BC_1	Genotype ID	Resistance genes	Agro-infiltration with effectors:				DLA (mean ± SD)	*Phytophthora* attack Δ-rAUDPC (2009–2016)
			Avrblb1	***Avr2***	***Avr3a***	***Avr3b***		
Wild parent	*Blb*41	*Rpi-blb1, Rpi-blb3, R3a, R3b*	HR(9)	HR(9)	HR(9)	HR(9)	1.0 ± 0.00	-0.124_{13}; -0.405_{14}
Cultivar	Delikat	none	0(9)	0(9)	0(9)	0(9)	5.73 ± 1.17	0.311_{12}; 0.164_{15}; 0.477_{16}; 0.491_{18}
Cultivar	Quarta	*R3a, R3b*	0(9)	0(9)	HR(9)	HR(9)	4.80 ± 1.16	0.159_{09}; -0.008_{10}
Standardcultivar	Sarpo Mira	*R3a, R3b*	0(9)	0(9)	0(9)	HR(9)	2.11 ± 0.81	-0.573_{11}; -0.495_{12}; -0.332_{13}; $-0,567_{14}$; -0.286_{15}; -0.199_{16}; -0.128_{17}, -0.084_{18}
Standardcultivar	Adretta	nd	nd	nd	nd	nd	6.48 ± 1.57	0.246_{11}; 0.499_{12}; 0.255_{13}; 0.308_{14}; 0.416_{15}; 0.414_{16}; 0.364_{17}; 0.635_{18}
SH (Delikat + *blb*41)	82/4	*Rpi-blb1, -blb3, R3a, R3b*	HR(9)	HR(9)	HR(9)	HR(8)	1.38 ± 0.36	-0.352_{12}; -0.075_{13}
BC_1 (SH x Quarta)	82/4/8	*Rpi-blb1*	HR(9)	HR(9)	0(9)	HR(8)	1.94 ± 0.26	0.096_{14}; 0.119_{15}; -0.012_{16}; 0.253_{17}; -0.072_{18}
	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
	82/4/23	*Rpi-blb1, R3a, R3b*	HR(9)	0(9)	HR(9)	HR(9)	1.25 ± 0.45	-0.056_{13}; 0.094_{14}
	82/4/27	*Rpi-blb1*	nd	nd	nd	nd	1.43 ± 0.34	0.009_{14}; -0.055_{15}; -0.035_{16}
	82/4/30	*Rpi-blb1, R3a, R3b*	HR(9)	nd	HR(9)	HR(9)	1.53 ± 0.79	-0.122_{14}; -0.122_{15}; 0.039_{16}
	82/4/38	*Rpi-blb1, -blb3,*	HR(9)	HR(7)	0(9)	0(9)	1.45 ± 0.26	-0.282_{12}; 0.040_{13}
	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
BC_2 (BC_1 x Sarpo Mira)	82/4/46/6	*R3a, R3b*	HR(6)*	HR(6)*	HR(6)*	HR(6)*	1.90 ± 0.19	0.152_{17}; -0.103_{18}
	82/4/46/14	*Rpi-blb1, -blb3, R3a, R3b*	HR(8)	HR(9)	HR(9)	0(9)	1.53 ± 0.25	-0.202_{17}; 0.044_{18}
BC_1 (SH x Quarta)	82/4/57	*Rpi-blb1*	HR(9)	HR(9)	0(9)	0(9)	2.60 ± 0.66	-0.159_{13}; 0.203_{14}
	82/4/68	*Rpi-blb1, -blb3, R3b*	HR(9)	HR(9)	0(9)	HR(9)	3.36 ± 1.58	-0.145_{13}; 0.164_{14}; 0.022_{15}; 0.174_{16}
BC_2 (BC_1 x Quarta)	82/4/68/3	*Rpi-blb1, -blb3*	HR(9)	0(9)	0(9)	0(9)	4.13 ± 0.81	0.508_{14}
	82/4/68/29	*Rpi-blb1, -blb3, R3a, R3b*	HR(9)	HR(8)	HR(9)	HR(9)	2.36 ± 0.84	0.154_{14}
	82/4/68/45	*Rpi-blb1, -blb3, R3a*	HR(9)	HR(9)	HR(9)	0(9)	1.88 ± 0.24	0.935_{14}
BC_1 (SH x Quarta)	82/4/69	*Rpi-blb1, -blb3*	HR(9)	HR(9)	0(9)	0(9)	1.75 ± 0.62	-0.074_{12}; 0.113_{14}; 0.017_{15}; 0.003_{16}
BC_2 (BC_1 x Sarpo Mira)	82/4/69/7	? *R3b*	HR(9)	0(9)	0(9)	HR(9)	2.88 ± 1.26	-0.537_{17}; -0.130_{18}
BC_1 (SH x Quarta)	82/4/76	*Rpi-blb1, -blb3, R3a*	HR(6)*	HR(6)*	HR(6)*	0(6)*	1.53 ± 0.25	-0.041_{13}

Progenies with four stacked resistance genes, showing HR reaction and improved resistance to Pi in DLA and the field are highlighted in red; nd- not determined.

Aardevo Exhibit 1010
Page 0726

TABLE 2 | Assessment of the somatic hybrid (SH) 83/9, between potato cv. 'Delikat' and *Solanum bulbocastanum* (*blb*41) and derived back-crossed progenies BC_1 (the cultivar used for crossing is indicated), for the presence of the resistance genes as revealed by gene specific markers, functional profiling of *Avr* genes by agro-infiltration, resistance to late blight in a detached leaf assay (DLA) and in the field (Δ-rAUDPC); standard varieties 'Adretta' (susceptible) and 'Sarpo Mira' (resistant to late blight) were used; the year of the field assays is given as a subscript to each value of Δ-rAUDPC; from each genotype three plants and three leaflets were infiltrated, hypersensitive reaction is indicated by HR and lack of reaction by 0: the numbers in brackets indicate the number of repetitions with the same reaction.

Parents/ SH/BC_1	Genotype ID	Resistance genes	Agro-infiltration with effectors				DLA (mean ± SD)	*Phytophthora* attack Δ-rAUDPC (2009–2018)
			Avrblb1	*Avr2*	*Avr3a*	*Avr3b*		
Wild parent	*Blb*41	*Rpi-blb1, -blb3, R3a, R3b*	HR(9)	HR(9)	HR(9)	HR(9)	1.00 ± 0.00	-0.124_{13}; -0.405_{14}
Cultivar	Delikat	none	0(9)	0(9)	0(9)	0(9)	5.73 ± 1.17	0.311_{12}; 0.164_{15}; 0.477_{16}; 0.491_{18}
Cultivar	Quarta	*R3a, R3b*	0(9)	0(9)	HR(9)	HR(9)	4.80 ± 1.16	0.159_{09}; -0.008_{10}
Standard cultivar	Sarpo Mira	*R3a, R3b*	0(9)	0(9)	0(9)	HR(9)	2.11 ± 0.81	-0.573_{11}; -0.495_{12}; -0.332_{13}; -0.567_{14}; -0.286_{15}; -0.199_{16}; -0.128_{17}, -0.084_{18}
Standardcultivar	Adretta	nd	nd	nd	nd	nd	6.48 ± 1.57	0.246_{11}; 0.499_{12}; 0.255_{13}; 0.308_{14}; 0.416_{15}; 0.414_{16}; 0.364_{17}; 0.635_{18}
SH (Delikat + *blb*41)	83/9	*Rpi-blb1, -blb3, R3a, R3b*	HR(9)	HR(9)	HR(9)	HR(7)	1.34 ± 0.37	-0.324_{12}; 0.130_{13}
BC_1 (SH x Quarta)	83/9/3	*Rpi-blb1, R3a, R3b*	HR(9)	0(9)	HR(9)	HR(9)	1.40 ± 0.28	0.223_{14}
	83/9/8	*Rpi-blb1, -blb3, R3a, R3b*	HR(9)	0(9)	0(9)	HR(9)	3.06 ± 0.57	-0.115_{14};-0.130_{15}; 0.045_{16}
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	83/9/12	*Rpi-blb1, -blb3*	nd	nd	nd	nd	1.14 ± 0.15	-0.196_{14}; -0.234_{15}
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	83/9/39	*Rpi-blb3, R3a, R3b*	0(9)	HR(9)	HR(8)	HR(9)	1.60 ± 0.59	0.133_{14}
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	83/9/55	*Rpi-blb1, R3a, R3b*	HR(9)	0(9)	0(8)	HR(9)	1.80 ± 0.33	-0.230_{14}; -0.119_{15}; -0.292_{16}; 0.089_{17}; -0.198_{18}
	83/9/63	*Rpi-blb1, -blb3, R3a, R3b*	HR(9)	nd	HR(9)	HR(9)	1.87 ± 0.38	-0.103_{14}; -0.065_{15}, -0.170_{16}; 0.022_{17}; 0.047_{18}
	83/9/70	*Rpi-blb1, R3a, R3b*	HR(9)	0(9)	0(9)	HR(9)	2.25 ± 0.68	0.039_{14}
	83/9/73	*R3a, R3b*	0(9)	0(9)	HR(9)	HR(9)	2.25 ± 1.29	-0.083_{15}

Progenies with four stacked resistance genes, showing HR reaction and improved resistance to Pi in DLA and the field are highlighted in red; nd-not determined.

Aardevo Exhibit 1010
Page 0727

TABLE 3 | Assessment of the somatic hybrid (SH) 95/1, between potato cv. 'Delikat' and *Solanum bulbocastanum* (*blb*41) and derived back-crossed progenies BC_1 and BC_2 (the cultivar used for crossing is indicated), for the presence of the resistance genes as revealed by gene specific markers, functional profiling of *Avr* genes by agro-infiltration, resistance to late blight in a detached leaf assay (DLA) and in the field (Δ-rAUDPC); standard varieties 'Adretta' (susceptible) and 'Sarpo Mira' (resistant to late blight) were used; the year of the field assays is given as a subscript to each value of Δ-rAUDPC; from each genotype three plants and three leaflets were infiltrated, hypersensitive reaction is indicated by HR and lack of reaction by 0; the numbers in brackets indicate the number of repetitions with the same reaction.

Parents/ SH/BCs	Genotype ID	Resistance genes	Agro-infiltration with effectors				DLA (mean ± SD)	*Phytophthora* attack Δ-rAUDPC (2012–2018)
			Avrblb1	***Avr2***	***Avr3a***	***Avr3b***		
Wild parent	*Blb*41	*Rpi-blb1, -blb3, R3a, R3b*	HR(9)	HR(9)	HR(9)	HR(9)	1.0 ± 0.00	-0.124_{13}; -0.405_{14}
Cultivar	Delikat	none	0(9)	0(9)	0(9)	0(9)	5.73 ± 1.17	0.311_{12}; 0.164_{15}; 0.477_{16}; 0.491_{18}
Cultivar	Baltica	*R3a, R3b*	nd	0(9)	HR(9)	HR(9)	3.92 ± 0.71	nd
Cultivar	Romanze	*R3b*	0(9)	0(9)	0(9)	HR(9)	5.20 ± 1.12	nd
Standard cultivar	Sarpo Mira	*R3a, R3b*	0(9)	0(9)	0(9)	HR(9)	2.11 ± 0.81	-0.573_{11}; -0.495_{12}; -0.332_{13}; $-0,567_{14}$; -0.286_{15}; -0.199_{16}; -0.128_{17}; -0.084_{18}
Standard cultivar	Adretta	nd	nd	nd	nd	nd	6.48 ± 1.57	0.246_{11}; 0.499_{12}; 0.255_{13}; 0.308_{14}; 0.416_{15}; 0.414_{16}; 0.364_{17}; 0.635_{18}
SH (Delikat + *blb*41)	95/1	*Rpi-blb1, -blb3, R3a, R3b*	HR(9)	HR(9)	HR(9)	HR(9)	1.40 ± 0.35	0.084_{15}
BC_1 (SH x Baltica)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	95/1/4	*Rpi-blb1, -blb3, R3a, R3b*	HR(9)	HR(9)	HR(9)	HR(9)	2.94 ± 1.81	nd
BC_2 (BC_1 x Romanze)	95/1/4/11	*R3a, R3b*	HR(9)	HR(9)	HR(9)	HR(9)	1.19 ± 0.40	-0.138_{14}; -0.257_{15}; -0.051_{16}; -0.091_{17}; -0.096_{18}
BC_2 (BC_1 x Romanze)	95/1/4/59	*Rpi-blb1, R3a, R3b*	HR(9)	HR(9)	HR(9)	HR(9)	2.25 ± 1.65	0.147_{14}; 0.291_{15}
BC_1 (SH x Baltica)	95/1/7	*Rpi-blb1, -blb3, R3a, R3b*	HR(9)	HR(9)	HR(9)	HR(9)	3.13 ± 2.74	0.045_{12}

BC_1 clone with four stacked resistance genes, showing HR reaction and improved resistance to Pi in DLA and the field are highlighted in red; nd-not determined.

Aardevo Exhibit 1010
Page 0728

staining. For chromosome counts, at least five cells with well-spread metaphase chromosomes were used for each clone. Best spreads were photographed with a digital camera attached to an epifluorescent microscope (Olympus BX 60 with appropriate filter for DAPI). For all cytogenetic evaluations of the parental lines, the diploid *blb*41 and tetraploid cultivated potatoes were used as internal standards.

Determination of the Composition of the Genome Using mcGISH

A modified multicolour GISH protocol (Kruppa et al., 2013) was used to simultaneously visualize the chromosomes of *Solanum bulbocastanum* ($2n = 2x = 24$, A^bA^b) and *S. tuberosum* ($2n = 4x = 48$, AAAA). Therefore, total DNA was labeled with biotin-16-dUTP and digoxigenin-11-dUTP (Roche Diagnostics, Mannheim, Germany), respectively, using the random primed labeling protocol. The hybridization mixture contained 60 ng each of the labeled probes/slide, dissolved in a 15 µL mixture of 100% formamide, 20xSSC and 10% dextran-sulfate in the ratio of 5:1:4. In order to denature the probes DNA, the hybridization mixture was heated at 75°C for 8 min and then immediately put on ice. The chromosome spreads were denatured at 80°C in a mixture containing 100% formamide, 20xSSC and 10% dextran-sulfate in the ratio of 15:3:3. Hybridization was done at 42°C overnight. Streptavidin-FITC (Roche) and Anti-Digoxigenin-Rhodamine (Roche), respectively, were used in the detection phase. Counterstaining was performed using 2 µg/mL DAPI in Vectashield antifade mounting medium. The slides were screened using a Zeiss Axioskop-2 fluorescence microscope equipped with filters appropriate for DAPI, FITC, Rhodamine and the simultaneous detection of FITC and Rhodamine. Images were taken using a Spot CCD camera (Diagnostic Instruments) and processed using Image Pro Plus software (Media Cybernetics).

Resistance to Late Blight

Detached Leaf Assay (DLA)

This assay used five leaves per clone and was replicated at least twice. The description of the *Pi* strain is given in section "Field Test." One drop (1 µL) of a *Pi* suspension of 15×10^3 zoosporangia /ml was placed on the underside of the leaves, which were flipped after 1 day and incubated at 16°C and 95% relative humidity at a light intensity of 2.1 µmol m^2 s^{-1} and incubated for 5 days. After 5 days the size of the necrotic areas and mycelium development were estimated on a scale from 1 (no symptoms visible) to 9 (completely necrotic and covered with mycelium). The protocol for evaluating resistance using the DLA is described by Thieme et al. (2008). The *Pi* inoculum was maintained for 1 year on tuber slices of a highly susceptible variety without any R-genes (cv. 'Adretta') in order to maintain a high level of virulence.

Field Test

Field tests were carried out at the Julius Kühn-Institut (JKI) Federal Research Centre for Cultivated Plants Trial Station for Potato Research, Groß Lüsewitz 18190 Sanitz, Germany. Clones were cultivated in double-row plots with ten plants per plot. A strip of hemp 3 m in width was planted around the field to ensure wind protection and to maintain a humid environment. Irrigation was conducted in the early morning and in the evening if necessary. Control plots of varieties highly resistant (cv. 'Sarpo Mira') and susceptible (cv. 'Adretta') to late blight were also planted in the field. The inoculum of *Pi* consisted of a mixture of common races collected in the field in the year before and refreshed the following year with an inoculum collected from natural infections (Hammann et al., 2009). The virulence of the isolate was evaluated under field conditions using the SASA differential set carrying the *R* genes *R1* to *R11* (Black et al., 1953). It was found highly aggressive as it overcame the resistance genes *R1* to *R8* and *R10* and *R11*. Probably due to their later maturation, *R9* plants were less infected. A continuous monitoring of the composition of the clonal lineages of the pathogen was not possible throughout the test period, but analyses of samples from 2014 to 2017 identified EU_13 mating type A2 as the predominant genotype in local populations. The lowest leaves of each last plant in a row were inoculated with a 5 ml spore suspension (15×10^3 zoosporangia/ml) in the evening. The area of the potato tops attacked was scored as a percentage twice a week until the plants were mature. This was based on the Area Under Disease Progress Curve (AUDPC) and Relative Area Under Disease Progress Curve (rAUDPC) (Hammann et al., 2009). As rAUDPC is strongly associated with the maturity of the plants, it was transformed into delta (Δ) rAUDPC. RAUDPC represents the percentage of infection and Δ-rAUDPC the quantitative maturity-corrected transformation of rAUDPC (Shaner and Finney, 1977; Bormann, 2003; Truberg et al., 2009). The maturity of the parents, SHs and progenies was evaluated in a field trial that was treated with fungicides using a scale from 1 (very early) to 9 (very late). Fifteen plants per clone were cultivated and agronomic characters other than maturity were also assessed.

Evaluation of Flowering, Yield, Tuber Shape/Appearance and Tuber Processing Qualities

Assessment of Flowering, Yield, Tuber Shape and Appearance

The SHs and offspring were repeatedly cultivated in a greenhouse for 2–5 years depending on genotype and breeding step. In addition to the evaluation of their resistance to pathogens, morphological characteristics, such as, plant habitus, flowering and the fruit (berry) development were assessed. Crossbreeding experiments with pollen of different potato varieties were carried out. The number of flowers on a single greenhouse plant was assessed and expressed on a scale: + [low number ($n \leq 5$)], ++ [moderate number ($n = 6$–20)] and +++ [high number of flowers ($n > 21$)]. Fertile SHs and BC clones were selected for further use.

Tuber appearance in terms of shape, eye depth, skin texture were assessed and the results are summarized in **Table 4** [scale: + (poor), +++ (very good)].

Yield (kg of tubers per plot), total number of tubers and tuber shape were assessed of plants that developed from the 15 tubers planted in each plot in the field. Yield of SHs and

Aardevo Exhibit 1010
Page 0729

TABLE 4 | Assessment of the agronomic traits of BC progenies derived from *S. bulbocastanum*, GLKS 31741 (*blb*41) (+) cv. 'Delikat' hybrids grown in a field for 2 or 3 years, including flowering, tuber number, weight and starch content as well as processing qualities: Discolouration of raw tuber tissue, of cooked tuber flesh (average for 3 years); the BC clones with best yield, qualities for agronomic traits and processing are highlighted in green.

Genotype	Agronomic traits					Discolouration	
	Flower intensity	Appea-rance	Tubers (n/plant)	Tuber weight (kg/plant)	Starch content (%)	Raw tuber tissue	Cooking tuber flesh
Cv. Delikat	+	+++	11.4 ± 2.1	1.45 ± 0.5	17.5 ± 0.9	2.0 ± 0	4.7 ± 0.6
BC_1 82/4/8	+	+	13.1 ± 3.8	1.00 ± 0.2	16.3 ± 1.4	4.5 ± 0.7	7.7 ± 0.6
BC_1 82/4/27	+	+	14.3 ± 7.6	0.58 ± 0.3	12.5 ± 0.5	2.5 ± 0.7	6.0 ± 2.0
BC_1 82/4/30	(+)	+++	8.3 ± 3.8	0.52 ± 0.3	15.4 ± 2.2	2.5 ± 0.7	5.5 ± 2.1
BC_1 82/4/46	+	++	9.2 ± 1.7	1.23 ± 0.4	14.7 ± 2.3	2.0 ± 0	4.5 ± 2.1
BC_1 82/4/67	+	(+)	14.0 ± 0.9	1.38 ± 0.8	17.7 ± 1.4	5.0 ± 0	6.5 ± 2.1
BC_1 82/4/68	+	+	9.1 ± 1.9	0.87 ± 0.2	15.3 ± 2.0	2.0 ± 0	5.7 ± 1.2
BC_1 82/4/69	+	+	15.1 ± 6.6	0.52 ± 0.2	16.9 ± 0.3	3.5 ± 0.7	4.7 ± 0.6
BC_1 83/9/8	++	+	7.7 ± 1.3	0.94 ± 0.1	21.1 ± 0.6	3.5 ± 0.7	5.7 ± 1.5
BC_1 83/9/11	+++	+	6.8 ± 1.9	1.01 ± 0.2	17.6 ± 0.4	4.0 ± 0	6.0 ± 1.0
BC_1 83/9/35	+++	+	13.4 ± 5.5	0.53 ± 0.1	18.4 ± 1.1	3.5 ± 0.7	6.3 ± 1.2
BC_1 83/9/47	+	+	11.2 ± 2.0	0.95 ± 0.2	17.0 ± 1.0	4.5 ± 0.7	7.3 ± 0.6
BC_1 83/9/55	+	+++	8.8 ± 0.8	1.34 ± 0.6	17.6 ± 0.4	3.5 ± 0.7	5.3 ± 1.2
BC_1 83/9/63	(+)	++	10.1 ± 0.2	1.25 ± 0.2	15.8 ± 1.3	3.0 ± 0	4.7 ± 1.2
BC_2 82/4/68/11	+	+++	6.6 ± 1.2	2.67 ± 3.5	12.5 ± 1.1	3.5 ± 0.7	4.8 ± 1.3
BC_2 95/1/4/11	++	+	14.2 ± 1.6	0.75 ± 0.2	15.5 ± 0	4.5 ± 0	2.5 ± 0.7
BC_2 95/1/4/59	++	++	16.8 ± 0.6	1.10 ± 0.1	17.4 ± 2.6	4.0 ± 0	5.5 ± 0.7

Initial number, 15–20 plants per genotype and plot; tuber harvest per plot. Flower intensity on a scale: (+), +, low number ($n \leq 5$); ++, moderate number ($n = 6–20$), and +++, high number of flowers ($n > 21$); Tuber appearance (including tuber shape, eye depth, skin texture) is summarized on a scale: (+), +, poor; +++, very good; Discolouration: 1 (best) – 9 (low quality).

derived progenies were compared to that of the parental potato cultivar 'Delikat'.

Processing quality was based on the discolouration of raw tuber tissue, flesh after cooking and the starch content.

Assessment of Discolouration of Raw Tuber Tissue

Four tubers per genotype were used 4–5 months after harvest. Using a cork-borer two tissue cylinders were cut out parallel to the long axis of each tuber. After 24 h, the discolouration was visually estimated in terms of intensity of gray to black on a scale from 1 (not discolored) to 9 (black). Cultivar 'Delikat' was used as a reference, as its reaction is well known.

Discolouration of Tuber Flesh After Cooking

Four medium-sized tubers per genotype were cooked, hot peeled, cut longitudinally and the cut surface placed facing down on a plate. Discolouration was estimated on a scale from 1 to 9 after 24 h and compared with the reference samples.

Starch Content

Starch content was determined, by calculating the difference between the weight of a tuber in air and in water using an under-water balance EORO-KUW-2000 (Fischer KG Bielefeld, Germany).

Statistical Analysis

The free statistical software R (R Development Core Team, 2010) was used for statistical calculations. The function mean() was used to calculate means and the function sd() was used to calculate standard deviations.

RESULTS

Identification of *Rpi-blb1*, *Rpi-blb3*, *R3a*, and *R3b* Genes in *S. bulbocastanum blb*41, SHs and Derived BC Progenies and Assessment of Resistance to Foliage Blight

Using specific markers it was possible to demonstrate the presence of the following resistance genes in *blb*41: *Rpi-blb1*, *Rpi-blb3*, *R3a*, and *R3b* and confirm their transfer into SHs and introgression into derived BC progenies (**Figure 1**, **Tables 1–3**, and **Supplementary Figure S1**). The fertile and best performing SH clones were further selected for ploidy (chromosome counts) and field assessments. All three SHs selected and the majority of their derived BCs carried both *Rpi* resistance genes (**Tables 1–3**). In one BC_1 (83/9/39) gene specific markers indicated the presence of only the *Rpi-blb3* gene (**Figure 1** and **Table 2**). This clone had a good score for resistance in the DLA (mean of 1.6), but a plus value for Δ-rAUDPC in the field (**Table 2**). The race-specific genes *R3a* and *R3b* were detected using gene specific markers in *blb*41 and the cultivars 'Baltica', 'Quarta' and 'Sarpo Mira', which were used as pollinators in back-crosses. Only *R3b* was detected in cv. 'Romanze' (**Tables 1–3** and **Supplementary Figure S1**). The selected SHs 82/4, 83/9 and 95/1 carried the

Aardevo Exhibit 1010
Page 0730

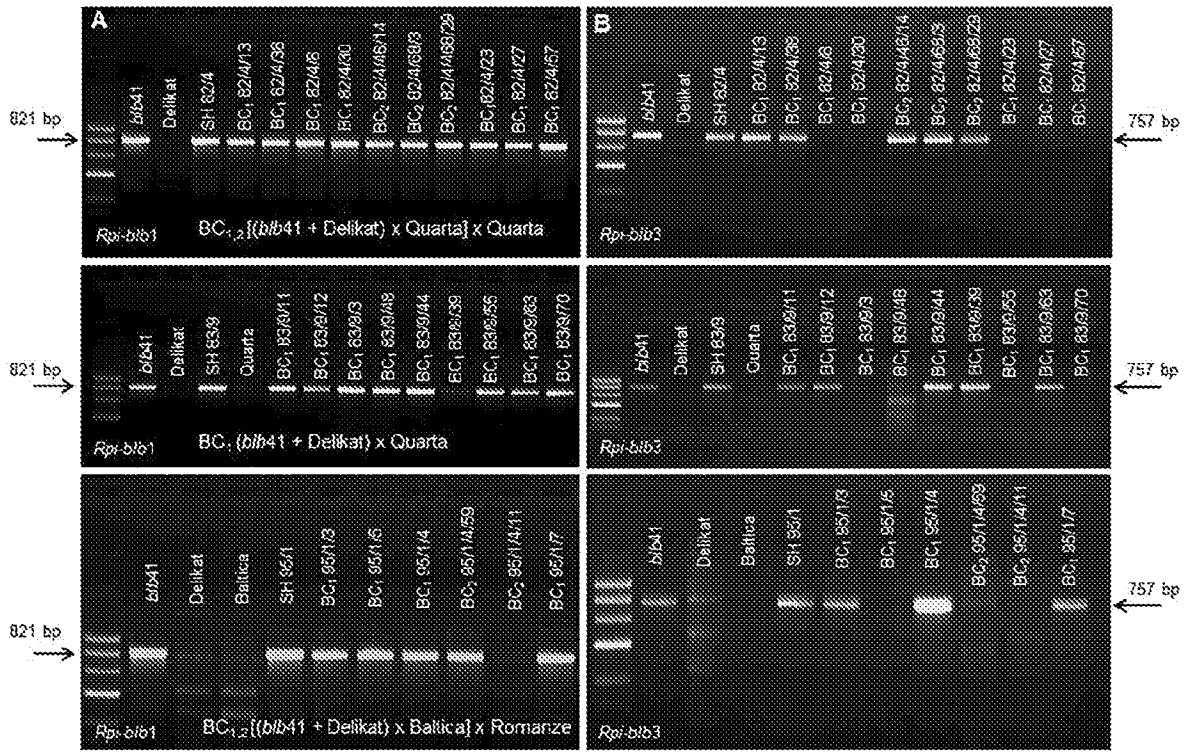


FIGURE 1 | Molecular analysis using gene specific markers to determine the presence of **(A)** *Rpi-blb1* and **(B)** *Rpi-blb3* genes in selected somatic hybrids, their parental lines *S. bulbocastanum* GLKS 33741 (*blb*41), cv. 'Delikat' and different segregating BC_1 progenies after back-crossing with cvs. 'Quarta' and 'Baltica' (from above to below, arrows indicate the target fragments).

two durable resistance genes *Rpi-blb1* and *Rpi-blb3* and race-specific *R3a* and *R3b* genes (**Tables 1–3**). Hence, eight clones of the BCs derived from the three selected SHs and had specific fragments of all the resistance genes (**Tables 1–3–highlighted** in red, **Figure 1**. The three selected SHs and 30 of the 36 derived BC clones (listed in **Tables 1–3**), had a significantly high degree of *Pi* resistance in field trials, during the 1–4 years in which the intensity of the foliage blight attack varied (**Tables 1–3** and **Figures 3, 4A,B**). In the first years, a large number of SHs was evaluated in the field (**Figure 4A**) and the BC clones of the best three SHs were evaluated in the years 2014 to 2016 (**Figure 4B**). The level of resistance varied from year to year and the susceptible cultivar 'Delikat' had a rAUDPC of only 50% in 2013. The maturity-corrected values of Δ-rAUDPC for these SH clones are lower than that of the susceptible standard cv. 'Adretta' and cv. 'Delikat', but rather similar to the wild species *blb*41, used as a control, which did not show any symptoms of foliage blight attack (rAUDPC = 0%) or cv. Sarpo Mira, used as resistant standard cultivar. The area under the disease progress curve (rAUDPC in %) and maturity data are shown in **Figures 4A,B**, and Δ-rAUDPC in **Tables 1–3**, for each SH family. Minus values of Δ-rAUDPC indicate a low level of susceptibility. Three of the SHs selected and eleven BC_1–BC_2 clones had all the genes and were only slightly susceptible with minus values of Δ-rAUDPC in the field (see **Tables 1–3**). Other BCs (18) with only one, two or three resistance genes had minus values for Δ-rAUDPC in the field trials in at least 1 year in **Tables 1–3**. The maturity of SHs and derived BC_1 clones was intermediate between that of potato cultivar 'Delikat', with a maturity value of 5 and the very late maturing (value 9) wild species *blb*41 (**Figures 4A,B**). The majority of the BCs had values between 4.7 and 7.8, with some BC_1 clones maturing later with values 8 and 9 (82/4/8, /27, /67 and 83/9/11, /35). Many of the BC_1 clones had a low percentage rAUDPC with the last ones having low values in some years (**Figure 4B**). The SHs and their derived BC_1 had minus values for Δ-rAUDPC when tested in the field, which was confirmed by the DLA results with one exception, clone 82/4/68/29, which had a good value in DLA (2.36 ± 0.84), but was susceptible in the field with a plus value of Δ-rAUDPC in 1 year (**Table 1**). It needs to be verified by more assays in the future.

Agro-Infiltration With Effectors

The genes detected by gene specific markers after agro-infiltration with corresponding effectors were seen as elicitors of the hypersensitive response (HR) (**Figure 2**). Generally, after agro-infiltration these genes were associated with incompatible interactions and necrosis (**Tables 1–3**). There were also cases where genes were present but there was no hypersensitive reaction to the agro-infiltration of the *Avr* gene, as for example: BC_2 82/4/68/3 with the genes *Rpi-blb1* and *Rpi-blb3* but *Rpi-blb3* shows no HR (**Table 1**); or BC_1 83/9/8 with both *Rpi-blb3* and *R3a* genes showing no activity after agro-infiltration with *Avr2* and

Aardevo Exhibit 1010
Page 0731

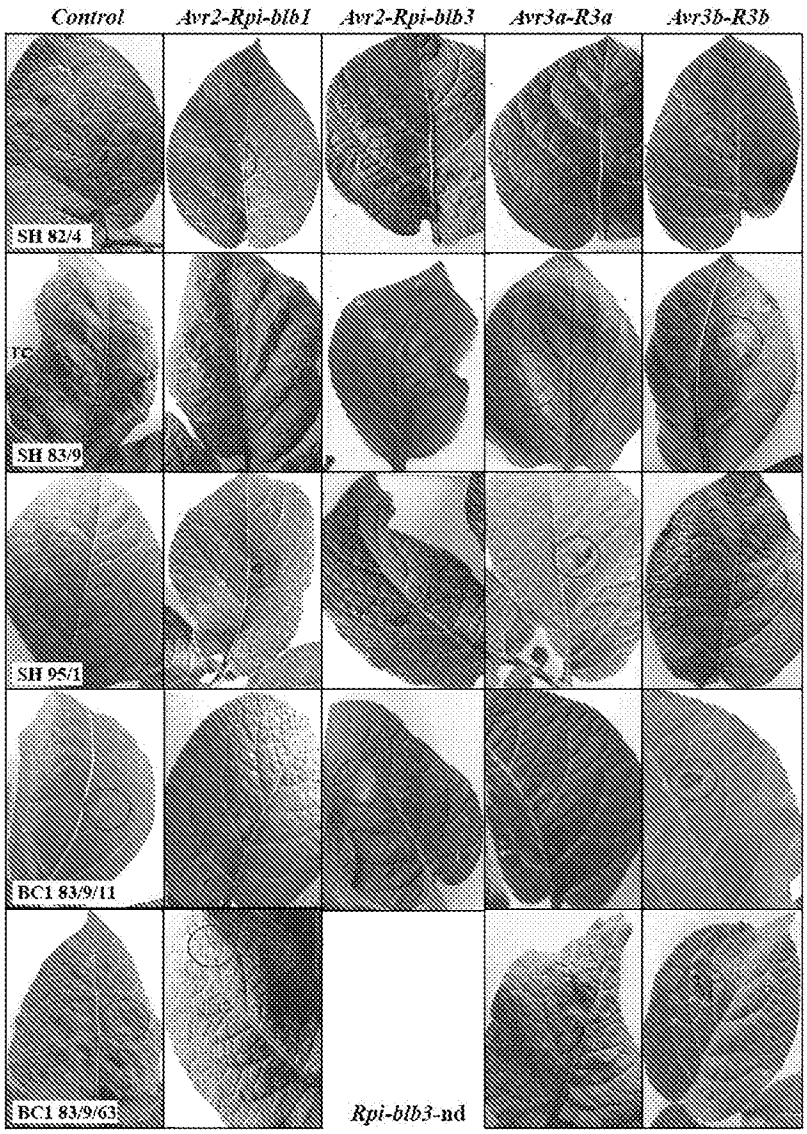


FIGURE 2 | Hypersensitive reaction of leaves of *Solanum bulbocastanum* (GLKS 33741, *blb*41) (+) cv. 'Delikat somatic hybrids (SHs) and selected back-cross progenies after agro-infiltration of *Avr*-effectors at 4*dpi*, which reveals the activity of corresponding resistance genes: *Rpi-blb1*, *Rpi-blb2*, *R3a*, or *R3b*. No visible reaction on leaves of SHs, BC_1 by infiltration of the vector without effectors (empty vector control), nd-not determined.

Avr3a; BC_1 83/9/55 and 83/9/70 in which the *R3a* gene, although present was inactive (**Table 2**). The eight BC clones with stacks of four genes that showed a HR are highlighted. Those are also best performing BCs in the DLA with constant minus values of Δ-rAUDPC in different years in the field after inoculation with *Pi* (**Tables 1–3**).

Ploidy and Genome Constitution of SHs and Derived BCs

As expected from previous investigations (Rakosy-Tican et al., 2015), the ploidy levels of the SH clones differed, one was hexaploid and two between pentaploid and hexaploid, i.e., aneuploids and asymmetric in their nuclear genetic constitutions (**Supplementary Table S1** and **Figures 5A–E**). Our results indicate that after back-crossing with the following tetraploid cultivars: 'Quarta', 'Baltica', 'Romanze', and 'Sarpo Mira', all BC_1 and BC_2 clones have lower chromosome numbers (**Supplementary Table S1**). Nevertheless, mcGISH indicates that between 4 and 24 of the chromosomes were from the wild parent *blb*41 and the introgression of the DNA of wild species into potato chromosomes (**Figure 5C**).

Yield of BC Clones in the Field and Processing Quality

The BC clones grown in the field varied in terms of yield and shape of the tubers at harvest (**Table 4** and **Figure 6**). The

Aardevo Exhibit 1010
Page 0732

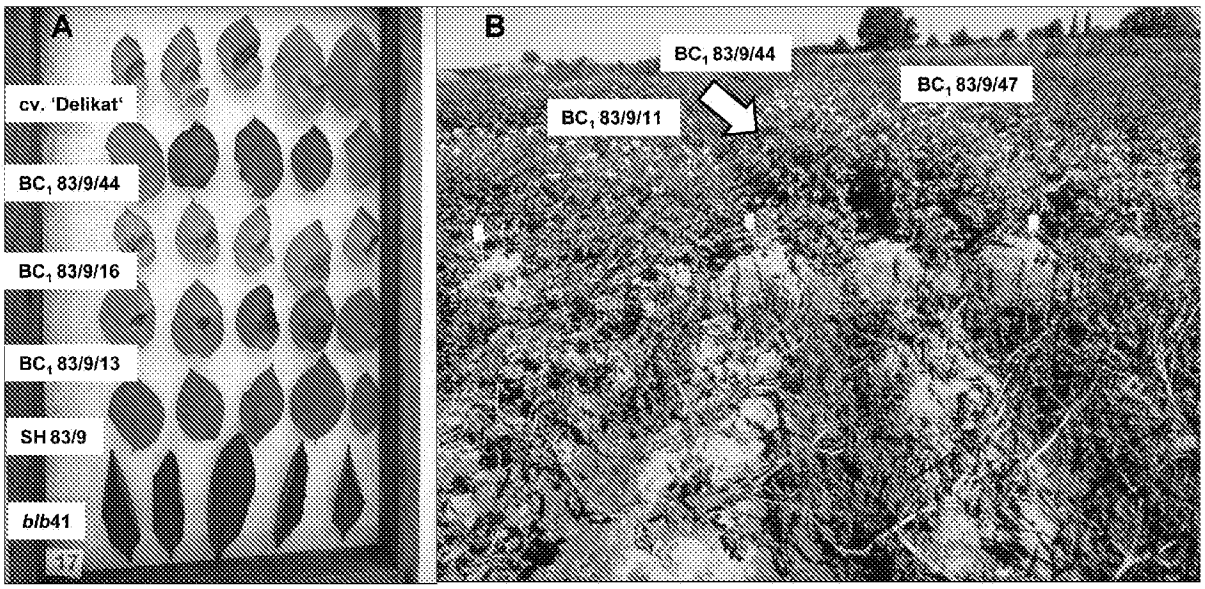


FIGURE 3 | Evaluation of resistance to late blight in a greenhouse and the field **(A)** Detached leaf assay (DLA) showing resistant *S. bulbocastanum* GLKS 33741 (*blb*41), a resistant somatic hybrid, two susceptible and one resistant BC_1 clones and the susceptible cv. 'Delikat' after infection with *Phytophthora infestans* (from below upward) **(B)** BC_1 clones 83/9/44 (arrow), 83/9/11 (left) and 83/9/47 (right) derived from *blb*41 (+) cv. 'Delikat' somatic hybrid 83/9 in a field trial in 2015 expressed improved horizontal resistance to foliage blight.

majority of the BC clones flowered and produced a satisfactory yield and number of tubers (**Table 4** and **Figure 6**). Moreover, three BC clones (BC_1 82/4/30; BC_1 83/9/55; BC_2 82/4/68/11) produced tubers of good appearance and similar to those of cultivar 'Delikat' (**Table 4** and **Figure 6**). The yield of two BC_1 clones (82/4/67, 83/9/63) and one BC_2 clone (95/1/4/59) was higher or similar to that of this cultivar, but the number of flowers and appearance of the tubers were low and acceptable, respectively. The starch content of the tubers of these clones varied from 12.5 to 21.1% and processing quality varied between scores 2.0 to 7.7 (**Table 4**). The BC_1 clones 82/4/46; 82/4/68; 83/9/35; 83/9/55 produced tubers with good processing qualities (**Figure 7**). Useful pre-breeding clones are also highlighted in green (**Table 4**).

DISCUSSION

Somatic hybridization and the identification of resistance genes using gene-specific markers were used to transfer a stack of genes determining durable resistance to late blight (*Rpi-blb1* and *Rpi-blb3*) and the race-specific genes (*R3a* and *R3b*) into the potato cv. 'Delikat'. This cultivar was shown to be a good candidate for somatic hybridization programs (Rakosy-Tican et al., 2015). By using *blb* accession GLKS 31741 (*blb*41), in which four R genes were identified and were introgressed into potato pre-breeding clones. Previously, it has not been possible to transfer this gene combination into potato either by classical breeding or by gene transfer.

The exploitation of the R genes for breeding research in potatoes has also been tested, in particular using genetic engineering approaches. Recently, more than 30 R genes found in species of wild potato resistant to late blight were mapped and 20 were cloned (Rodewald and Trognitz, 2013; Haverkort et al., 2016). These genes are available for the transfer into potato varieties and breeding lines. The company BASF developed the potato variety "Fortuna" derived from the cv. 'Fontane' by genetic transformation with two late blight R genes, *Rpi-blb1* and *Rpi-blb2* from *S. bulbocastanum* (Storck et al., 2012). This variety was not approved for the European market and it ceased to be cultivated. Zhu et al. (2012) have successfully stacked three other late blight resistance genes: *Rpi-sto1* (*S. stoloniferum*) homolog of *Rpi-blb1*, *Rpi-vnt1.1* (*S. venturii*) and *Rpi-blb3* (*S. bulbocastanum*). The susceptible cv. 'Désirée' became more resistant by introgressive hybridization and DLA indicated the presence of the stacked genes in the plants grown in a field over a period of 2 years (Zhu et al., 2012; Haesaert et al., 2015). In addition, two genes from *S. bulbocastanum* (*RB*, *Rpi-blb2*) and one R gene *Rpi-vnt1.1* from *S. venturii* were stacked using genetic transformation and transferred into the varieties preferred by farmers, 'Désirée' and 'Victoria', and transgenic clones of these plants were cultivated in Sub-Saharan Africa. These potato lines were completely resistant to late blight over three seasons in the field in Southwest Uganda (Gishlain et al., 2019). Currently, transgenic potato varieties are approved for cultivation in the United States, Canada and Bangladesh. In a 10 years research project on the durable resistance of potato to *Phytophthora* at Wageningen University and a Research Centre genetic engineering was used to produce potato plants resistant to late blight using R genes mainly from *Solanum bulbocastanum* (*blb*), *S. edinense*, *S. stoloniferum* (*sto*), *S. venturii* (*vnt*), and *S. chacoense* (*chc*). Four varieties were transformed with one to

Aardevo Exhibit 1010
Page 0733

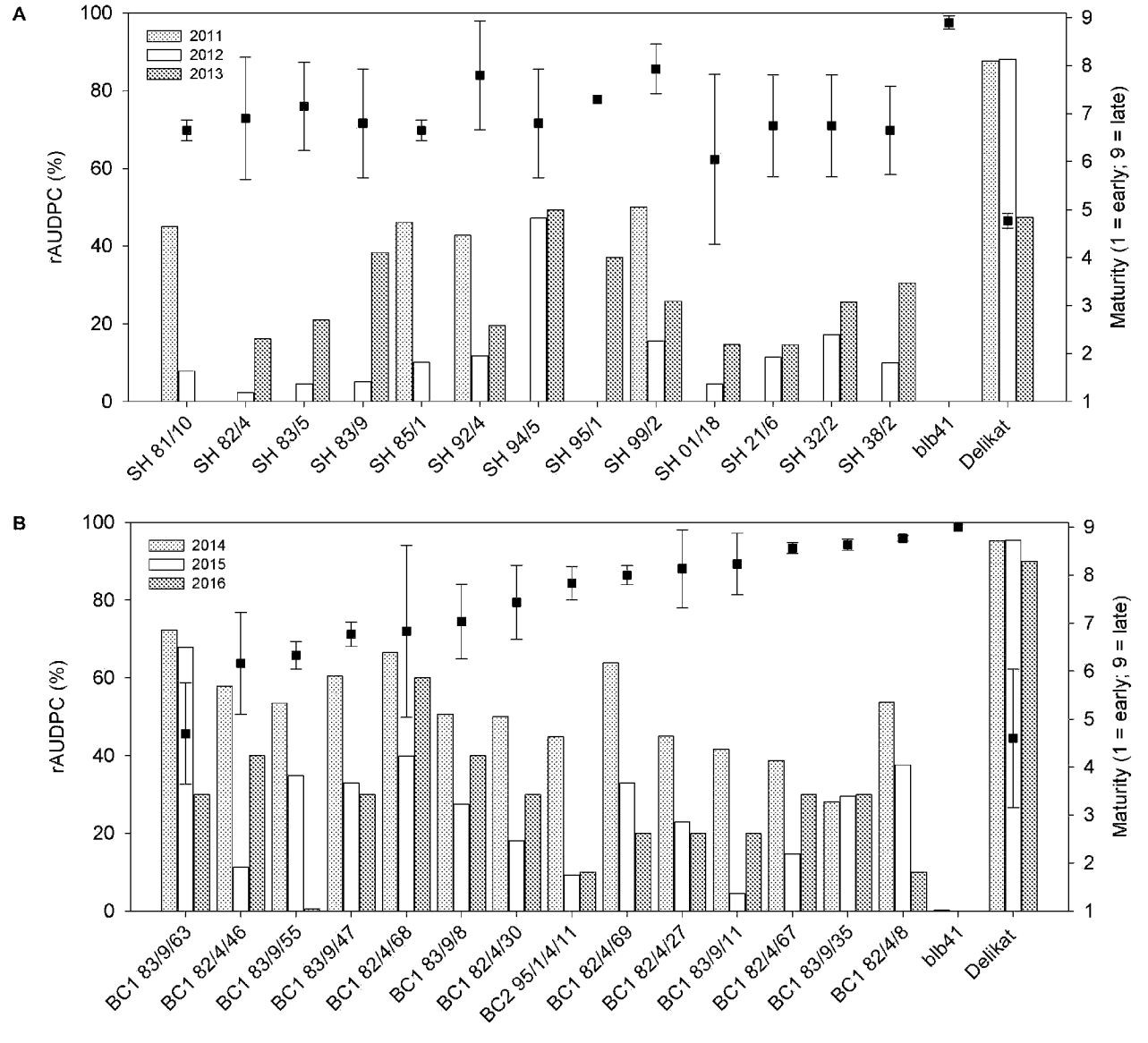


FIGURE 4 | Percentage of foliage area infected by late blight expressed as relative Area Under Disease Progress Curve (rAUDPC) and maturity (1 = very early; 9 = very late), evaluated in the field, after artificial inoculation of parents, **(A)** Somatic hybrids and **(B)** Their back-crossed progenies, potato (+) *Solanum bulbocastanum* GLKS 33741 (*blb*41); **(A)** years: 2011, 2012, 2013; **(B)** Values for years 2014–2016).

three R genes that originated from these wild potato species. The 'Désirée' clones containing stacks of either two R genes (*Rpi-blb3:sto1*; *Rpi-vnt1:chc1, Rpi-vnt1:sto1*) or three R genes (*Rpi-blb3:vnt1:sto1*) were not infected with *Pi* in field trials that lasted for 2 years (Haverkort et al., 2016). Recently, after using biotechnological improvement Simplot Plant Sciences has reported 'Innate' Gen 2 potatoes that are of high quality and show a strong resistance to late blight in the second generation[1]. Consequently, the use of biotechnology and MAS will increase the speed with which other genes conferring resistance can be incorporated into potato cultivars and their subsequent cultivation (Rietman, 2011; Chandel et al., 2015). Extensive investigations indicate that stacking of multiple R genes and monitoring how to deploy these stacks spatially and temporally could reduce fungicide use by over 80% (Haverkort et al., 2016).

Thus, genetic engineering could prove very useful if exempted from GMO rules in Europe. We used somatic hybridization, as the resulting plants are not genetically modified (directive 2001/18/EC–annex 1B)[2]. The use of somatic hybridization does enable to transfer new assortments of nuclear and cytoplasmic genes into regenerated plants and to overcome sexual incompatibility barriers (Rakosy-Tican et al., 2015; Thieme

[1]http://www.innatepotatoes.com/gen-two

[2]http://www.biosafety.be/PDF/2001_18.pdf

Aardevo Exhibit 1010
Page 0734

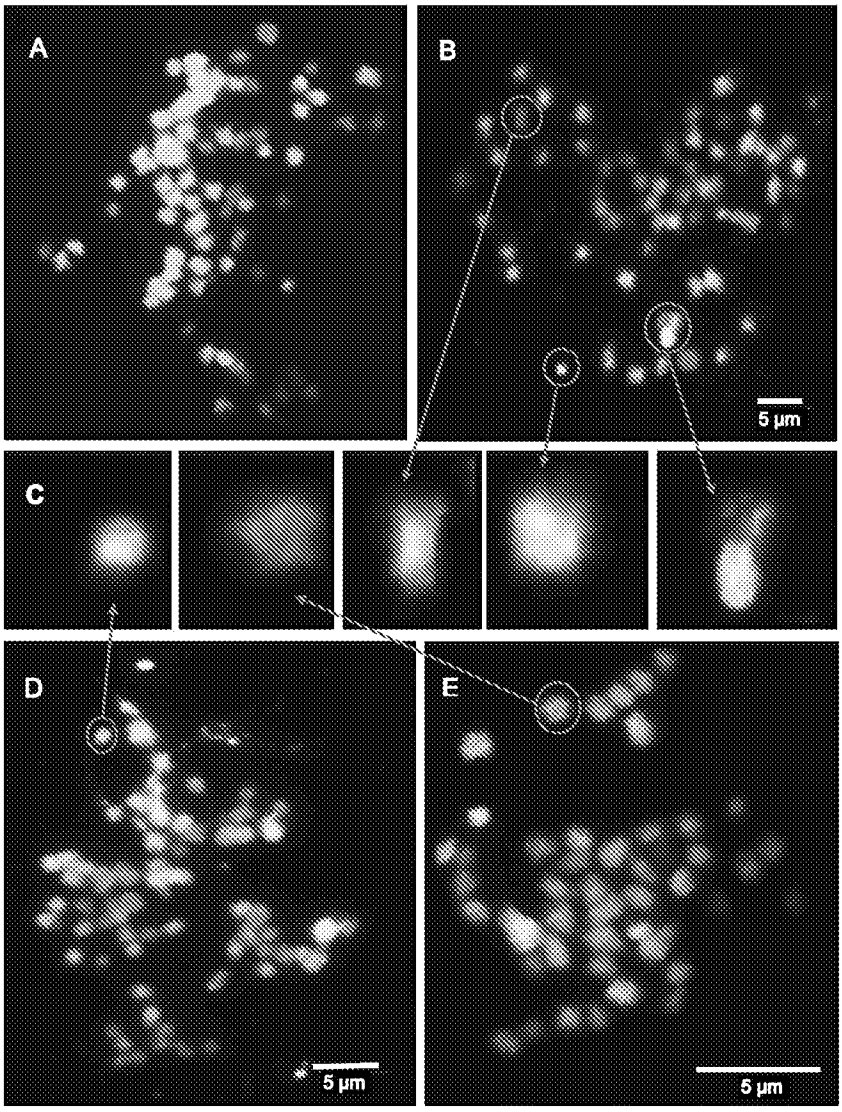


FIGURE 5 | The results of a mcGISH analysis of the genetic composition of somatic hybrids (SH), back-cross progenies (BC_1 and BC_2) – examples; green labels *S. tuberosum*, red labels *S. bulbocastanum* GLKS 33741 (*blb*41) chromosomes. **(A)** SH 83/9 - total 72 chromosomes, in red *S. bulbocastanum* = 24; **(B)** BC_1 83/9/3 - total chromosomes 54, 10 red from *blb*41 **(C)** Detailed magnifications of the introgressions from *blb*41 (red) into potato chromosomes (green) in BC_1 83/9/3, SH 95/1 and BC_2 95/1/4/59 (as indicated by red arrows); **(D)** SH 95/1 – total 64 chromosomes with 24 *blb*41 chromosomes in red; **(E)** BC_2 95/1/4/59 (total 48 chromosomes, 4 red from *blb*41 and recombinations – with red and green); Yellow regions denote the complementarity of the respective sequences; bar = 5 µm.

and Rakosy-Tican, 2017). Moreover, when asymmetric SHs are produced because of somatic incompatibilities (Rakosy-Tican et al., 2015), only a part of the nuclear DNA from the wild parent is present in the hybrids and derived BCs. In this study, mcGISH confirmed the introgression of *blb*41 DNA into BC clones, but more detailed analyses of chromosomes using FISH would identify the transferred resistance gene(s) and their location. Nevertheless, this study showed the introgression of DNA from wild species into potato cultivars using mcGISH.

The use of molecular markers and cytogenetic methods revealed that many of the SHs evaluated for resistance to *Pi* are asymmetric (Rakosy-Tican et al., 2015), which is confirmed in this study using indirect and direct cytogenetics and mcGISH. From the high number of the SHs between *blb*41 and five potato cultivars produced only those that had the basic potato characters in terms of morphology and fertility were selected (Rakosy-Tican et al., 2015) for assessing resistance to foliage blight and the important traits for a pre-breeding program. The DLA revealed that the three SH clones (*blb*41 + cv. 'Delikat') and BC_1 derived progenies with both *Rpi-blb1* and *Rpi-blb3* genes showed little symptoms of infection with *Pi*. The analysis of the specific alleles at the *Rpi-blb1*, *Rpi-blb3*, *R3a*, and *R3b* loci indicate that in the following generations

Aardevo Exhibit 1010
Page 0735

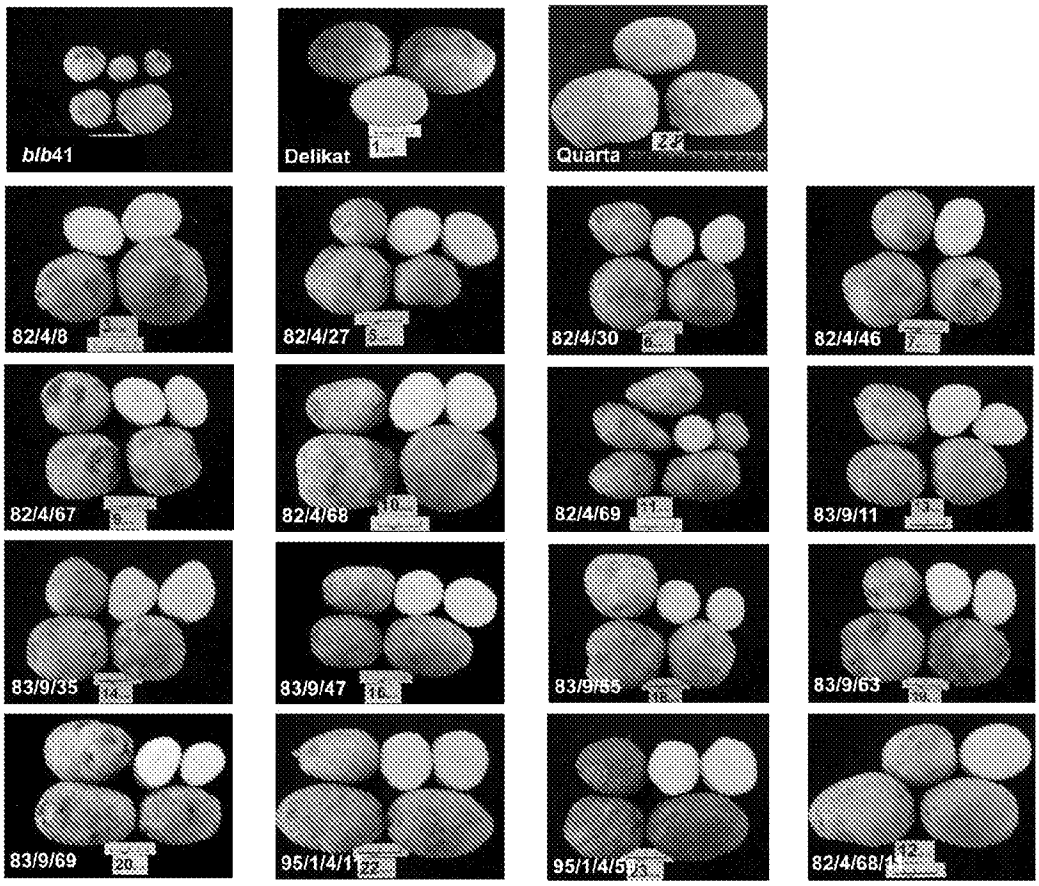


FIGURE 6 | Size and appearance of tubers of parental lines and selected BC_1 and BC_2 (the last three below) progenies of *S. bulbocastanum* GLKS 31741 (*blb*41) (+) cv. 'Delikat' somatic hybrids after cultivation in a field.

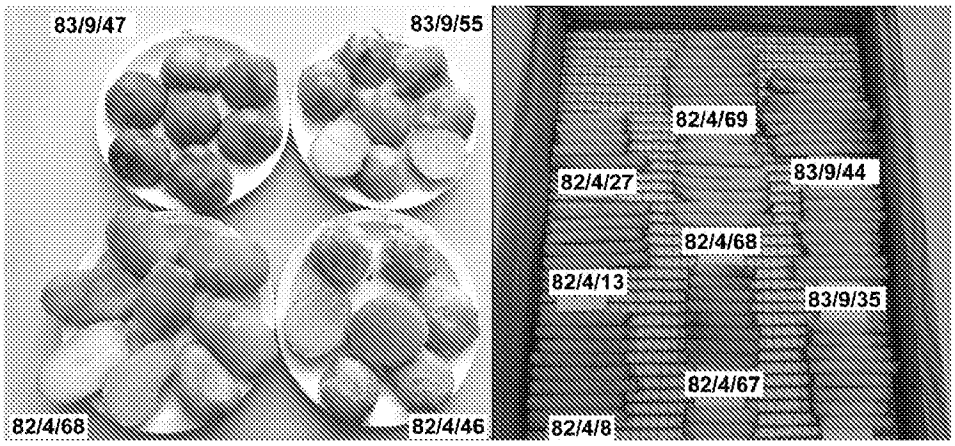


FIGURE 7 | Assessment of quality traits: Discolouration of cooked tuber flesh (left) and raw tuber tissue of different BC progenies derived from *S. bulbocastanum* GLKS 31741 (*blb*41) (+) cv. 'Delikat' somatic hybrids 82/4 and 83/9 (back-crossed with cv. 'Quarta').

the resistance genes segregated. Presence of race-specific genes in the following BC generations was also caused by their presence in some of the cultivars used for back-crossing, like 'Baltica', 'Sarpo Mira', and 'Quarta' with *R3a* and *R3b* genes, and 'Romanze' with only the *R3b* gene. In a few cases, a lack of a direct correlation between the presence of one of the

Aardevo Exhibit 1010
Page 0736

resistance genes and the level of resistance or functionality after agro-infiltration with the corresponding effector, indicate that the relevant genes were inactivated and the markers detected not only these resistance genes but also their analogs. Resistance is a complex trait with many effectors, epistatic or pleiotropic interactions (Lokosson et al., 2010; Aguilera-Galvez et al., 2018). It is known that in the susceptible haplotype (*rb*) there is a 18 bp deletion in the RB/*Rpi-blb1* gene (Bradeen et al., 2003). When the *Rpi-blb2* gene from *S. bulbocastanum* was transferred into potato cv. 'Désirée', there was not a positive correlation between gene expression in RT-qPCR assays and resistance to *Pi* in some cases (Orbegozo et al., 2016). Bearing in mind the genetic complexity of the SHs and BC clones (with many asymmetric nuclear constitutions in the SHs and a tendency for genetic stabilization in BC progenies), it is likely that the results of the RT-qPCR analysis will not always be correlated with the phenotypic resistance in DLA or field tests. Moreover, the evaluation of AUDPC in the field is also dependent on climate, which is the reason for presenting the data separately for each year in this study. The Δ-rAUDPC, however, confirmed the results of DLA in the field. It is likely that the tetrasomic inheritance in cultivated potato and the diverse nature of the genomic compositions of the SHs and derived progenies increased the complexity of the interactions, both genetically and between pathogen and host. Nevertheless, in the field trials SHs and four BC_1/BC_2 clones performed well in terms of tuber yield (**Table 4**) and resistance to foliage blight (**Tables 1–3**). For some of the hybrid clones the yield and number of tubers per plot was similar to those of the corresponding cultivar grown in the field trial, but tuber morphology in BC_2 progenies need further improvement by breeding. The negative values of the Δ-rAUDPC of SHs and BC_1 clones indicate that their resistance to foliage blight in the field assays was higher in at least 2 years. Compared to their parents more of the hybrids with both *Rpi-blb1* and *Rpi-blb3* genes survived an outbreak of late blight in the field in 2015 (data not shown). Moreover, the functionality of the genes identified by gene-specific markers was proven by agro-infiltration with corresponding *Avr*-effectors. The results demonstrate the functionality of stacked genes in the eight BCs, which were the most significant also in DLA and resistance in field assessments. The results demonstrate that the usage of somatic hybridization in combination with gene specific markers allow the transfer of multiple resistance traits (Thieme et al., 2008, 2010), which can also be tracked and stacked directly into potato cultivars. Incorporating more *R* genes into the potato might be the only way to increase the durability and level of resistance against late blight (Tan et al., 2010; Orbegozo et al., 2016). Other resistance mechanisms and traits can also be transferred along with those for which genes are already sequenced and, hence, can be easily tracked. Other authors (Davis et al., 2012) also report multiple resistances to late blight, viruses and aphids in the somatic fusion of potato and *S. bulbocastanum* and their crosses. In this study, tracking of these genes is not reported. Previous studies reported other resistant hybrids, which were fertile/infertile and either symmetric or asymmetric (Rakosy-Tican et al., 2015), which represent valuable material for further characterization and biotechnological improvement.

However, it is likely that *P. infestans* will eventually overcome the resistance to the newly released R gene variants. Therefore, various available strategies to control this insidious pathogen should be considered. It is necessary to deploy more cultivars that are both resistant and productive and our experiments showed that this is feasible. Moreover, some of our BC progenies of the three selected SHs are also good for producing chips and French Fries. Discolouration of the tissue of tubers, especially after cooking, and their starch content determine their table quality and value for the production of non-food products. Discolouration of the raw tuber flesh is caused by oxidation of phenolic compounds in the presence of polyphenol oxidase resulting in quinones, which is transformed into a dark pigment (Friedman, 1997). The level of phenolic compounds, activity of polyphenol oxidase and level of free amino acids also affect this process. Discolouration of tuber flesh after cooking is a key factor in determining their suitability for fresh consumption and processing (Wang-Pruski, 2007). The evaluation of the processing qualities of fusion hybrids and BC progenies will promote the exploitation of resistant clones as pre-breeding valuable genotypes. The results presented here underline the value of potato genotypes that are resistant and suitable for further processing.

In conclusion, this analysis of selected SHs and their BC_1 and BC_2 progenies has revealed eight progenies with a stack of four resistance genes, which are hypersensitive to corresponding *Avr*-effectors and resistant to late blight in DLA and the field (Δ-rAUDPC), the yield and processing qualities of some of which are good. These genotypes are valuable pre-breeding lines to produce potato cultivars with potentially durable resistance against foliage blight.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

ER-T and RT developed the idea, produced the plant material, wrote the manuscript and contributed to the DLA tests, agro-infiltration, and data analysis. MN carried out the marker analysis. T-ED, KK, and MM-L did the cytogenetics and mcGISH analysis. TH and JK performed the resistance in the field, yield evaluations and functional profiling of resistance genes by agro-infiltration. All authors read and approved the final manuscript.

FUNDING

ER-T acknowledges the financial support of a grant from the Romanian Authority for Scientific Research, CNCS-UEFISCDI

Aardevo Exhibit 1010
Page 0737

project number PNII-ID-PCE-2011-3-0586. The DAAD scholarship for 2018 awarded to ER-T is also acknowledged.

ACKNOWLEDGMENTS

Vivianne Vleeshouwers kindly provided *Agrobacterium tumefaciens* with effectors for checking the functionality of the stacked resistance genes. The authors wish to thank technical staff for excellent assistance, T. Dixon and N. Bernhardt for their helpful revision of the manuscript and Bernd Truberg for help with the statistical analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00699/full#supplementary-material

FIGURE S1 | An example of molecular analysis using gene specific markers to determine the presence of *R3a* and *R3b* genes in *S. bulbocastanum* GLKS 31741 (*blb*41), somatic hybrids *blb*41 (+) cv. 'Delikat' (SH) and their offspring (BC_1); M: DNA Ladder 100 bp.

TABLE S1 | Results of direct and indirect (flow cytometry) ploidy determination of selected somatic hybrids between potato cv. 'Delikat' and *Solanum bulbocastanum* (*blb*41) and their derived BC_1 and BC_2 progenies (nd-not determined).

REFERENCES

Aguilera-Galvez, C., Champouret, N., Rietman, H., Lin, X., Wouters, D., Chu, Z., et al. (2018). Two different R gene loci co-evolved with Avr2 of *Phytophthora infestans* and confer distinct resistance specificities in potato. *Stud. Mycol.* 89, 105–115. doi: 10.1016/j.simyco.2018.01.002

Black, W., Mastenbroek, C. W., Mills, R., and Peterson, L. C. (1953). A proposal for an international nomenclature of races of *Phytophthora infestans* and of genes controlling immunity in *Solanum demissum* derivatives. *Euphytica* 2, 173–179. doi: 10.1007/bf00053724

Bormann, C. A. (2003). *Genetic and Molecular Analysis Of Quantitative And Qualitative Late Blight Resistance In Tetraploid Potato.* Dissertation, Universität Hohenheim, Stuttgart.

Bradeen, J. M., Naess, S. K., Song, J., Haberlach, G. T., Wielgus, S. M., Buell, C. R., et al. (2003). Concomitant reiterative BAC walking and fine genetic mapping enable physical map development for the broad-spectrum late blight resistance region. *RB. Mol. Genet. Genomics* 269, 603–611. doi: 10.1007/s00438-003-0865-8

Bradshaw, J. E., Pande, B., Bryan, G. J., Hackett, C. A., McLean, K., Stewart, H. E., et al. (2004). Interval mapping of quantitative trait loci for resistance to late blight [*Phytophthora infestans* (Mont.) de Bary], height and maturity in a tetraploid population of potato (*Solanum tuberosum* subsp. *tuberosum*). *Genetics* 168, 983–995. doi: 10.1534/genetics.104.030056

Chakrabarti, S. K., Xie, C., and Tiwari, J. K. (eds) (2017). *The Potato Genome.* Berlin: Springer.

Chandel, P., Tiwari, J. K., Ali, N., Devi, S., Sharma, S., Sharma, S., et al. (2015). Interspecific potato somatic hybrids between *Solanum tuberosum* and *S. cardiophyllum*, potential sources of late blight resistance breeding. *Plant Cell Tiss. Organ. Cult.* 123, 579–589. doi: 10.1007/s11240-015-0862-8

Colton, L. M., Groza, H. I., Wielgus, S. M., and Jiang, J. M. (2006). Marker-assisted selection for the broad-spectrum potato late blight resistance conferred by gene RB derived from a wild potato species. *Crop Sci.* 46, 589–594. doi: 10.2135/cropsci2005.0112

Danan, S., Veyrieras, J.-B., and Lefebvre, V. (2011). Construction of a potato consensus map and QTL meta-analysis offer new insights into the genetic architecture of late blight resistance and plant maturity traits. *BMC Plant Biol.* 11:16. doi: 10.1186/1471-2229-11-16

Davis, J. A., Radcliffe, E. B., Thill, C. A., and Ragsdale, D. W. (2012). Resistance to aphids, late blight and viruses in somatic fusion and crosses of *Solanum tuberosum* L. *and Solanum bulbocastanum. Am. J. Pot. Res.* 89, 489–500. doi: 10.1007/s12230-012-9272-1

Doležel, J., Greilhuber, J., and Suda, J. (2007). Estimation of nuclear DNA content in plants using flowcytometry. *Nat. Protoc.* 2, 2233–2244. doi: 10.1038/nprot.2007.310

Du, J., Rietman, H., and Vleeshouwers, V. G. A. A. (2014). Agroinfiltration and PVX agroinfection in potato and *Nicotiana benthamiana. J. Visual Exp.* 2014:e50971. doi: 10.3791/50971

Friedman, M. (1997). Chemistry, biochemistry, and dietary role of potato polyphenoles. A review. *J. Agric. Food Chem.* 45, 1523–1540. doi: 10.1021/jf960900s

Gebhardt, C., and Valkonen, J. P. T. (2001). Organization of genes controlling disease resistance in the potato genome. *Annu. Rev. Phytopathol.* 39, 79–102.

Gishlain, M., Byarugaba, A. A., Magembe, E., Njoroge, A., Rivera, C., Román, M. L., et al. (2019). Stacking three late blight resistance genes from wild species directly into African highland potato varieties confers complete field resistance to local blight races. *Plant Biotechnol. J.* 17, 1119–1129. doi: 10.1111/pbi.13042

Haas, B. J., Kamoun, S., Zody, M. C., Jiang, R. H. Y., Handsaker, R. E., Cano, L. M., et al. (2009). Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans. Nature* 46, 393–398.

Haesaert, G., Vossen, J. H., Custers, R., De Loose, M., Haverkort, A., Heremans, B., et al. (2015). Transformation of potato variety Désirée with single or multiple resistance genes increases resistance to late blight under field conditions. *Crop Prot.* 77, 163–175. doi: 10.1016/j.cropro.2015.07.018

Hammann, T., Truberg, B., and Thieme, R. (2009). "Improving resistance to late blight (*Phytophthora infestans*) by using interspecific crosses in potato (*Solanum tuberosum* spp.)," in *Crop Plant Resistance to Biotic and Abiotic Factors*, eds F. Feldmann, D. V. Alford, and C. Furk (Braunschweig: Gesellschaft), 407–414.

Hanson, P., Sitathani, K., Sadashiva, A., Yang, R.-Y., Graham, E., and Ledesma, D. (2007). Performance of Solanum habrochaites LA1777 introgression line hybrids for marketable tomato fruit yield in Asia. *Euphytica* 158, 167–178. doi: 10.1007/s10681-007-9440-4

Haverkort, A. J., Boonekamp, P. M., Hutten, R., Jacobsen, E., Lotz, L. A. P., Kessel, G. J. T., et al. (2016). Durable late blight resistance in potato through dynamic varieties obtained by cisgenesis: scientific and societal advances in the DuRPh project. *Potato Res.* 59, 35–66. doi: 10.1007/s11540-015-9312-6

Haverkort, A. J., Struik, P. C., Visser, R. G. F., and Jacobsen, E. (2009). Applied biotechnology to combat late blight in potato caused by *Phytophthora infestans. Potato Res.* 52, 249–264. doi: 10.1007/s11540-009-9136-3

Hein, I., Birch, P. R. J., Lefebvre, S. D. V., Odeny, D. A., Gebhardt, C., Danan, S., et al. (2009). Progress in mapping and cloning qualitative and quantitative resistance against *Phytophthora infestans* in potato and its wild relatives. *Potato Res.* 52, 215–227. doi: 10.1007/s11540-009-9129-2

Hermsen, J. G. T., and Ramanna, M. S. (1973). Double-bridge hybrids of *Solanum bulbocastanum* and cultivars of *Solanum tuberosum. Euphytica* 22, 457–466. doi: 10.1007/bf00036641

Huang, S., van der Vossen, E. A. G., Kuang, H., Vleeshouwers, V. G. A. A., Zhang, N., Borm, T. J. A., et al. (2005). Comparative genomics enabled the isolation of the R3a late blight resistance gene in potato. *Plant J.* 42, 251–261. doi: 10.1111/j.1365-313x.2005.02365.x

Huang, S., Vleeshouwers, V. G., Werij, J. S., Hutten, R. C., van Eck, H. J., Visser, R. G. F., et al. (2004). The R3 resistance to *Phytophthora infestans* in potato is conferred by two closely linked R genes with distinct specificities. *Mol. Plant Microb. Interact* 17, 428–435. doi: 10.1094/mpmi.2004.17.4.428

Jo, K.-R., Zhu, S., Bai, Y., Hutten, R. C. B., Kessel, G. J. T., Vleeshouwers, V. G. A. A., et al. (2016). "Problematic crops: 1. potatoes: towards sustainable potato late blight resistance by cisgenic R gene pyramiding," in *Plant Pathogen Resistance Biotechnology*, ed. D. B. Collinge (Hoboken, NJ: John Wiley & Sons Inc), 171–191. doi: 10.1002/9781118867716.ch9

Aardevo Exhibit 1010
Page 0738

Johnston, S. A., den Nijs, T. P. M., Peloquin, S. J., and Hanneman, R. E. Jr. (1980). The significance of genic balance to endosperm development in interspecific crosses. *Theor. Appl. Genet.* 57, 5–9. doi: 10.1007/bf00276002

Kamoun, S., and Smart, C. D. (2005). Late blight of potato and tomato in the genomics era. *Plant Disease* 89, 692–699. doi: 10.1094/pd-89-0692

Kruppa, K., Türkösi, E., Szakács, É, Cseh, A., and Molnár-Láng, M. (2013). Development and identification of a 4HL.5DL wheat/barley centric fusion using GISH, FISH and SSR markers. *Cereal Res. Commun.* 41, 221–229. doi: 10.1556/crc.2012.0038

Li, G. C., Huang, S. W., Guo, X., Li, Y., Yang, Y., Guo, Z., et al. (2011). Cloning and characterization of R3b members of the R3 superfamily of late blight resistance genes show sequence and functional divergence. *Mol. Plant Microb. Interact* 24, 1132–1142. doi: 10.1094/mpmi-11-10-0276

Lokossou, A. A., Park, T.-H., van Arkel, G., Arens, M., Ruyter-Spira, C., Morales, J., et al. (2009). Exploiting knowledge of R/Avr genes to rapidly clone a new LZ-NBS-LRR family of late blight resistance genes from potato linkage group IV. *Mol. Plant Microb. Interact* 22, 630–641. doi: 10.1094/mpmi-22-6-0630

Lokossou, A. A., Rietman, H., Wang, M., Krenek, P., van der Schoot, H., Henken, B., et al. (2010). Diversity, distribution, and evolution of *Solanum bulbocastanum* late blight resistance genes. *Mol. Plant Microb. Interact* 23, 1206–1216.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol. Plant.* 15, 473–497. doi: 10.1111/j.1399-3054.1962.tb08052.x

Oosumi, T., Rockhold, D., Maccree, M., Deahl, K., McCue, K., and Belknap, W. (2009). Gene Rpi-bt1 from *Solanum bulbocastanum* confers resistance to late blight in transgenic potatoes. *Am. J. Potato Res.* 86, 456–465. doi: 10.1007/s12230-009-9100-4

Orbegozo, J., Roman, M. L., Rivera, C., Gamboa, S., Tovar, J. C., Forbes, G. A., et al. (2016). Rpi-blb2 gene from *Solanum bulbocastanum* confers extreme resistance to late blight disease in potato. *Plant Cell Tiss. Organ. Cult.* 125, 269–281. doi: 10.1007/s11240-016-0947-z

Paro, R., Triboni, G. M., Buccione, R., Rossi, G., Cellini, V., Canipari, R., et al. (2012). The fungicide mancozeb induces toxic effects on mammalian granulosa cells. *Toxicol. Appl. Pharmacol.* 260, 155–161. doi: 10.1016/j.taap.2012.02.005

R Development Core Team (2010). *Version 2.11.1.* Available online at: www.lsw.uni-heidelberg.de/users/christlieb/teaching/./R-refman.pdf (accessed September 2016).

Rakosy-Tican, E., Thieme, R., Nachtigall, M., Molnar, I., and Denes, T.-E. (2015). The recipient potato cultivar influences the genetic makeup of the somatic hybrids between potato cultivars and sexually incompatible species *Solanum bulbocastanum* Dun. *Plant Cell Tiss. Organ. Cult.* 122, 395–407. doi: 10.1007/s11240-015-0777-4

Rietman, H. (2011). *Putting the Phytophthora Infestans Genome Sequence At Work; Multiple Novel Avirulence And Potato Resistance Gene Candidates Revealed.* Ph. D. thesis, Wageningen University. Wageningen.

Rodewald, J., and Trognitz, B. (2013). *Solanum* resistance genes against *Phytophthora infestans* and their corresponding avirulence genes. *Mol. Plant Pathol.* 14, 740–757. doi: 10.1111/mpp.12036

Shaner, G., and Finney, R. E. (1977). The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. *Phytopathology* 67, 1051–1056.

Shi, X., Tian, Z., Liu, J., van der Vossen, E. A. G., and Xie, C. (2012). A potato pathogenesis-related protein gene, StPRp27, contributes to race-nonspecific resistance against *Phytophthora infestans*. *Mol. Biol. Rep.* 39, 1909–1916. doi: 10.1007/s11033-011-0937-5

Simko, I. (2002). Comparative analysis of quantitative trait loci for foliage resistance to *Phytophthora infestans* in tuber-bearing *Solanum* species. *Am. J. Potato Res.* 79, 25–132.

Song, J., Bradeen, J. M., Naess, S. K., Raasch, J. A., Wielgus, S. M., Haberlach, G. T., et al. (2003). Gene RB cloned from *Solanum bulbocastanum* confers broad spectrum resistance to potato late blight. *Proc. Natl. Acad. Sci. U.S.A.* 100, 9128–9133. doi: 10.1073/pnas.1533501100

Storck, T., Böhme, T., and Schultheiss, H. (2012). Status and perspectives of GM approaches to fight late blight. Thirteenth EuroBlight workshop, St. Petersburg (Russia). *PPO Spec. Rep.* 15, 45–48.

Tan, M. Y. A., Hutten, R. C. B., Visser, R. G. F., and van Eck, H. J. (2010). The effect of pyramiding *Phytophthora infestans* resistance genes RPi-mcd1 and RPi-ber in potato. *Theor. Appl. Genet.* 121, 117–125. doi: 10.1007/s00122-010-1295-8

Thieme, R., and Rakosy-Tican, E. (2017). "Somatic cell genetics and its application in potato breeding," in *The Potato Genome*, eds S. K. Chakrabarti, C. Xie, and J. K. Tiwari (Cham: Springer International Publishing), 217–269.

Thieme, R., Rakosy-Tican, E., Gavrilenko, T., Antonova, O., Schubert, J., Nachtigall, M., et al. (2008). Novel somatic hybrids and their fertile BC1 progenies of potato (*Solanum tuberosum* L.) + *S. tarnii*, extremely resistant to potato virus Y and resistant to late blight. *Theor. Appl. Genet.* 116, 691–700. doi: 10.1007/s00122-007-0702-2

Thieme, R., Rakosy-Tican, E., Nachtigall, M., Schubert, J., Hammann, T., Antonova, O., et al. (2010). Characterization of multiple resistance traits of somatic hybrids between *Solanum cardiophyllum* Lindl. and two commercial potato cultivars. *Plant Cell Rep.* 29, 1187–1201. doi: 10.1007/s00299-010-0905-x

Truberg, B., Hammann, T., Darsow, U., and Piepho, H.-P. (2009). Empirischer Vergleich verschiedener Methoden zur Reifekorrektur von Daten zum Befall mit Krautfäule (*Phytophthora infestans* (Mont.) de Bary) in Selektionsexperimenten bei der Kartoffel (*Solanum tuberosum* subsp. *tuberosum*). *J. Kulturpfl.* 61, 77–81.

Van der Vossen, E. A. G., Gros, J., Sikkema, A., Muskens, M., Wouters, D., Wolters, P., et al. (2005). The Rpi-blb2 gene from *Solanum bulbocastanum* is an Mi-1 gene homolog conferring broad spectrum late blight resistance in potato. *Plant J.* 44, 208–222. doi: 10.1111/j.1365-313x.2005.02527.x

Van der Vossen, E. A. G., Sikkema, A., Hekkert, B., Gros, J., Stevens, P., Muskens, M., et al. (2003). An ancient R gene from the wild potato species *Solanum bulbocastanum* confers broad-spectrum resistance to *Phytophthora infestans* in cultivated potato and tomato. *Plant J.* 36, 867–882.

Vleeshouwers, V. G. A. A., Raffaele, S., Vossen, J. H., Champouret, N., Oliva, R., Segretin, M. E., et al. (2011). Understanding underexploiting late blight resistance in the age of effectors. *Annu. Rev. Phytopathol.* 49, 507–531. doi: 10.1146/annurev-phyto-072910-095326

Vleeshouwers, V. G. A. A., Rietman, H., Krenek, P., Champouret, N., Young, C., Oh, S.-K., et al. (2008). Effector genomics accelerates discovery and functional profiling of potato disease resistance and *Phytophthora infestans* avirulence genes. *PLoS One* 3:e2875. doi: 10.1371/journal.pone.0002875

Vreugdenhil, D. (ed.) (2007). *Potato Biology and Biotechnology*. Amsterdam: Elsevier.

Wang, J., Jiang, J., and Wang, Y. (2013). Protoplast fusion for crop improvement and breeding in China. *Plant Cell Tissue Organ. Cult.* 112, 131–142. doi: 10.1007/s11240-012-0221-y

Wang, M., Allefs, S., van den Berg, R. G., Vleeshouwers, V. G. A. A., van der Vossen, E. A. G., and Vosman, B. (2008). Allele mining in solanum: conserved homologues of Rpi-blb1 are identified in *Solanum stoloniferum*. *Theor. Appl. Genet.* 116, 933–943. doi: 10.1007/s00122-008-0725-3

Wang-Pruski, G. (2007). The canon of potato science: 47. After-cooking darkening. *Potato Res.* 50, 403–406. doi: 10.1007/s11540-008-9061-x

Zhu, S., Li, Y., Vossen, J. H., Visser, R. G. F., and Jacobsen, E. (2012). Functional stacking of three resistance genes against *Phytophthora infestans* in potato. *Transgen. Res.* 21, 89–99. doi: 10.1007/s11248-011-9510-1

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Rakosy-Tican, Thieme, König, Nachtigall, Hammann, Denes, Kruppa and Molnár-Láng. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Aardevo Exhibit 1010
Page 0739

EXHIBIT B

Dissection of

the major late blight resistance cluster

on potato linkage group IV

Anoma A. Lokossou

Aardevo Exhibit 1010
Page 0741

Thesis committee

Thesis supervisors

Prof. Dr. Richard G.F. Visser

Professor of Plant Breeding,

Wageningen University

Prof. Dr. Ir. Evert Jacobsen

Professor of Plant Breeding (Genetical Variation and Reproduction)Wageningen University

Other members

-Prof. Dr. Ir. Rudy Rabbinge, Wageningen University

-Dr. Ir. Bart P. H. J. Thomma, Wageningen University

-Dr. Theo A. J. van der Lee, Wageningen University

-Dr. Ing. Frank L. W. Takken, University of Amsterdam

This research was conducted under the auspices of the graduate School of Experimental Plant Sciences

Aardevo Exhibit 1010
Page 0742

Dissection of

the major late blight resistance cluster

on potato linkage group IV

Anoma A. Lokossou

Thesis

Submitted in fulfilment of the requirements for the degree of doctor

at Wageningen University

by the authority of the Rector Magnificus

Prof. dr. M.J. Kropff,

in the presence of the

Thesis committee appointed by the Academic Board

to be defended in public

on Monday 7th June 2010

at 4 p.m. in the Aula

Aardevo Exhibit 1010
Page 0743

Dissection of the major late blight resistance cluster on potato linkage group IV

Anoma A. Lokossou

PhD Thesis Wageningen University, The Netherlands, 2010

With references- with summaries in English, Dutch and French

ISBN 978-90-8585-654-2

Aardevo Exhibit 1010
Page 0744

Content

Chapter 1 7

General introduction

Chapter 2 15

Exploiting knowledge of R/Avr genes to rapidly clone a new LZ-NBS-LRR family of late blight resistance genes from potato linkage group IV

Chapter 3 41

Diversity, distribution and evolution of *Solanum bulbocastanum* Late Blight resistance genes

Chapter 4 71

Phytohthora infestans resistance gene *Rpi-mcd1.1* from the Argentinean *Solanum microdontum* is functionally distinct from other *R2* homologues of Mexican origin

Chapter 5 91

Exploration of sequence polymorphism in *R2* gene homologues permitted the identification of a single amino acid which triggers the potato/ *P. infestans* interaction R2/PiAVR2

Chapter 6 107

General discussion

Literature cited 114

Summary 129

Sammenvatting 131

Résumé 134

Acknowledgment 137

Curriculum vitae 140

Aardevo Exhibit 1010
Page 0745

Chapter 1

General introduction

Aardevo Exhibit 1010
Page 0747

General introduction

Potato (*Solanum tuberosum*) history and importance

The commercial potato derived from the wild species *Solanum tuberosum* originates from the Andes in South America. Originally it was first cultivated next to the present border separating Peru and Bolivia some 8,000 years ago. The Spanish took the potato from Latin America to Europe in the 16th century. Potato was first admired for its flowers before being appreciated for its tubers and since then potato became a major carbohydrate source in human and animal diets around the world. Adaptation to long days (Brown 1990; Hawkes 1978; 1994) and generations of breeding led to a panel of potato varieties differing in taste, skin color, shape, starch content, cooking type, etc… The fast growing characteristic of potato allowed poor families to cultivate it on small plots and break the circle of poverty. Hundred millions of people around the world depend on potato to survive. Potato is grown in more than 100 countries, under temperate, subtropical and tropical conditions and ranks as the world's third most important food crop, after rice and wheat. China is now the largest potato producer followed by India, Russian Federation and USA.

Late Blight and *Phytophthora infestans*

Monoculture and the lack of genetic diversity helped the emergence and dominance of the most significant threat to potato production worldwide: the Late Blight (LB) disease, caused by the oomycete pathogen *Phytophthora infestans*, which destroys leaves, stems and tubers. One memorable event happened in the 19th century, when Ireland was cultivating a few potato varieties sharing the same genetic background which were devastated by a Late Blight epidemic. Widespread famine followed with the death of millions of people and the migration of more than a million of Irish to the USA.

Current disease control relies on multiple (up to 20) fungicide applications per season (Hanson et al. 2007) and costs associated with chemical control and yield loss amount to €5 billion globally per year (Whisson et al. 2001). Such pesticide inputs are both environmentally and economically undesirable and have a negative impact on the sustainability of potato production. To prevent spray of too large quantities of fungicides, the focus of different scientific groups is to find an efficient decision support system for sustainable cultivation of potato (Savenkov et al. 2003). The purpose is to gather information about the emergence and the progress of *Phytophtora infestans* in the field, weather forecast, cultivars planted, date of fungicide spreads and spatial epidemiology (spore release, escape,

Aardevo Exhibit 1010
Page 0749

transport, deposition and survival), with the aim to give a prediction about the risk of regional spore dispersal and survival helping the potato growers in an online service for decision making about when, which and how much fungicide to spray (Midilis program; Simcast (Skelsey et al. 2009).

Introgression of Late Blight Resistance by traditional breeding

Early in the 20th century the potential of the wild Mexican hexaploid species *Solanum demissum* was recognized for conferring extreme levels of Late Blight resistance and hybrids with *S. tuberosum* were generated. Subsequently, 11 major dominant resistance (*R*) genes were identified, and introgressed into potato cultivars by interspecific hybridization and back crosses with cultivated potato (Malcolmson 1969; Malcolmson and Black 1966; Müller and Black 1952). The set of eleven genes (*R1* to *R11*) was exploited in monoculture, which besides providing an efficient crop protection, was exerting a strong selection pressure on the pathogen resulting in its rapid adaptation and consequently the defeat of those *R* genes (Fry 2008; McDonald and Linde 2002);. Even, the combination of different R genes in one cultivar did not always extend the life time of the cultivar in the field. The Scottish cultivar Pentland Dell containing three R genes, *R1, R2* and *R3* was infected six years after being released (Hein et al. 2009a). There are examples of cultivars, like cv Escort, with at least 3 combined R-genes which are most of the years showing a good protection against Late Blight (Dr. R. Hutten, Wageningen UR Plant Breeding pers. comm.).

Horizontal resistance

An alternative strategy to control Late Blight is to identify and combine loci harboring genes responsible for quantitative or partial resistance traits. Quantitative resistance loci (QRL) have been largely studied and are believed to provide a more stable durable resistance (Gebhardt 1994; Leonards-Schippers et al. 1994; Oberhagemann et al. 1999); so called horizontal resistance, provided by partial resistance genes in contrast to the vertical resistance provided by major R-genes. However, Vleeshouwers et al. (2000)) showed that in backgrounds with partial resistance also HR reactions are found, suggesting the presence of R-genes with a partial effect. Late Blight QRL have been identified in many wild *Solanum* species. The Argentinean wild diploid species *S. microdontum* harbors PiQRL (Bisognin et al. 2005). *S. paucissectum* has PiQRL identified on linkage group X, XI and XII (Villamon et al. 2005). Ghislain et al (2001) mapped PiQRLs on linkage group VII and XII of *S. phureja*. Linkage group VII contains a PiQRL, identified in a population derived, from *S. demissum* (Meyer et al. 1998). Sarah Danan (2009) describes the identification in cross populations derived from *S. sparsipulim* and *S. spegazzinii*, a total of 30 Late Blight QRL present on 13 distinct genomic regions, confirming previous studies (Leonards-Schippers et al. 1992; Oberhagemann et al. 1999).

Aardevo Exhibit 1010
Page 0750

Pyramiding of R-genes and Cisgenesis

Another way to control Late Blight disease is to learn from local farmers in the Andean region who have domesticated thousands of varieties during generations. Nowadays, over 50 varieties are cultivated on their farms using the polyculture method acting as a genetic resistance shield against Late Blight. In modern potato culture, pyramiding is pictured in mixing individual *R*-gene containing cultivars or creating a cultivar containing stacked *R*-genes.

Conventional introgression of resistance often involves inter-specific crosses and repeated backcrosses with cultivated potato, which is a very slow and inefficient process. The result is introgression of a piece of DNA with the gene of interest, surrounded by many other alleles of the donor species. This is called linkage drag and is almost always connected with linked alleles coding for traits with a negative impact (McGillivary and Clemente 1956). For durable Late Blight resistance strategies, efficient stacking of *Rpi* genes from one or several species is essential, but in practice this will enlarge the linkage drag problems considerably. Their introduction by genetic modification (GM) is a much more efficient way to improve resistance in one step and in a short period. It can even be applied to existing varieties with a long history of safe use. Currently, *Rpi* genes of natural origin, so called cisgenes (Jacobsen and Schouten 2007; Schouten and Jacobsen 2008) can be introduced using marker-free transformation systems leading to cisgenic plants with only the gene(s) of interest and without linkage drag of other genes from the wild species or from antibiotic resistance genes normally present at the T-DNA (de Vetten et al. 2003; Schaart et al. 2004; Yu et al. 2006). To date many *Rpi* genes have been cloned: *R1* and *R3a* from *S. demissum* (Ballvora et al. 2002; Huang et al. 2005a), *RB/Rpi-blb1, Rpi-blb2* and *Rpi-bt1* from *S. bulbocastanum* (Oosumi et al. 2009; Song et al. 2003; van der Vossen et al. 2003; van der Vossen et al. 2005), *Rpi-sto1* and *Rpi-pta1* from *S. stoloniferum* (Vleeshouwers et al. 2008) and *Rpi-vnt1.1, Rp-vnti1.2* and *Rpi-vnt1.3* from *S. venturii* (Foster et al. 2009; Pel et al. 2009). All belong to the NBS-LRR class of plant *R* genes, which are thought to be cytoplasmic (van der Biezen and Jones 1998). Additional *Rpi* genes that have been identified and genetically mapped, are *Rpi-blb3, Rpi-abpt, R2, R2-like* and *Rpi-mcd1* on chromosome 4 (Li et al. 1998; Park et al. 2005a; Park et al. 2005b; Park et al. 2005c; Tan et al. 2008) but also *Rpi1*from *S. pinnatissectum* on chromosome 7 (Kuhl and Hanneman 2001); *Rpi-phu1* and *Rpi-mcq1* on chromosome 9 (Sliwka et al. 2006; Smilde and Brigneti 2005) R_{ber} on chromosome 10 (Ewing and Simko 2000; Rauscher et al. 2006) and *R3b, R5, R6, R7, R8, R9, R10* and *R11* on chromosome 11 (Bradshaw et al. 2006; Huang et al. 2005b; Huang et al. 2004).

Aardevo Exhibit 1010
Page 0751

The resistance pathway

Phytophtora infestans is an obligate biotroph which infects potato by an invaginating feeding structure (haustoria) into the host cell plasma membrane, establishing an intimate contact with this host, and delivering effector molecules (virulence effectors) into the plant cell to reprogram the plant cell in order to enhance microbial fitness. The plant reacts to this invasion by recognizing the avirulent effectors and activating the innate immunity via complex pathways that arrest proliferation of the pathogen, thus conferring disease resistance (McHale et al. 2006). The ZIGZAG model (Fig 1) in the oomycete-plant interaction (Hein et al. 2009b; Jones and Dangl 2006), nicely describes most important potato- *P-infestans* interactions. In its infection process, *P.infestans* first secretes the so-called pathogen-associated molecular patterns (PAMPs) like CBEL (the cellulose binding elicitor lectin) (Dumas et al. 2008; Gaulin et al. 2006) or elicitors like INF1 (Kamoun 2006) to trigger immunity (PTI). This process can be suppressed by secretion of effectors like the glucanase or protease inhibitor GIP2 (Damasceno et al. 2008) or EPI1 (Tian et al. 2004) which act either outside or inside host cells and re-program host metabolism to promote 'effector-triggered susceptibility' (ETS). When a successful *P. infestans* isolate has suppressed PTI, the products of plant, *Rpi* genes, as a second 'layer' of defense, can directly or indirectly detect avirulence proteins like AVR3a (Armstrong et al. 2005; Bos et al. 2006) or AVR1b (Dou et al. 2008; Shan et al. 2004). *Rpi* proteins subsequently activate resistance responses (effector-triggered immunity; ETI), often including programmed cell death during the hypersensitive response (Jones and Dangl 2006).

AVR protein detection is possible in a direct way or indirectly by the resistance genes harboring key domains for pathogen recognition, the nucleotide binding site (NBS) and the leucine rich repeat (LRR) domains (Catanzariti et al. 2010; Collier and Moffett 2009; Ellis et al. 2007; Jones and Dangl 2006; Rairdan and Moffett 2006). Although, the N terminal domain of NBS-LRR protein has been demonstrated to play a crucial role in AVR protein recognition (Moffett et al. 2009), multiple studies showed that the LRR domain is the region of the protein that confers recognition specificity (Hulbert et al. 2001; Meyers et al. 1998; Mondragon Palomino and Meyers 2002; Noel et al. 1999). In silico secondary structure of the NBS-LRR protein shows that the LRR repeats with the motif xxLxLxx form a β-strand/β-turn structure in which the x residues are exposed to solvent and are available for interactions with potential ligands (Kobe and Deisenhofer 1995). These solvent exposed residues are hyper variable and subject to diversifying selection, suggesting that these residues have evolved to detect variation in pathogen-derived ligands (Michelmore and Meyers 1998). So far five matching pairs of *PiAVR* and potato *R*-gene have been identified, *PiAVR3a/R3a* (Armstrong et al. 2005), *AVR4/ R4* (van Poppel et al. 2008), *AVR-blb1/Rpi-blb1* (Vleeshouwers et al. 2008), *AVR-blb2/ Rpi-blb2* (Oh et

Aardevo Exhibit 1010
Page 0752

al. 2009), and *AVRvent1 /Rpi-vent1-1* (Pel 2010). Detailed investigation of the PiAVR/R interaction was so far orientated to the effector side to identify the crucial domain necessary for the activation of the hypersensitive reaction. The C terminal domains were found indispensable for PiAVR3a and AVR4 recognition (Bos et al. 2006; Van Poppel et al. 2009).

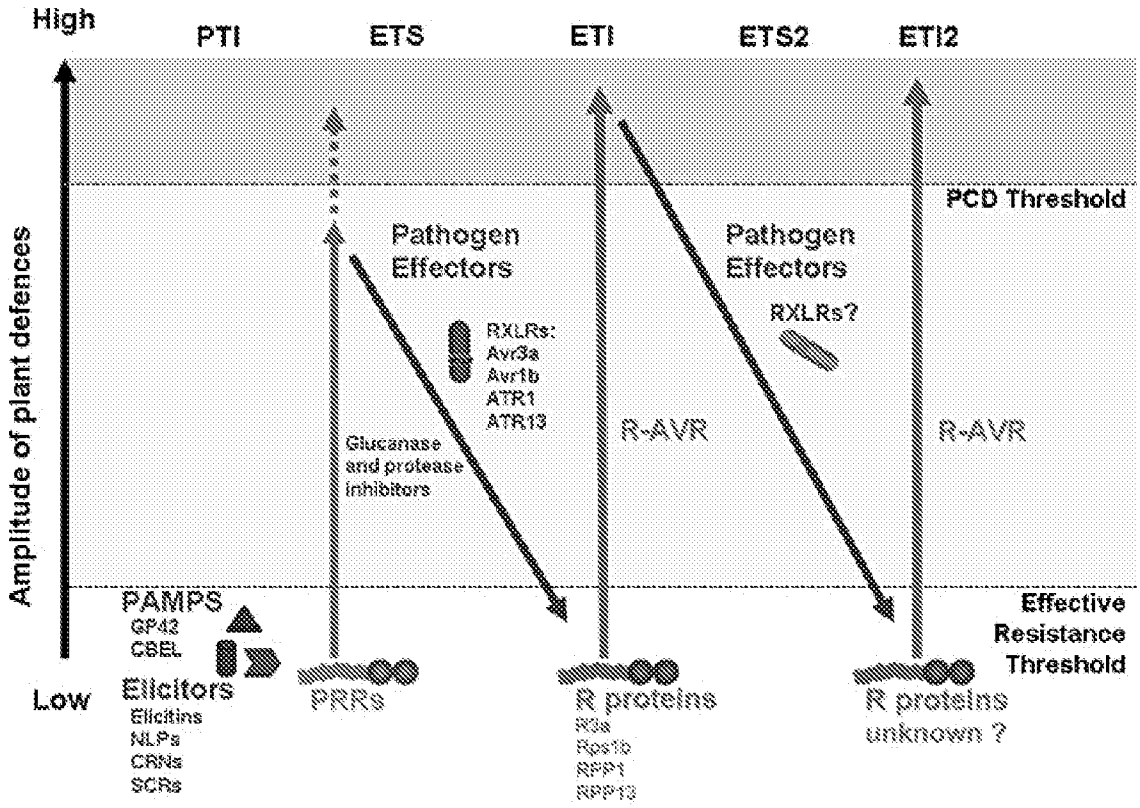


Fig. 1 The zig-zag-zig in oomycete–plant interactions. Published in Hein et al (2009b)

Outline of the thesis

The aim of the research described in this thesis was to study the major Late Blight resistance locus on linkage group IV, following the fine mapping and the characterization of the four orthologues resistance genes *Rpi-blb3, Rpi-abpt, R2* and *R2-like* (Park et al. 2005a, 2005b, 2005c; Li et al. 1998).

Cloning and functional characterization of the four genes is described in **Chapter 2,** using map based cloning and candidate gene allele mining approaches in combination with transient complementation in the heterologous *Nicotiana benthamiana* 'model' plant system. All 4 *Rpi* genes code for proteins of the NBS-LRR class of R proteins and share the highest similarity (34.9% aa) with RPP13 from *Arabidopsis thaliana* (Bittner-Eddy et al. 2000), which confers resistance to the oomycete

Aardevo Exhibit 1010
Page 0753

H. arabidopsidis. Despite apparent differences between the four functional R proteins, all four induce a hypersensitive response upon co-expression with the *P. infestans* derived RxLR effector PiAVR2 (Gilroy et al., in preparation) in *N. benthamiana* leaves, indicating that they recognize the same effector protein

Solanum bulbocastanum is well known for its resistance to Late Blight (Niederhauser and Mills 1953), from which four Rpi-genes were so far cloned, *Rpi-blb1, Rpi-blb2, Rpi-blb3* and *Rpi-bt1* (Oosumi et al. 2009; Song et al. 2003; van der Vossen et al. 2003; van der Vossen et al. 2005). As population genetic processes and dynamics might affect the evolution of *R*-genes, a better understanding of variation within and between populations is crucial. **Chapter 3** reports about the allelic frequencies of the *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* genes in several wild tuber-bearing *Solanum* accessions by performing allele mining with *R*-gene-specific primers. Additionally, Avr expression assays as well as detached leaf assays with key *P. infestans* isolates were utilized to strengthen the evidence for the presence of functional alleles. We related the occurrence of the genes to the geographical origin of the accessions.

Interestingly, several PiQRL mapped to the short arm of linkage group IV (Bradshaw et al. 2004; Bradshaw et al. 2006; Leonards-Schippers et al. 1994; Sandbrink et al. 2000), on the R2 locus (Hein et al. 2009a; Hein et al. 2007). Exploring the putative homology with R2GHs we attempted, in **Chapter 4**, to molecularly identify the *Rpi* genes responsible of the field resistance evaluated in a F1 population with *S. microdontum* BGRC 18302-34 (CGN 21342) as source of resistance in the earlier parentage. Molecular and phenotypic characterization of the population permitted the identification of at least three genes segregating in the population. *Rpi-mcd1.1* and *Rpi-mcd1.2* were originating from *S. microdontum* with resistance to PIC99177 and IPO-0, respectively, and *Rpi-phu2* from *S. phureja* providing only resistance to PIC99177. The use of allelic mining strategies resulted in the identification of 27 *R2* homologues, among which *Rpi-mcd1.1* was identified to be functional by using a transient complementation assay in *Nicotiana benthamiana*.

In **Chapter 5** we explored the information provided by the alleles generated in different *Rpi-blb3* allele mining strategies and used the transient co-expression system in *N. benthamiana* to identify the repeats in the LRR domain necessary for PiAVR2 recognition. Domain swaps between R genes interacting or not with PiAVR2 permitted the identification of a region spanning three LRR repeats of the cloned *Rpi* genes that determine the *R2* resistance specificity. Site directed mutagenesis pinpointed one solvent exposed amino acid to be crucial for PiAVR2 recognition.

Aardevo Exhibit 1010
Page 0754

Chapter 2

Exploiting knowledge of R/Avr genes to rapidly clone a new LZ-NBS-LRR family of late blight resistance genes from potato linkage group IV

Anoma A Lokossou, Tae-ho Park, Gert van Arkel, Marjon Arens, Carolien Ruyter-Spira, Juan Morales, Steve C. Whisson, Paul R. J. Birch, Richard G. F. Visser, Evert Jacobsen and Edwin A. G. van der Vossen

ABSTRACT

Additionally to *Rpi-blb1* and *Rpi-blb2*, *S. bulbocastanum* appears to harbor *Rpi-blb3* located at a major late blight resistance locus on LG IV, which also harbors *Rpi-abpt, R2, R2-like* and *Rpi-mcd1* in other *Solanum* spp. Here we report the cloning and functional analyses of 4 *Rpi* genes, using a map-based cloning approach, allele mining strategy, Gateway™ technology and transient complementation assays in *N. benthamiana*. *Rpi-blb3, Rpi-abpt*, *R2* and *R2-like* contain all signature sequences characteristic of LZ-NBS-LRR proteins, and share amino-acid sequences 34.9% similar to RPP13 from *Arabidopsis thaliana*. The LRR domains of all 4 Rpi proteins are highly homologous whilst LZ and NBS domains are more polymorphic, those of R2 being the most divergent. Clear blocks of sequence affiliation between the four functional R proteins and those encoded by additional *Rpi-blb3* gene homologs, suggest exchange of LZ, NBS and LRR domains, underlining the modular nature of these proteins. All four Rpi genes recognize the recently identified RXLR effector PiAVR2.

Published in Molecular Plant-microbe interactions (2009) 22:630-641

Aardevo Exhibit 1010
Page 0755

INTRODUCTION

The potato (*Solanum tuberosum)* ranks as the world's third most important food crop, after wheat and rice. Compared to these other staple crops a greater proportion of the potato crop is edible and a higher yield per hectare is achieved, which, combined with its high nutritional value, underlines its importance and explains ongoing increases in global potato production. The most significant threat to potato production worldwide is the late blight disease, caused by the oomycete pathogen *Phytophthora infestans*, which destroys leaves, stems and tubers. Current disease control relies on multiple (up to 20) fungicide applications per season (Hanson et al. 2007) and costs associated with chemical control amount to €5 billion globally per year (Whisson et al. 2001). Such pesticide inputs are both environmentally and economically undesirable and have a negative impact on the sustainability of potato production. Host resistance is a more environmentally benign means of restricting late blight infection, but the success of this management practices hinge on effective and durable host resistance and an understanding of the pathogen population.

Early in the 20th century the potential of the wild Mexican hexaploid species *Solanum demissum* was recognized for conferring extreme levels of late blight resistance, and hybrids with *S. tuberosum* were generated. Subsequently,11 major dominant resistance (*R*) genes were identified, and introgressed into potato cultivars (Müller and Black 1952; Malcolmson and Black 1966; Malcolmson 1969) However, deployment of these single genes did not provide durable resistance due to the rapidly changing populations of *P. infestans*. As an alternative approach, breeders started to select for quantitative 'field' resistance using races of *P. infestans* that were compatible with the *R* genes present in their breeding material (Toxopeus 1964; Black 1970). Despite considerable progress in genetic analysis of quantitative resistance to late blight using molecular markers (Gebhardt and Valkonen 2001; Simko 2002) under long day conditions breeders have achieved little progress, the major draw backs being tetrasomic inheritance of potato and the strong linkage between foliage resistance and late foliage maturity (Oberhagemann et al. 1999; Visker et al. 2003; Bradshaw et al. 2004). It is therefore anticipated that breeding for late blight resistance in potato, aimed at substantially contributing to disease management, requires the deployment of major resistance to *P. infestans* (*Rpi*) genes. Recent germplasm screens are revealing a wealth of *Rpi* genes in wild *Solanum* species and the challenge now is how to select, judiciously combine, and deploy sets of *Rpi* genes that can confer durable late blight resistance in modern potato varieties.

Key to durable exploitation of *Rpi* genes is also a better understanding of their interactions with effectors from *P. infestans.* All microbes trigger immune responses in plants *via* host receptor-mediated recognition of pathogen-associated molecular patterns (PAMPs). Such PAMP-triggered

Aardevo Exhibit 1010
Page 0757

immunity (PTI) can be suppressed by secretion of effectors which act either outside or inside host cells and re-program host metabolism to promote 'effector-triggered susceptibility' (ETS). When a successful pathogen has suppressed PTI, the products of plant, *R* genes, as a second 'layer' of defense, can directly or indirectly detect effectors (termed avirulence proteins; AVRs). R proteins subsequently activate resistance responses (effector-triggered immunity; ETI), often including programmed cell death during the hypersensitive response (Jones and Dangl 2006). The ability of a pathogen to evade recognition in the *R*-gene-for-*Avr*-gene concept (Flor 1971), can be explained by an evolutionary 'arms race' (Bergelson et al. 2001; Holub 2001). The generation, through mutation, and selection of *AVR* alleles that evade recognition is matched by strong selection pressure for *R* gene alleles that retain recognition. This is particularly evident in the case ATR1-RPP1 and ATR13-RPP13 interaction in the *Hyaloperonospora arabidopsidis* (formerly *H. parasitica*)-Arabidopsis pathosystem, where considerable allelic diversity has been revealed by studying natural host and pathogen populations (Rehmany et al. 2005; Allen et al.2008). In naturally co-evolving *P. infestans*-potato populations, similar high levels of allelic diversity are expected to be observed. So far, studies of the PiAVR3a-R3a interaction reveal diversifying selection for both genes (Amstrong et al. 2005; Huang et al, 2005). Recently, several oomycete *AVR* genes were identified (Allen et al. 2004; Shan et al. 2004; Armstrong et al. 2005; Rehmany et al. 2005; Vleeshouwers et al. 2008). Although each of the AVR proteins is distinct at the level of primary sequence similarity, they all share a secretion signal peptide followed by the motif RXLR and an acidic region, often ending in the sequence EER (Whisson et al. 2007; Birch et al. 2008a; Birch et al. 2008b).The RXLR-EER region is required for translocation of PiAVR3a, following its secretion from *P. infestans* haustoria, to the inside of host plant cells (Whisson et al. 2007). Translocation of RXLR-containing proteins inside the plant cell is consistent with their recognition by host NBS-LRR resistance proteins (Birch et al. 2006). As all oomycete AVR proteins identified to date have the RXLR motif, it provides a signature to identify AVR candidates from the *P. infestans* genome. Future knowledge of RXLR effectors diversity and their survey in *Phytophtora infestans* population could predict the span life of Rpi-genes in specific areas.

Conventional introgression of resistance often involves inter-specific crosses and repeated backcrosses with cultivated potato, which is a very slow and inefficient process. Currently, *Rpi* genes of natural origin, so called cisgenes (Jacobsen and Schouten 2007; Schouten and Jacobsen 2008), can be introduced using marker-free transformation systems leading to cisgenic plants with only the gene(s) of interest and without linkage drag of other genes from the wild species or from antibiotic resistance genes normally present at the T-DNA (de Vetten et al. 2003; Schaart et al. 2004; Yu et al. 2006). To date six *Rpi* genes have been reported: *R1* and *R3a* from *S. demissum* (Ballvora et al. 2002; Huang et al. 2005) *RB/Rpi-blb1* and *Rpi-blb2* from *S. bulbocastanum* (Song et al. 2003; van der Vossen

Aardevo Exhibit 1010
Page 0758

et al. 2003; van der Vossen et al. 2005),and *Rpi-sto1* and *Rpi-pta1* from *S. stoloniferum* and *S. papita,* respectively (Vleeshouwers et al. 2008). All belong to the NBS-LRR class of plant R proteins, which are thought to be cytoplasmic (van der Biezen and Jones 1998). Various loci are reported to contain *Rpi* genes on linkage group (LG) VII (Kuhl and Hanneman 2001), LG IX (Smilde and Brigneti 2005; Sliwka et al. 2006), LG X (Ewing and Simko 2000; Rauscher et al. 2006) and LG XI (Huang et al. 2004; Huang 2005; Bradshaw et al. 2006).

Here we describe the cloning and functional characterization of *Rpi-blb3, Rpi-abpt, R2* and *R2-like* from the major late blight resistance locus on LG IV of potato (Park et al. 2005a, 2005b, 2005c; Li et al. 1998). Upon map based cloning of *Rpi-blb3*, an efficient candidate gene allele mining approach in combination with transient complementation in the heterologous *Nicotiana benthamiana* 'model' system lead to the rapid cloning of the other three genes. All 4 *Rpi* genes code for proteins of the NBS-LRR class of R proteins. Interestingly, with respect to known functional R proteins, they share the highest similarity (34.9% aa) with RPP13 from *Arabidopsis thaliana* (Bittner-Eddy et al. 2000), which confers resistance to the oomycete *H. arabidopsidis.* Despite apparent differences between the four functional R proteins, all four induce a hypersensitive response upon co-expression with the *P. infestans* derived RxLR effector PiAVR2 (personal communication, E. Gilroy) in *N. benthamiana* leaves, indicating that they recognize the same effector protein.

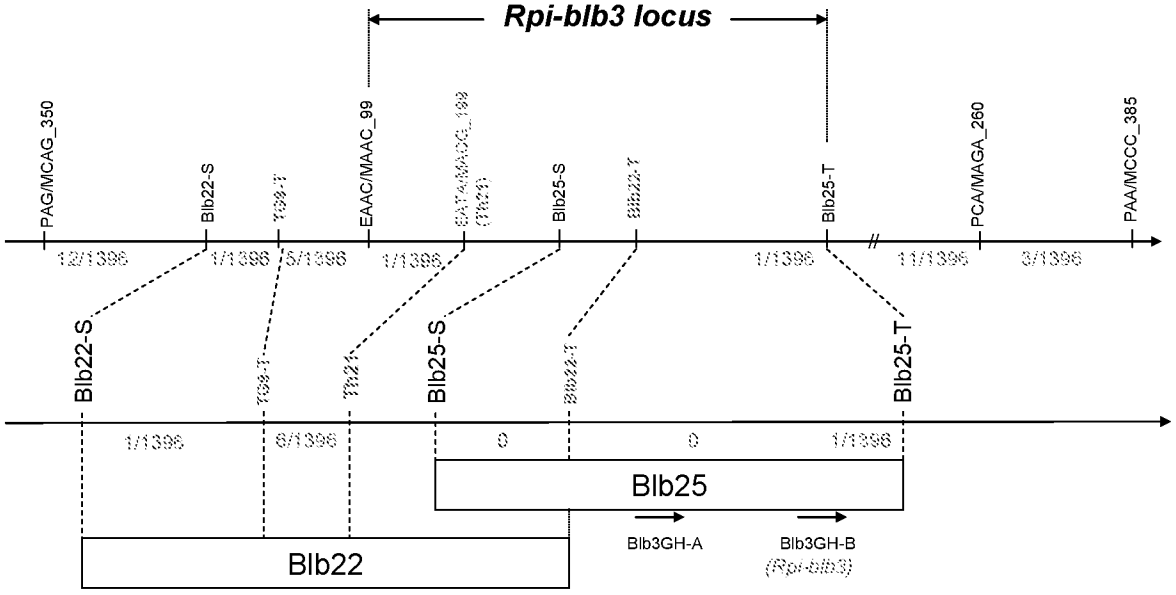
RESULTS

Cloning of Rpi-blb3 and Rpi-abpt

In order to clone *Rpi-blb3* and *Rpi-abpt*, two BAC libraries were constructed using DNA derived from the resistant clones Blb99-256-3 and 707TG11-1, respectively. Approximately 74000 clones with an average insert size of 85 kb, corresponding to 8 genome equivalents, were obtained for each library. These libraries were screened initially with SCAR marker Th21, which co-segregated with resistance in mapping populations of 1396 and 1383 F1 progeny plants, respectively (Park et al. 2005a). In this way BAC clones Blb22 and TG9 were identified, respectively (Fig. 1A and 1B). By sequencing the ends of these two BACs, new markers were developed which were used to define the genetic intervals of the R loci more precisely and to re-screen the BAC libraries to identify clones that overlapped with the initial BAC clones. In this way the *Rpi-blb3* locus was delimited to a 0.1 cM interval (EAAC/MAAC_99- Blb25-T; 2/1396 recombinants) that is physically spanned by the two partially overlapping BAC clones Blb22 and Blb25 (Fig. 1A). In the case of the *Rpi-abpt* locus, the partially

Aardevo Exhibit 1010
Page 0759

A.



B.

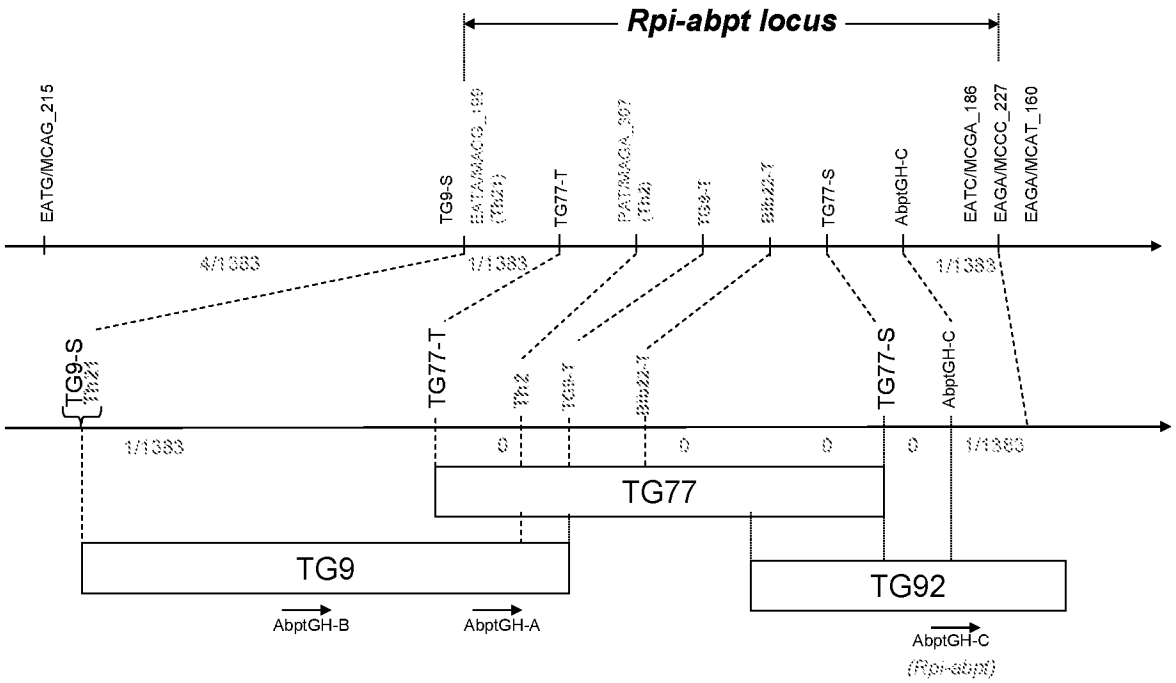


Figure 1: Genetic and physical map of the *Rpi-blb3* (A) and the *Rpi-abpt* (B) loci. Indicated are the relative positions of markers (in green are the one used in the mapping of both genes), the number of recombinants identified between markers, the overlapping BAC clones defining the two loci, and the relative positions of the *R* gene candidates that have been targeted for complementation analysis

Aardevo Exhibit 1010
Page 0760

overlapping BAC clones TG9 and TG77 were identified. One end of the contig co-segregated with resistance (TG77-S) whilst the other mapped 0.1 cM proximal to *Rpi-abpt* (TG9-S) (Fig. 1B).

Table 1. Overview of markers and primers used for mapping and cloning of *Rpi-blb3*

Name	Type	Polymerase chain reaction primer (5′ to 3′)	Tm[a]	Enzyme[b]
Th21	SCAR	F: ATTCAAAATTCTAGTTCCGCC		a.s.
		R: AACGGCAAAAAAGCACCAC		
Blb22-S	CAPS	F: GTTTGATGTATGTTTGTTCTTGC	56	Msp1
		R: TAATGCACTAATAACTAACTAGG		
Blb22-T	SCAR	F: CTTTATTAGTTCCAAGAGCTAC	56	
		R: ACCCATCCCTTTTTCCTTATC		
Blb25-S	CAPS	F: ACAGATGCTACGTCCATCAC	56	Alu1
		R: CTCCACATGCGATGCAAAAAG		
Blb25-T	CAPS	F: TTTCGATTATGGTGAGCCTTC	56	Hpy 188
		R: TAGAAAAAGGGTGGTTGTGAC		
RGH1	CAPS	F: GGSAAGACCACTCTTGCAAG	50	HpyCH4IV
		R: GGTTTTTAAGCTGCTAATGTTG		
RGH2	SCAR	F: GGSAAGACCACTCTTGCAAG	50	a.s.
		R: TGGTYATAATYACTCTGCTGC		
RGH3	CAPS	F: ATGRCTGATGCMTTTRTGTC	50	HaeIII
		R: CCYAAGTASAGAAAACACTGC		
4-PLOOP		F: GGiATGGGiGGiYTiGGiARGAC	68	
4-GLPL		R: TACiACAATiGCAAGiGGTAAMCC		
4-GLO2		F: GTGTCTCTCAAGAGTACAACAC	56	
		R: GCTCGAACATCAAGTAGTTTCC		
Blb3-start		F: ATGGCTGATGCCTTTCTRTCATTTG	55	
Blb3-end		R: TCAGGAATCTCCTTTAAATTTGGAC		
Blb3-prom		F: TCTTCCTTAGCATTCGTAGC	55	
		R: CTTTAGGAATACTAGTTTTGATTG		
Blb3-ter		F: AGCTTTTCTGCCAAGCACATTGG	55	
		R: GTACCCTCCGTTTGTCGTTTGATC		
Blb3-LRR-1-8		F: CTCTTTATGTATCAGACATGGC	55	
		R: CAACATCTTTCCACTGATCAC		
Blb3-prom -end		F: CCCCAAGTTGTATAATGGTTG	55	
Blb3-orf-bg		R: TGCTTGAGTGATTGAATCTCC		
Sto-orf-bg		R: GGCCATATTCAGACTGGGAG		
Blb3-spe		F: AGCTTTTTGAGTGTGTAATTGG	55	
		R: GTAACTACGGACTCGAGGG		

[a] Annealing temperature. In AF411807R, annealing temperature was 48°C for the first 7 cycles and 54°C for the last 30 cycles.

[b] Restriction enzymes that reveal polymorphism between resistant and susceptible linked alleles of the marker. a.s: allele-specific marker showing polymorphism without digestion

To gain insight into the molecular structure of the *R* loci under study, BAC clones Blb22 and TG9 were sequenced to 6x coverage. This revealed that clone Blb22 did not contain any *R* gene

Aardevo Exhibit 1010
Page 0761

homologues (RGH) whereas clone TG9 contained two RGH, which shared significant similarity to RGHs present in the sequenced tomato BAC clone AF411807L (Van der Hoeven et al. 2002) (Fig. 1). BAC clones Blb25 and TG77 were subsequently screened for the presence of RGH-specific sequences through PCR analysis using the primers 4-PLOOP-F and 4-GLPL-R (Table 1) which were designed by aligning the RGH sequences of clone AF411807L with those present on BAC clone TG9. Southern analysis of BAC clones Blb22, Blb25, TG9 and TG77 using an RGH-specific PCR fragment amplified from BAC clone Blb25 as a probe identified a minimum of two RGHs on BAC Blb25 and TG9 and one RGH on TG77 located on the overlapping part with TG9 (Fig. 1).Libraries harboring random overlapping binary subclones of 8-10 kb were therefore generated from BAC clones Blb25 and TG9. A total of 1152 clones per library were screened for the presence of RGHs using primers 4-GLO2-F and 4-GLO2-R (Table 1). Based on restriction analyses of the PCR fragments, RGH positive subclones were divided into separate classes: *Blb3GH-A* and *Blb3GH-B* for *Rpi-blb3*, *AbptGH-A* and *AbptGH-B* for *Rpi-abpt*. After determining the relative positions of the RGHs within the 8-13 kb subclones, candidates from each class were targeted for transformation to the susceptible potato cultivar Desiree. Transformation experiments carried out with subclones harboring candidates *Blb3GH-A*, *Blb3GH-B*, *AbptGH-A* and *AbptGH-B*, respectively, resulted in numerous primary transformants. Detached leaf assays using isolates IPO-0 and 90128 revealed that all of the plants transformed with subclones harboring candidates *Blb3GH-A*, *AbptGH-A* and *AbptGH-B* were susceptible to both isolates but that the majority (7/8) of the tested transgenic plants harboring *Blb3GH-B* reacted to both isolates with a hypersensitive response (HR) (Fig. 2). In view of the differential response between the primary transformants harboring *Blb3GH-A* and *Blb3GH-B*, we designated *Blb3GH-B* as the *Rpi-blb3* gene.

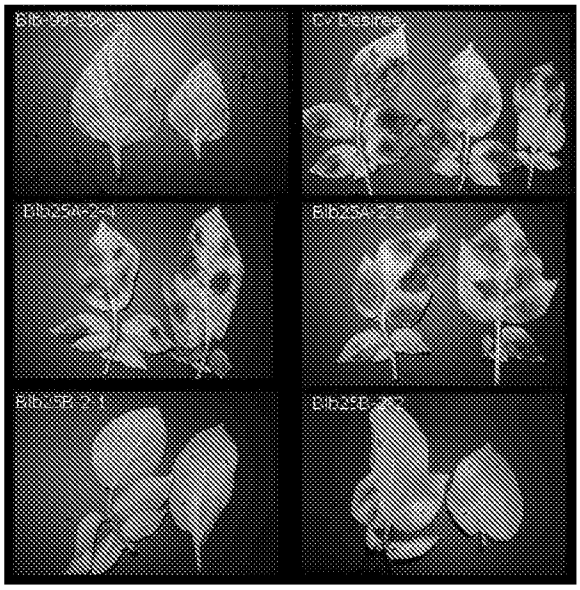


Figure 2: Genetic complementation for late blight resistance. Eight days after inoculation with *Phytophtora infestans* isolate 90128, large necrotic lesions and massive sporulation were noted for potato cultivar Desiree, and derived transformant Blb25A-2-4 and Blb25A-2-5, both expressing RGH-Blb25A. Typical resistance phenotype, i.e hypersensitive response (HR) was observed for Blb25B-2-1 and Blb25B-2-2 expressing RGH-Blb25B (*Rpi-blb3*)

Aardevo Exhibit 1010
Page 0762

In order to identify additional candidate genes for *Rpi-abpt*, the *Rpi-abpt*-specific BAC library was screened with TG77-S, leading to the identification of the TG77 overlapping BAC clone TG92 (Fig. 1B). Screening of this BAC clone with different sets of primers designed to amplify *AbptGH-A, AbptGH-B, Blb3GH-A* or *Rpi-blb3*, resulted in the identification of a third *Rpi-abpt* candidate gene, *AbptGH-C* (Fig. 1B). *AbptGH-C* LRR domain was amplified, cloned and sequenced using the primers Blb3-LRR-1-8 (Table 1). Sequence comparison of the three *abpt-GHs* enabled to design *AbptGH-C* specific marker (Fig. 1B) which co-segregated with resistance. Southern blot analysis using the AbptGH-C amplicon as a probe revealed that clone TG92 contained one resistance gene encompassing an LRR domain highly similar to the one of *AbptGH-C*. Primers designed on the start and stop codon of *Rpi-blb3* were subsequently used to amplify a full-length AbptGH-C amplicon from clone TG92, which was cloned into the Gateway® entry vector pDONR221. Using Multisite Gateway® technology, the *AbptGH-C* amplicon was subsequently cloned into the binary pKGW-MG destination vector between *Rpi-blb3* derived promoter and terminator sequences of 2723 nt and 883 nt, which were cloned into pDONR™ P4-P1R and pDONR™ P2R-P3 (Suppl. Fig. S1).

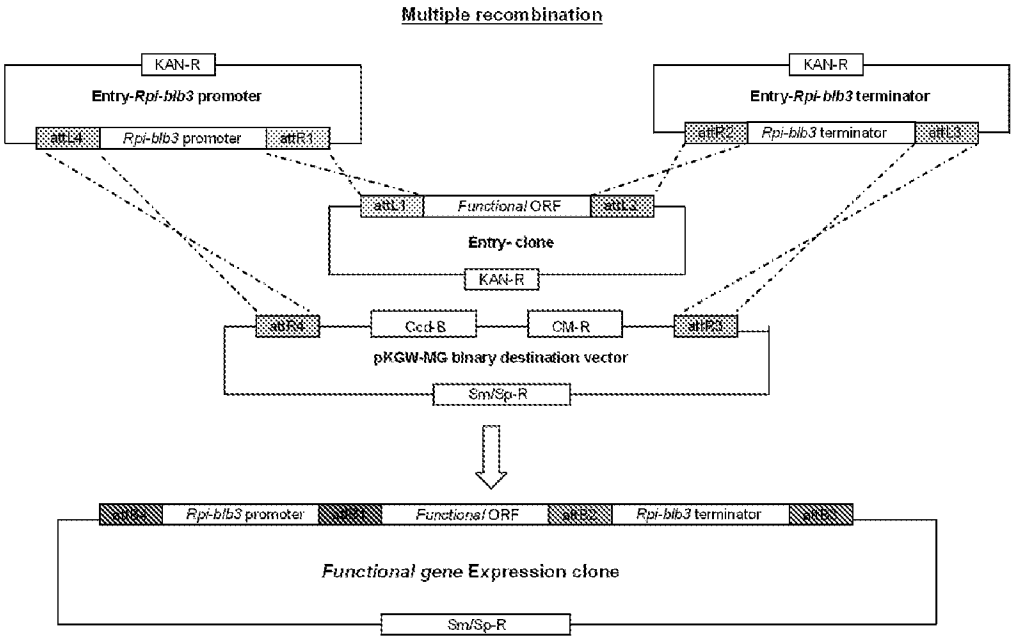


Figure S1: Gateway strategy used to clone *Rpi-abpt*, *R2* and *R2-like*. LR recombination of the three entry clones bearing the DNA fragments *Rpi-blb3* promoter, *Functional* ORF and *Rpi-blb3* terminator with the Multisite Gateway destination binary vector pKGW-MG, leading to the functional gene expression clone.

Complementation analysis was carried out in *N. benthamiana* using the *Agrobacterium tumefaciens* transient assay (ATTA) whereby leaves from 4-week-old wild type *N. benthamiana* plants were infiltrated with the *Agrobacterium* strain COR308 containing pKGW-AbptGH-C. The binary

Aardevo Exhibit 1010
Page 0763

clones pBP-Rpi-blb3 and pKGW-Rpi-blb3, comprising the genomic *Rpi-blb3* gene and a Multisite Gateway® reconstituted *Rpi-blb3* gene construct, respectively, were used as positive controls, and pBP-AbptGH-A as a negative control. After two days, infiltrated leaves were inoculated with *P. infestans* strain PY23, IPO-0 and IPO-C in detached leaf assays. Leaves infiltrated with pKGW-AbptGH-C, pBP-Rpi-blb3 and pKGW-Rpi-blb3 developed HRs at the inoculation sites whereas non infiltrated leaves and those infiltrated with pBP-AbptGH-A were susceptible to isolate PY23 and IPO-0. As expected, all leaves inoculated with IPO-C were susceptible (Fig. 3 and Table 2). In view of these results, *AbptGH-C* was designated *Rpi-abpt*.

Allele mining for R2 and R2-like

Rpi-blb3 and *Rpi-abpt* belong to the major late blight resistance locus on LG IV that also harbors *R2* and *R2-like* (Li et al. 1998; Park et al. 2005c). In view of the conserved marker order and observed allelic conservation between the genetic maps of *Rpi-blb3, Rpi-abpt*, *R2*, and *R2-like* (Park et al. 2005c), and the high sequence conservation between *Rpi-blb3* and *Rpi-abpt*, we hypothesised that *R2* and *R2-like* were orthologues of *Rpi-blb3* and thus attempted to clone them through an allele mining strategy. The same primers used to amplify the *Rpi-abpt* candidate gene from BAC clone TG92 were used to amplify full-length Blb3GH from the resistant parental genotypes CEBECO44158-4 (MaR2) and AM3778-16, harboring *R2* and *R2-like*, respectively. Amplicons of the expected size were cloned into pDONR221 and fully sequenced. In total, eight unique sequences were obtained from AM3778-16 (R2-likeGH) and nineteen from CEBECO44158-4 (R2GH), with amino acid identities between Rpi-blb3 and the novel Blb3GH ranging from 86.4% to 97.3% for R2-likeGH and 83.8% to 94.2% for R2GH (Supplementary Table S1 and S2). Phylogenetic analysis of all the available amino acid sequences clustered one R2-like-GH and five R2GH in a clade together with the functional proteins Rpi-blb3 and Rpi-abpt (Fig. 4). The amino acid sequence of R2-likeGH-8 shares 97.3 % amino acid identity with Rpi-blb3. R2GH-2, R2GH-8, R2GH-G3, R2GH-D3 and R2GH-65 share 94.2, 91, 92.6, 89.7, and 92.8% amino acid identity with Rpi-blb3, respectively. This set of candidate genes was targeted for functional analysis and therefore cloned into the binary vector pKGW-MG between the *Rpi-blb3* promoter and terminator sequences, as described above for the *Rpi-abpt* gene.

Transient complementation assays in *N. benthamiana* indicated that R2GH-G3 and R2-likeGH-8 conferred resistance to Pi isolate IPO-0, whereas R2GH-2, R2GH-8, R2GH-D3 and R2GH-65 were non-functional (Fig. 3). R2GH-G3 and R2-likeGH-8 were therefore designated *R2* and *R2-like*, respective.

Aardevo Exhibit 1010
Page 0764

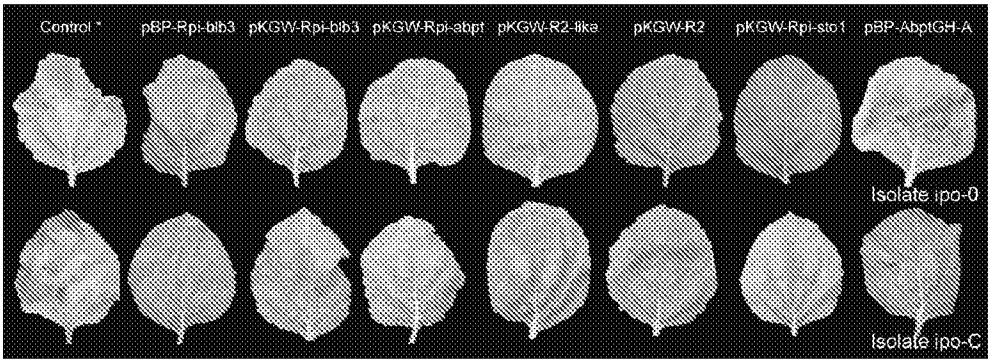


Figure 3. Complementation of *Rpi-blb3, Rpi-abpt , R2, R2-like, Rpi-sto1* by transient expression in *Nicotiana benthamiana*, followed by inoculation by *Phytophtora infestans* isolate Ipo0 (avirulent) and Ipo-C (virulent on all except *Rpi-sto1*). The prefix pBP stand for pBINplus, and pKGW stand for the gateway destination vector containing *Rpi-blb3* regulatory elements. In leaves expressing pB-Rpi-blb3, pKGW-Rpi-blb3, pKGW-Rpi-abpt, pKGW-R2-like, pKGW-R2, a HR was observed at seven days post inoculation with Pi isolate Ipo-0, whereas large necrotic lesions and massive sporulation were evident for Pi isolate Ipo-C. Wild type *N. benthamiana* (Control *) and the non functional AbptGH-A (pBP-AbptGH-A) were included as susceptible controls, and Rpi-sto1 (pKGW-Rpi-sto1) was included as a resistant control **.**

Table 2 Overview of resistance screening with a set of 16 *P. infestans* isolates[a].

spp	Genotype	Gene	Chr.	py 23	IPO-0	90128	H30P04	VK98014	IPO428-2	Dintel	Katshaar	F95573	EC1	89148-09	99183	99189[b]	IPO-C	USA618	99177
BLB	Blb99-256-3	*Rpi-blb3*	4	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S
BLB	707TG11-1	*Rpi-abpt*	4	R	R	R	R	R	R	R	R	R	R	R	R	**R**	S	S	S
BLB	AM3778-16	*R2-like*	4	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S
DMS	CEBECO44158-4	*R2*	4	R	R	R	R	Rq	R	Rq	R	R	R	R	R	S	S	S	S
	cv. Desiree	-	-	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	cv. Bintje	-	-	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

[a] Differential specificity are in bold. Chr.=chromosome, S= susceptible; R= resistant and Rq= quantitatively resistant.

[b] Isolate differentially recognized

All four R genes interact with the RXLR-EER effector PiAVR2

Using a map-based cloning approach, markers flanking the *PiAVR2* locus (van der Lee et al., 1997) were used to delimit a physical region of 742,635 bp of the *P. infestans* genome sequence spanning the genetic interval containing *PiAVR2*. Within this region, two candidate *PiAVR2* genes on supercontig 16 of the *P. infestans* genome (16_7987 and 16_8044), each encoding a secreted RXLR-EER effector, were identified. The cloning and analyses of these gene candidates will be reported in detail in a separate publication (personal communication, E. Gilroy).

To functionally analyze the two RxLR-EER candidate genes identified at the *PiAVR2* locus in this study, *N. benthamiana* leaves were co-infiltrated with *A. tumefaciens* strains carrying a binary construct expressing one of the four *Rpi* genes under study together with strains carrying a binary construct expressing each of the two candidate RxLR-EER genes. Co-infiltration of *Rpi-blb3, Rpi-abpt, R2* and *R2-like* with *16_7987* resulted in a hypersensitive cell death response (Fig. 5), whereas co-expression of these *Rpi* genes with the *16_8044* candidate or *PiAVR3a*, failed to elicit visible cell death (Fig. 5; personal communication, E. Gilroy). Based on these results *16_7987* was designated *PiAVR2*, and clearly all four *Rpi* genes under study interact with *PiAVR2*.

Gene structure and functionality

Rpi-blb3, Rpi-abpt, *R2* and *R2-like* are intronless genes which encode ORFs of 2538-2544 nucleotides (nt) that code for proteins of 845-847 amino acids harboring all the signature sequences characteristic of LZ-NBS-LRR R-proteins (Fig. 6). Interestingly, with respect to known functional R proteins, Rpi-blb3, Rpi-abpt, R2, and R2-like share the highest similarity (34.9% aa) with RPP13 from *Arabidopsis thaliana* (Bittner-Eddy et al. 2000), which confers resistance to *H. arabidopsidis* isolates containing *ATR13*. The highest similarity with RPP13 resides in the NBS domain with 49.3% sequence identity, and the lowest within the LRR domain (34.3%). The LRR domains of Rpi-blb3, Rpi-abpt, R2, and R2-like are highly homologous and comprise 14 imperfect repeats (Fig. 6). The LZ and NBS domains are more polymorphic, those of R2 being the most divergent. Rpi-abpt and R2-like are identical except for the sequence between LRR2 and LRR3, where Rpi-abpt contains a stretch of amino acids that is identical to that of R2. The LRR domain of R2 is identical to that of Rpi-abpt (Fig. 6).

Aardevo Exhibit 1010
Page 0766

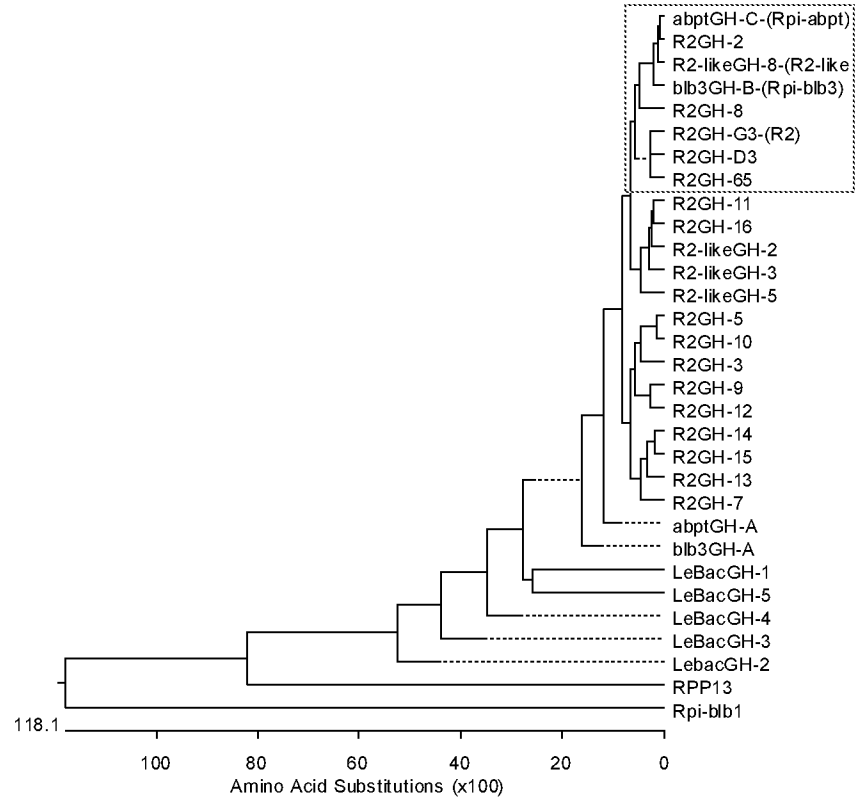


Figure 4: Phylogenetic tree at the amino acid level of Rpi-blb3, RGHs amplified from late blight resistant potato clones harboring *Rpi-abpt*, *R2-like or R2*, RGHs present on the tomato Bac clone AF411807, *RPP13-Nd* and *Rpi-blb1*. Boxed is the group containing Rpi-blb3, Rpi-abpt, R2 and R2-like.

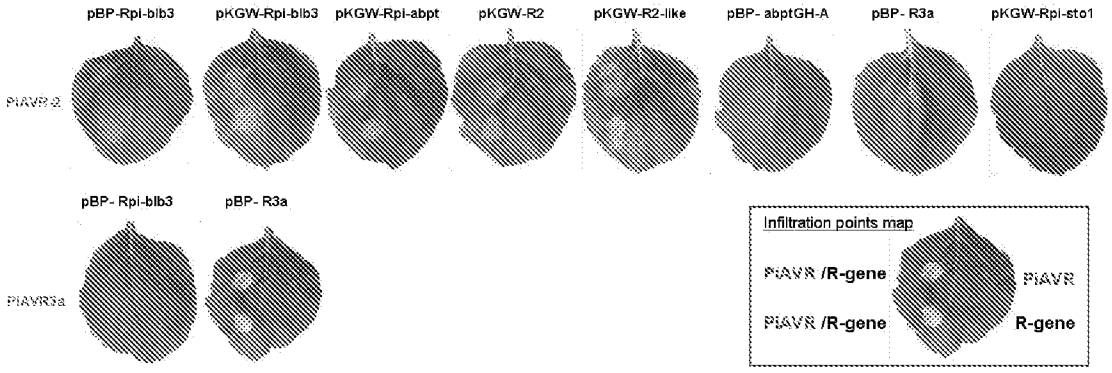
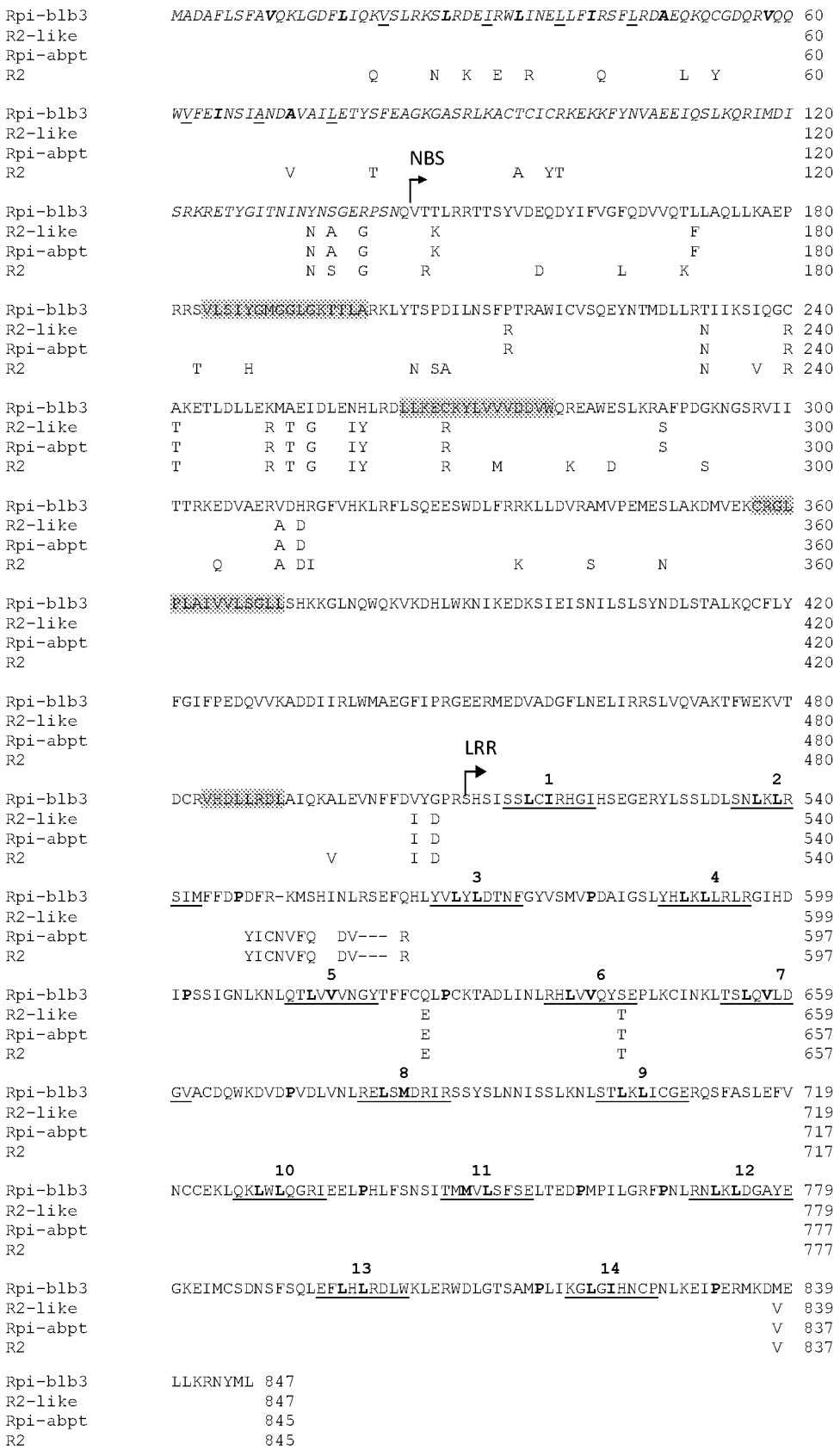


Figure 5. Agrobacterium-mediated co-expression of *PiAvr2* or *PiAvr3a* together with *Rpi-blb3, Rpi-abpt , R2, R2-like, Rpi-sto1* , the non functional *abptGH-A* or *R3a*. The different infiltration points are indicated on the map. On each leaf, four infiltration spots were performed, duplicated co-infiltration of the *Avr* and the *R* gene on the left , the *Avr* on the top right and the *R* gene on the bottom right. *Rpi-blb3, Rpi-abpt , R2 and R2-like* in combination with *PiAvr2* as well as *R3a* and *PiAvr3a* induced the hypersensitive response (HR). Pictures were taken 6 days post-infiltration.

Aardevo Exhibit 1010
Page 0767



Aardevo Exhibit 1010
Page 0768

Figure 6: Alignment of the deduced protein product encoded by the functional genes *Rpi-blb3*, *Rpi-abpt*, *R2-like*, and *R2*. The full amino acid sequence of *Rpi-blb3* and in comparison, differing amino acids of *Rpi-abpt, R2-like*, and *R2* are shown. In italics is the leucine zipper domain with the first and the fifth hydrophobic residues from the heptads in bold and underlined respectively. Positions of the nucleotide binding (NB)-ARC and the leucine reach repeat (LRR) are indicated by arrows. The NB-ARC domain has its characteristic conserved domains highlighted in grey (kinase 1a, kinase 2 and kinase 3). The LRR domain domain is composed of 14 repeats with the motif xx**L**x**L**xxx .

Alignment of the nucleotide sequences of the four functional genes and those of four additional *Blb3GHs* and subsequent analysis of informative polymorphic sites (IPS), i.e. sites where two or more genes carry the same nucleotide (Parniske et al. 1997), reveals clear blocks of sequence affiliation between different members of the gene family (Fig. S2), indicating that sequence exchange events between *Blb3GHs* have been involved in the evolution of the gene family (Fig. S2). Interestingly, the observed sequence affiliations in the 5'-terminal half of the genes extend throughout the LZ and NBS domains whereas sequence affiliations in the LRR domain suggest exchange of specific combinations of LRRs, underlining the modular nature of R proteins.

In an attempt to assess the biological relevance of the observed sequence exchange events in relation to resistance spectrum, detached leaves of the parental clones harboring *Rpi-blb3* (Blb99-256-3), *Rpi-abpt* (707TG11), *R2* (CEBECO44158-4(MaR2)) and *R2-like* (AM3778-16) were inoculated with 16 different *P. infestans* isolates (Table 2). For 15 isolates all four clones displayed the same specificity. However, one isolate, PIC99189, displayed a differential interaction; Blb99-256-3, AM3778-16 and CEBECO44158-4(MaR2) were susceptible to this isolate whereas 707TG11-1 was resistant (Table 2). Previously it was reported that 707TG11-1 contained tuber specific *R1-like* (Park et al. 2005) and foliage specific *R3-haplotype* (Huang . 2005). To dissect the resistance specificity of 707TG11-1 we inoculated 48 F1 progeny plants of the *Rpi-abpt* mapping population RH4x103 with isolates 90128 and PIC99189. For both isolates a clear 1:1 segregation of resistance was observed but there was no correlation between resistance to both isolates, confirming that the resistance to isolate PIC99189 is indeed the result of a second gene other than *Rpi-abpt*, and not located on LG IV.

In order to confirm the functionality of *Rpi-abpt*, *R2-like*, and *R2*, transgenic Desiree were generated. Detached leaves of 6, 5, and 2 independent transgenic lines expressing *R2-like, Rpi-abpt*, and *R2,* respectively, were inoculated with *P. infestans* isolates IPO-0 and IPO-C. All transformants reacted with a hypersensitive response (HR)to isolate IPO-0 and were susceptible to isolate IPO-C (Fig S3). This result confirms that *Rpi-abpt*, *R2-like*, and *R2* are functional *R* genes with expected specificity to *P. infestans* isolates. Moreover, the use of the *N. benthamiana* transient complementation assay as an efficient tool for rapid functional screen of putative R-genes is verified."

Aardevo Exhibit 1010
Page 0769

Supplementary Table S2: Amino acid sequence similarity between the LZ, NBS or LRR domains of the different homologues depicted in the phylogenetic tree (Fig. 4) (Rpi-abpt, R2-like, R2, blb3GH-A, abptGH-A, 3 R2likeGHs, 15 R2GHs, 5 LeBacGHs, RPP13, Rpi-blb1) and Rpi-blb3. Highlighted in grey are the genes depicted in the crustal W alignment (Fig. 6).

blb3GH-B -(Rpi-blb3)	blb3GH-B-(Rpi-blb3)	abptGH-C -(Rpi-abpt)	R2-likeGH-1 -(R2-like)	R2GH-G3 -(R2)	blb3GH-A	abptGH-A	R2-likeGH-2	R2-likeGH-3	R2-likeGH-5	R2GH-2	R2GH-3	R2GH-5	R2GH-7	R2GH-8	R2GH-9	R2GH-10
LZ-NBS-LRR	100	95.9	97.3	92.6	81.7	86.3	86.4	89.9	91.4	94.2	84.7	87.9	88.2	91	86.6	86.8
LZ	100	98	98	90.1	87.5	90.8	84.2	84.2	84.2	98	90.1	90.1	97.4	89.5	90.1	89.5
NBS	100	95.5	95.5	91.2	91.8	93.6	89.9	89.7	91.8	94.7	89.4	93.4	92.6	91	89.7	93.4
LRR	100	95.9	99	95.9	81.9	80.4	85.3	92.8	94	93	81.4	83.8	82.6	93.7	84.5	81.9

blb3GH-B-(Rpi-blb3)	blb3GH-B-(Rpi-blb3)	R2GH-11	R2GH-12	R2GH-13	R2GH-14	R2GH-15	R2GH-16	R2GH-D3	R2GH-65	LeBacGH-1	LebacGH-2	LeBacGH-3	LeBacGH-4	LeBacGH-5	RPP13	Rpi-blb1
LZ-NBS-LRR	100	87	85	86.9	84	83.8	86.7	89.7	92.8	75.6	55.7	70.9	78.3	47.6	34.9	24.4
LZ	100	90.8	90.1	98	88.8	90.8	85.5	89.5	90.1	80.9	79.6	76.3	78.3	50	34.9	25
NBS	100	89.4	89.1	88.9	89.1	88.1	89.4	90.2	92.3	80.6	59.7	81.7	86.7	54.9	49.3	31.8
LRR	100	85.7	81.9	83.1	80.4	80.4	85.7	90.8	95.4	73.1	58.6	64.6	74.6	63	34.3	14.5

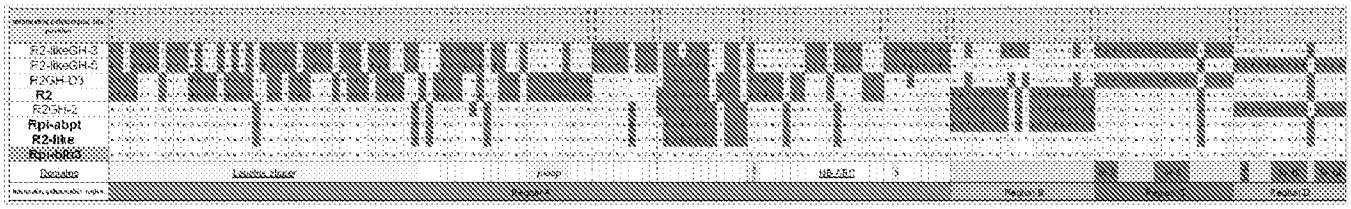


Figure S2: Nucleotide polymorphism analysis of *Rpi-blb3* and 7 homologues sharing informative polymorphic or homologous sequences. In bold are the functional genes *R2*, *Rpi-abpt*, *R2-like*, *Rpi-blb3*. Only informative polymorphic sites are shown, which reduces the sequence size from 2444 bp to 170 bp. Highlighted in red are the positions polymorphic to *Rpi-blb3* sequence. The vertical number at the top of each row indicates the corresponding nucleotide position in the full consensus sequence, the limits of the recombination regions positioned on the bottom of the figure are indicated in bold. The positions of the different domains characterizing LZ-NBS-LRR genes and the informative polymorphic regions A to D are indicated down to the sequences.

Aardevo Exhibit 1010
Page 0770

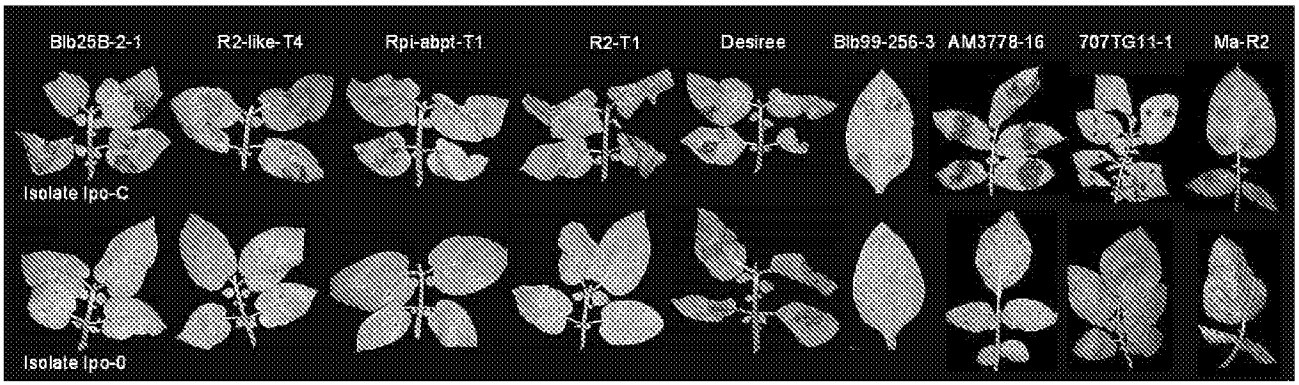


Figure S3: Genetic complementation for late blight resistance. Six days after inoculation with *Phytophtora infestans* isolate IPO-0, the hypersensitive response (HR) was observed for the positive control Blb25B-2-1 carrying *Rpi-blb3*, the transformants expressing *R2*-like (R2-like-T4), *Rpi-abpt* (Rpi-abpt-T1), *R2* (R2-T1) and the parental clones harboring *Rpi-blb3* (Blb99-256-3), *R2-like* (AM3778-16), *Rpi-abpt* (707TG11) and *R2* (MaR2); large necrotic lesions and massive sporulation were noted for potato cultivar Desiree. All the tested leaves were susceptible to the virulent isolate IPO-C.

DISCUSSION

In this study we describe the cloning and functional characterization of four homologous *R* genes from the major late blight resistance locus on LG IV of potato. Previously, marker order and allelic conservation suggested that *Rpi-blb3, Rpi-abpt, R2* and *R2-like* reside at the same locus on LG IV (Park et al. 2005a, 2005b, 2005c). *Rpi-blb3* and *Rpi-abpt* were cloned using a map-based cloning approach whereby physical maps of both loci were generated by screening appropriate BAC-libraries with a highly linked molecular marker, followed by subsequent rescreening of the libraries with novel BAC-end markers until the outer ends of the BAC-contigs exceeded the genetic boundaries of the *R* loci. In the case of *Rpi-blb3,* two candidate NBS-LRR genes were identified and subcloned into a binary vector and targeted for complementation analysis through stable transformation of a susceptible potato variety.

For *Rpi-abpt,* a NBS-LRR candidate was cloned from the BAC using a homology-based PCR cloning strategy using primers designed on the functional *Rpi-blb3* gene. In addition complementation was carried out using an *Agrobacterium*-mediated transient expression assay in *N. benthamiana* followed by detached leaf assays with appropriate *P. infestans* isolates (Rietman et al. in preparation).

Development of the transient complementation assay together with the successful *in planta* expression of *Rpi-abpt* and other *R* genes (Rietman et al. in preparation) using the DNA regulatory elements of *Rpi-blb3*, facilitated for the rapid cloning of *R2* and *R2-like* through an efficient functional allele mining strategy. A prerequisite for such an allele mining strategy is prior knowledge of candidate gene sequences at the locus of interest. Observations within the *Solanaceae* indicate that *R* gene clusters are conserved across species, thus enabling comparative genomics (Grube et al. 2000; Huang. 2005; van der Vossen et al. 2005). As more and more *R* genes are mapped and cloned, the greater the chance that novel *R* genes reside at known and well-characterized loci. Moreover, ongoing potato and tomato genome sequencing projects by international consortia promise to provide a complete survey of the distribution of *R* gene clusters in the *Solanaceae,* enabling even faster cloning of *R* genes through allele mining strategies.

Interestingly, with respect to known functional R proteins, Rpi-blb3, Rpi-abpt, R2, and R2-like share the highest similarity (34.9% aa) with RPP13 from *A. thaliana* (Bittner-Eddy et al. 2000), which confers resistance to *H. arabidopsidis*. Mining of *RPP13* alleles in a diverse set of *A. thaliana* accessions revealed both extreme sequence diversity and functional diversity in pathogen recognition (Rose et al. 2004). In line with this observation a significant excess of amino acid polymorphism was

Aardevo Exhibit 1010
Page 0772

localized within the LRR domain of *RPP13*. In the case of *Rpi-blb3, Rpi-abpt, R2,* and *R2-like*, the LRR domains are nearly identical despite significant polymorphism at the CC and NBS domains, suggesting conservation of function, and indeed we have shown that all four proteins interact with the same effector protein, PiAVR2, which was recently cloned through a map-based cloning approach (personal communication, E. Gilroy). Although the origin of *Rpi-abpt* and *R2-like* remain unclear, this finding is a second example of how *R* gene recognition specificities can be conserved across *Solanum* species originated from different potatoe superseries, in this case *S. bulbocastanum* (*Rpi-blb3*) belongs to the *Potatoe Stellata Bulbocastana* and *S. demissium* (*R2*) to the *Potatoe Rotata demissa*. Recently we demonstrated both gene conservation and specificity conservation between the *S. bulbocastanum*-derived gene *Rpi-blb1* and the *S. stoloniferum* and *S. papita*-derived orthologues *Rpi-sto1* and *Rpi-pta1,* respectively (Vleeshouwers et al. 2008). Despite apparent positive selection in the LRR domain, the LZ and NBS domains are more polymorphic, those of R2 being the most divergent. Clear blocks of sequence affiliation between the four functional R proteins and additional *Rpi-blb3* gene homologs, suggest exchange of LZ, NBS and LRR domains, and also of specific LRRs, underlining the modular nature of these proteins. *R2* has a particular gene structure likely to be the product of a relatively recent recombination event. The LZ-NBS domain (nucleotides 1-1149) is highly affiliated to that of the non-functional *R2-GH-D3*, whereas the LRR domain (nucleotides 1059-2544) is highly affiliated to that of *Rpi-abpt* and the non-functional *R2-GH-65*, revealing a sequence exchange track between nucleotides 1059 and 1149. Sequence exchange between RGHs has been proposed as a major mechanism shaping *R* gene diversity in plants (Michelmore and Meyers 1998; Meyers et al. 1999; Kuang et al. 2004). Interestingly, the approximate position of this recombination site corresponds to the one described by Rose et al. (2004) for the *RPP13* allele *ksk*-2, a region between the conserved GLPL and VHD motifs of the NB-ARC domain. This is in line with the expectation that in multi-domain proteins, sequence exchange tracks will be concentrated at specific sites that separate domains with independent functions.

Preliminary results from ongoing *Rpi-blb3* allele mining studies in *Solanum* germplasm indicate that *Rpi-blb3* is present in several other species, e.g. *S. brachistotrichum* and *S. pinnatisectum*. In addition several *Rpi* genes recently identified in our laboratory (data not shown) also map to the *Rpi-blb3* interval. The QTLs for late blight resistance (Pi-QTL) (Sandbrink et al 2000) explaining 40% of the phenotypic variation, was mapped to the *Rpi-blb3* locus, and renamed *Rpi-mcd1* (Tan et al. 2008). Similarly, high resolution mapping data of the major Pi-QTL identified in cv. Stirling strongly suggest that this Pi-QTL is also an *Rpi-blb3* homologue (Hein et al. 2007; Ingo Hein, personal communication). And various other examples of Pi-QTLs on the short arm of LG IV have been described (Leonards-Schippers et al. 1994; Sandbrink et al. 2000; Bradshaw et al. 2004; Bradshaw et al. 2006).

Aardevo Exhibit 1010
Page 0773

Rpi-blb3, Rpi-abpt, R2 and R2-like specifically detect PiAVR2. Do the Rpi-blb3 homologues putatively underlying the Pi-QTL described above also recognize PiAVR2 homologues? If so do they weekly recognize an allele, which is conserved throughout *P. infestans* or do they recognize multiple alleles, thus resulting in non-race-specific resistance?

Current models for NB-LRR R proteins suggest a dual role for the LRR domain, not only as recognition specificity determinant but also as a repressor of inappropriate NB activation (Belkhadir et al. 2004). Furthermore, evolutionary analyses of R proteins and their structure has shown selection pressure on the LRR region but also on other domains within R proteins, which, in light of the dual function, may point towards co evolution in R proteins to establish and maintain recognition of effectors while maintaining the inhibitory function (McDowell and Simon. 2006). Our findings are slightly different, contradictory, indicating conservation in the LRR domain but more variation in the NBS domain, that may point towards different signaling pathways or additional effectors being recognized by these R proteins.

MATERIALS AND METHODS

Plant material and *Phytophthora infestans isolates*

In this study we used the four resistant parental clones Blb99-256-3 harbouring *Rpi-blb3* (Park et al., 2005a), 707TG11-1 *Rpi-abpt* (Park et al., 2005c), CEBECO44158-4 (MaR2) *R2* and AM3778-16 *R2-like*, respectively (Park et al.2005b). F1 progeny plants of population RH4x103 were derived from a cross between the late blight resistant clone 707TG11-1 and the diploid potato clone RH89-039-16 (Park et al., 2005c).The susceptible potato cultivar Desiree was used for *Agrobacterium*-mediated transformation. The characteristics and origin of *P. infestans* isolates used in this study are indicated in supplementary Table S3.

Aardevo Exhibit 1010
Page 0774

Supplementary Table S3 Characteristics of *P. infestans* isolates used in this study. Resistance genes which didn't have a clear phenotype from one repeat to the other are in brackets in the virulence profile.

Isolate	Year	Geographic origin	Mating type	Obtained from	Virulence profile
90128	1990	Geldrop, Netherlands	A2	Govers, Phytopathology WUR	1.3.4.7.8.10.11
H30P04	unknown	Artificial hybrid	unknown	Govers, Phytopathology WUR	3a, 7, 10
USA618	n.d	Toluca Valley, Mexico	A2	Fry, Cornell, USA	1.2.3.6.7.10.11
89148-09	1989	Netherlands	A1	Govers, Phytopathology WUR	0
IPO-0	n.d	Netherlands	n.d	Kessel, PRI, WUR	0
IPO-C	1982	Belgium	A2	Kessel, PRI, WUR	1.2.3.4.5.6.7.10.11
VK98014	1998	Veenkolonién, Netherlands	A1	Kessel, PRI, WUR	1.(2).4.11
Dinteloord	n.d	Dinteloord, Netherlands	n.d	Kessel, PRI, WUR	1.(2).4
EC1	n.d	Ecuador	n.d	Birch, SCRI, Scotland	1.3.4.7.10.11
F95573	1995	Flevoland, Netherlands	A1	Govers, Phytopathology, WUR	1.3.4.7.10.11
IPO-428-2	1992	Ede, Netherlands	A2	Kessel, PRI, WUR	1.3.4.7.8.10.11
Katshaar	n.d	Katshaar, Netherlands	n.d	Kessel, PRI, WUR	1.3.4.7.10.11
PIC 99177	1999	Metepec, Mexico	A2	Kessel, PRI, WUR)	1.2.3.4.7.11
PIC 99183	1999	Metepec, Mexico	A2	Kessel, PRI, WUR	1.3.4.5.7.8.10.11
PIC 99189	1999	Metepec, Mexico	A2	Kessel, PRI, WUR	1.2.5.7.10.11
88069	1988	Netherlands	A1	Govers, Phytopathology, WUR	1.3.4.7
PY23*	1999	GGO (modified 88069)	A1	Govers, Phytopathology, WUR	1.3.4.7

* References: Kamoun et al. 1998; West et al. 199

BAC library construction

Clones Blb99-256-3 and 707TG11-1 were used as DNA sources for the construction of BAC libraries. High-molecular weight DNA preparation and BAC library construction were carried out as described previously (Rouppe van der Voort et al. 1999). Approximately 74000 clones with an average insert size of 85 kb, corresponding to 8 genome equivalents, were obtained per library. The BAC clones were stored as bacterial pools containing approximately 700 to 1000 white colonies. These were generated by scraping the colonies from the agar plates into LB medium containing 18% glycerol and 12.5 µg ml^{-1} chloramphenicol using a sterile glass spreader. These so-called super pools were stored at -80ºC. Marker screening of the BAC libraries was done, first by isolating plasmid DNA from each pool using the standard alkaline lysis protocol and PCR was carried out to identify positive pools. Bacteria corresponding to positive pools were diluted and plated on LB agar plate containing chloramphenicol (12.5 µg ml^{-1}). Individual white colonies were picked into 384-well microtitre plates and single positive BAC clones were subsequently identified by marker screening as described by Rouppe van der Voort (1999). Names of BAC clones isolated from the super pools carry the prefix Blb (e.g. Blb25) or TG (e.g. TG9).

Aardevo Exhibit 1010
Page 0775

Subcloning of candidate genes

Candidate RGAs were subcloned from BAC clone BLB25 and TG9 as follows. Aliquots of approximately 1 µg BAC DNA were digested with 0.03U of *Sau3A*l restriction enzyme for 10 min. The partially digested BAC DNA was subjected to electrophoresis at room temperature in 0.5X TAE using a linear increasing pulse time of 1-10 sec and a field strength of 90 V cm^{-1} for 6 h. After electrophoresis, the agarose gel was stained with ethidium bromide to locate the region of the gel containing DNA fragments of approximately 10 Kbp in size. This region was excised from the gel and treated with GELASE (Epicentre Technologies, Madison, WI, USA) according to the manufacturer's instructions. The size-selected DNA was ligated to the *BamH*I-digested and dephosphorylated binary vector pBINPLUS (van Engelen et al. 1995) followed by transformation to ElectroMAX E.coli DH10B competent cells (Life technologies, Paisley, UK).

DNA sequencing and computer analysis

BAC clone sequencing was carried out using a shotgun cloning strategy. Sequencing reactions were performed using a dye terminator cycle sequencing reaction kit (Perkin-Elmer, Pt Biosystem, Warrington, UK), M13 universal forward and reverse primers, and an ABI377 automated sequencer (Applied Biosystem, La Jolla, CA, USA). Sequence contigs were assembled using the STADEN sequence and analysis program (Dear and Staden 1991).

The binary constructs were sequenced using a primer walking strategy (700 bp by 700 bp). Gene structures were predicted using FGENESH++ (Softberry) and protein sequences were deduced by translation of ORF using the standard genetic code. Multiple sequence alignments were conducted using CLUSTALX 1.81 (Thompson et al. 1997). The search for genes homologous to *Rpi-blb3* was carried out using the Basic Local Alignment Search Tool (BLAST). Conserved domains were identified using Swiss-Prot (InterProScan, EMBL-EBI, ExPASy, SAPS).

Resistance assay

Detached leaf assays were used to determine the resistance phenotypes of primary transformants of potato and transiently transformed *N. benthamiana* leaves. For complementation analyses, primary transformants were tested with isolates IPO-0 and 90128 (Fig. 2 and Table S3). Inoculum preparations and inoculations were performed as described by Vleeshouwers et al. (1999). Six days after inoculation, plant phenotypes were determined. Leaves showing no symptoms or a localized necrosis at

Aardevo Exhibit 1010
Page 0776

the point of inoculation were scored as resistant and those with clear sporulating lesions (visible by naked eye) as susceptible.

Transient complementation in N. benthamiana

Infiltrations of *Agrobacterium* strains carrying the candidate RGH were performed in *N. benthamiana.* Recombinant COR308 *A. tumefaciens* cultures were grown in LB medium (10 gram bacteriological peptone, 10 gram NaCl and 5 gram yeast extract in 1 liter milli-Q water) supplemented with 5 mg/L tetracycline and 50 mg/L kanamycin. After one or two days a calculated amount of culture (according to OD 0.5 at 600 nm) was transferred to YEB medium (5 gram beef extract, 5 gram bacteriological peptone, 5 gram sucrose, 1 gram yeast extract, 2ml 1 M MgSO4 in 1 liter MQ water) supplemented with kanamycin for all strains. After 1 day overnight cells were centrifuged at 3500 rpm and re-suspended in MMA medium (20 gram sucrose, 5 gram MS salts and 1.95 gram MES) supplemented with 1 ml 200 mM acetosyringone to a final OD of 0.2 and infiltrated into leaves of 4 weeks old plants with a 3ml syringe. After two days, infiltrated leaves were detached and inoculated with *P. infestans* strains IPO-C (Table S3) and PY23 in detached leaf assays. Hypersensitive response (HR) or *P. infestans* sporulation was observed between 4 to 8 days post inoculation.

Agrobacterium-mediated coexpression in N. benthamiana

pBP-Rpiblb3, *pKGW-Rpi-blb3*, *pKGW-Rpi-abpt*, *pKGW-R2*, *pKGW-R2-like*, *pKGW-Rpi-sto1*, *pBP-abptGH-A* and *pBP-R3a* were transferred to the *Agrobacterium tumefaciens* strain AGL1 (Lazo et al. 1991) containing the helper plasmid pVirG and pSoup (van der Fits et al. 2000). Recombinant *A. tumefaciens* cultures were grown in LB medium supplemented with 5 mg/L tetracycline , 100 mg/L carbeniciline, 75 mg/L choramphenicol and 50 mg/L Kanamycin. The avirulence effectors were cloned into the pGRAB vector (kindly supplied by Sean Chapman, SCRI. Scotland. UK) and transferred to the same *A. tumefaciens* strain AGL1. Recombinant *A. tumefaciens* cultures were grown in LB medium supplemented with 30 mg/L choramphenicol and 50 mg/L kanamycin. The same protocol as the transient complementation was used for the sub-cultures. *PiAVR Agrobacterium* culture and *Rpi-gene Agrobacterium* culture were mixed at a final OD6oo of 0.1 for each and infiltrated into leaves from 5 to 7 week old plants. Specific recognition of the *AVR* gene by the *R* gene induces hypersensitive cell death response (HR) from 5 days post-infiltration.

Aardevo Exhibit 1010
Page 0777

Transformation of susceptible potato variety

Binary plasmids harboring the candidate genes *Blb3GH-A*, *Blb3GH-B*, *AbptGH-A* and *AbptGH-B* were transferred to *Agrobacterium tumefaciens* strain COR308 (Hamilton et al. 1996) and the constructs pKGW-Rpi-abpt, pKGW-R2-like, pKGW-R2 in AGL1 (Lazo et al. 1991). After verifying their stability in *Agrobacterium* these clones were transformed to the susceptible potato variety Desiree. Overnight cultures of the transformed *A.tumefaciens* strain were used to transform internodal cuttings from *in vitro* grown plants (Heilersig et al. 2006). A total of 100 to 200 explants were used for each transformation. Three to eight plants per transformant were transferred to the greenhouse.

Allele mining

Primers of 32 nucleotides were designed on Rpi-blb3 sequence, with the forward primer beginning at the start codon (Blb3-start) and the reverse primer beginning at the stop codon (Blb3-end; Table 1). The BAC clone TG92 containing *Rpi-abpt* and the genomic DNA of the parental resistant genotype AM-(3778-16) containing R2-like, were used as template in a long range PCR reaction (95 C: 2'40'', 30X [94 C: 20'', 56.8 C: 25'', 64.3 C:7'], 64.3 C: 25') using the high fidelity DNA polymerase *PfuTurbo*® (Stratagene, la Jolla, CA, U.S.A). PCR products were separated on agarose gel and purified using the QIAquick Gel Extraction Kit from Qiagen (Hilden, Germany). The purified pool of R2-likeGH ORFs and the purified Rpi-abpt ORF were used in a BP reaction together with the donor plasmid pDONR 221 according to the protocol described by (Untergasser 2006b). Subsequently, the BP reaction products were transferred to DH10B *E. coli* competent cells and plated on LB-agar plates containing the antibiotic kanamycin at the concentration of 100 µg/ml. Colonies were cultured in LB liquid medium supplemented with kanamycin. Plasmid DNA was subsequently extracted by mini-prep protocol adapted from Sambrook et al (2nd edition, 1989) using the P1, P2, P3 solutions from Qiagen. The first and last 700 bp of plasmids containing R2-likeGH ORFs were sequenced and classified. Then one clone of each group was entirely sequenced and compared to the *Rpi-blb3* sequence. The clones sharing high similarity with *Rpi-blb3* were cloned into the destination vector pKGW-MG (kindly provided by Andreas Untergasser) in between *Rpi-blb3* regulatory elements via a multiple LR reaction with the *Rpi-blb3* promotor in pDONR-P4P1R, the *Rpi-blb3* terminator in pDONR-P2RP3, the ORF of interest in pDONR221, and the binary destination, using the protocol of (Untergasser 2006a). The final expression constructs were then transferred to *A. tumefaciens* strain COR308 after the configuration of the constructs was confirmed by PCR.

Aardevo Exhibit 1010
Page 0778

ACKNOWLEDGEMENTS

AAL is supported by the Wageningen University Fund. PRJB is supported by the Scottish Government Rural and Environment Research and Analysis Directorate (RERAD) and JM is supported by EU Alβan.

We thank Vivianne Vleeshouwers and Ingo Hein for critically reading the manuscript, Mark Fiers and Pierre-Yves Chibon for their help in bio-informatics.

Supplementary Table S1: Genebank accessions numbers

Gene	accession number
Rpi-blb3	FJ536346
R2-like	FJ536323
Rpi-abpt	FJ536324
R2	FJ536325
blb3GH-A	FJ536326
R2-likeGH-2	FJ536327
R2-likeGH-3	FJ536328
R2-likeGH-5	FJ536329
abptGH-A	FJ536330
R2GH-2	FJ536331
R2GH-3	FJ536332
R2GH-5	FJ536333
R2GH-7	FJ536334
R2GH-8	FJ536335
R2GH-9	FJ536336
R2GH-10	FJ536337
R2GH-11	FJ536338
R2GH-12	FJ536339
R2GH-13	FJ536340
R2GH-14	FJ536341
R2GH-15	FJ536342
R2GH-16	FJ536343
R2GH-D3	FJ536344
R2GH-65	FJ536345

Aardevo Exhibit 1010
Page 0779

Chapter 3

Diversity, distribution and evolution of *Solanum bulbocastanum* Late Blight resistance genes

Anoma A. Lokossou, Hendrik Rietman, Miqia Wang, Pavel Krenek, Hanneke van der Schoot, Betty Henken, Roel Hoekstra, Vivianne G. A. A. Vleeshouwers, Edwin A. G. van der Vossen, Richard G. F. Visser , Evert Jacobsen, Ben Vosman

ABSTRACT

Knowledge on the evolution and distribution of Late Blight resistance genes is important for a better understanding of the dynamics of these genes in nature. We analyzed the presence and allelic diversity of the Late Blight resistance genes, *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3*, originating from *Solanum bulbocastanum* in a set of tuber-bearing *Solanum* species. These genes were only present in some Mexican diploid as well as polyploid species closely related to *S. bulbocastanum*. Sequence analysis of the fragments obtained from the *Rpi-blb1* and *Rpi-blb3* gene suggests an evolution through recombination and point mutations. For *Rpi-blb2* only sequences identical to the cloned gene were present in *S. bulbocastanum* accessions, suggesting that it has emerged recently. The three resistance genes occurred in different combinations and frequencies in *S. bulbocastanum* accessions and their spread is confined to Central America. A selected set of genotypes was tested for their response to the avirulence effectors IPIO-2, Avr-blb2 and Pi-Avr2, which interact with Rpi-blb1, Rpi-blb2 and Rpi-blb3, respectively, as well as by disease assays with a diverse set of isolates. Using this approach some accessions could be identified that contain novel, yet unknown, Late Blight resistance factors that in addition to the *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* genes.

Accepted in Molecular Plant-microbe interactions (2010)

Aardevo Exhibit 1010
Page 0781

INTRODUCTION

Solanum bulbocastanum is well known for its resistance to Late Blight (Niederhauser and Mills 1953), a disease caused by the oomycete *Phytophthora infestans*. The species belongs to the section *Petota* of the genus *Solanum*. It is a diploid, self-incompatible species native to Central America. It inhabits mountainous areas at an altitude between 1200-2300 m (Spooner et al. 2004) from northwestern Mexico (Durango and Nayarit regions) all the way south to Honduras..

Although characterized by Late Blight resistance, *S. bulbocastanum* resistance sources have not been widely exploited yet, which is mainly due to crossing barriers with cultivated potato (Helgeson et al. 1998; Hermsen and Ramanna 1973). Until now, 4 different NBS-LRR resistance genes have been identified in and cloned from *S. bulbocastanum*, namely *Rpi-Blb1* (van der Vossen et al. 2003) also known as *RB* (Song et al. 2003), *Rpi-Blb2* (van der Vossen et al. 2005) *Rpi-Blb3* (Lokossou et al. 2009) and *Rpibt1* (Oosumi et al. 2009), which opens the possibility to study the distribution and diversity of these genes in the section *Petota.*

The distribution of specific resistance genes in natural populations has been studied in a few cases only, *Rps2* and *Rpp13* in *Arabidopsis thaliana* (Mauricio et al. 2003; Rose et al. 2004), and *Cf-4* and *Cf-9* in tomato (Kruijt et al. 2005; Caicedo 2008) . Mauricio et al. (2003) studied the diversity of *Rps2*, a resistance gene against the bacteria *Pseudomonas syringae*, in a world wide sample of 27 *A. thaliana* accessions. They favored balancing selection as the explanation for the *Rps2* variation. This maintenance of variance by natural selection might be a general feature for the dynamics of *R*-gene evolution (Ellis et al. 1999; Luck et al. 2000). From the study on diversity for *Rpp13*, a resistance gene in *A. thaliana* against *Hyaloperonospora arabidopsis,* which is characterized by an extreme sequence diversity and resistance to various pathogen isolates; the suggestion was made that the co-evolutionary interactions between host and pathogen must be very active (Rose et al. 2004). Confirmation was provided with the analysis of the recently identified *Avr*-gene of *Rpp13*, *Atr13*, which reveals high sequence diversity for this effector as well (Allen et al. 2008) and moreover, one *Rpp13* allele seems to recognize another *H. arabidopsis* effector (Hall et al. 2009). Kruijt et al. (2005) showed that a high proportion of wild tomato plants specifically recognize the *C. fulvum* Avr4 and Avr9 elicitors, and functional homologs of the *C. fulvum* resistance genes *Cf-4* and *Cf-9* are conserved in diverged tomato species. The *Cf-4* and *Cf-9* genes appear to have been maintained by selection pressure imposed by the pathogen *Cladosporium fulvum*. Caicedo (2008) studied the distribution of *Cf-2* homologs in natural populations of *S. pimpinellifolium* and deduced that primarily the geographic distribution of *Cf-2* homolog diversity has been shaped by demographic factors or by selective pressure with a clinal geographic distribution. Levels of homologue diversity varied greatly between populations.

Aardevo Exhibit 1010
Page 0783

As population genetic processes and dynamics might affect the evolution of *R*-genes, a better understanding of variation within and between populations is crucial. Spooner (2009) evaluated several factors that might be predictive for identifying pest and disease resistant plants in wild relatives of the cultivated potato. For late blight resistance they identified taxonomic, climatic and geographic (latitude and altitude) variables. From these analyses it was clear that species level was the best predictor for resistance against *P. infestans*. These results stressed the need to use more focused evaluations of germplasm and to have a clear picture of geographical patterns of diversity in Late Blight resistance genes in wild *Solanum* species. To analyze these, here, use has been made of well studied *R*-genes like *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* which are terrific candidates since both *Avr* and *R*-genes have been identified and cloned.

The *Rpi-blb1* is located on linkage group VIII, near marker CT64 in a cluster of 4 Resistance Gene Analogues (RGA's) (van der Vossen et al. 2003). Its susceptible haplotype, rb, is characterized by an 18 bp deletion (Song et al. 2003). In an evaluation by Wang et al. (2008) using 44 species of *Solanum* section *Petota* for *Rpi-blb1* (*RGA2*) and its flanking *RGA's* (*RGA1* and *RGA3*) it was shown that the genes had a different distribution and diversity. The flanking *RGA1* appeared to be highly conserved within the tuber bearing *Petota* and was also found in the non tuber bearing species of the section, while *RGA3* was only present in some tuber bearing species. The *Rpi-blb1* gene was found in Mexican diploid species (*S. bulbocastanum* and *S. cardiophyllum*) and the Mexican polyploid species *S. stoloniferum* (including *S. papita* and *S. polytrichon*) (Wang et al. 2008). Functional analysis of the genes found in *S. stoloniferum* (*Rpi-sto1* and *Rpi-pta1*) indeed confirmed the functional homology with *Rpi-blb1* (Vleeshouwers et al. 2008). The occurrence of *Rpi-blb1* homologues in *S. stoloniferum* was suggested to be the result from common ancestry, e.g. *S. bulbocastanum* was suggested as one of the parents of the allopolyploid *S. stoloniferum* (Wang et al. 2008).

Rpi-blb2 is located on linkage group VI near marker CT119, in a locus harboring at least 15 *Mi-1* gene homologues (van der Vossen et al. 2005). The *Mi-1* gene of tomato confers resistance to the root knot nematode *Meloidogyne incognita* (Milligan et al. 1998) and shares 82% overall protein sequence identity with *Rpi-blb2* (van der Vossen et al. 2005). *Rpi-ver1,* a Late Blight *R*-gene from *S. verrucosum* also maps in this region (Jacobs et al. 2010). Other *R*-genes found at this locus in tomato include *Ol-1* and *Ol-3* (Bai et al. 2005), *Cf2* and *Cf5* (Dickinson et al. 1993), conferring resistance to *Oidium neolycopersici* and *Cladosporium fulvum,* respectively. Apart from *S. bulbocastanum*, no other species were found to contain *Rpi-blb2* (Wang et al. 2008).

Rpi-blb3 is located on linkage group IV, near marker TG339 in a major Late Blight resistance gene cluster containing *R2*, *Rpi-abpt* and *R2-like* (Park et al. 2005). Also Late Blight resistances with a

Aardevo Exhibit 1010
Page 0784

more QTL-like appearance like those of *S. microdontum* (Sandbrink et al. 2000; Tan et al. 2008) and those found in tomato (Brouwer et al. 2004) map into the same region. Tan et al. (2008) proposed that the QTL from *S. microdontum,* indentified as *Rpi-mcd1,* is a major *R*-gene. Other pathogen *R*-genes, mapping to the same region, include Ny_{tbr} conferring resistance to *Potato virus Y* (Celebi-Toprak et al. 2002) and the *Hero* resistance gene which confers resistance to all known pathotypes of *Globodera rostochiensis* and partial resistance to *Globodera pallida* (Ernst et al. 2002). Allele mining studies for *Rpi-blb3* are not yet reported.

Distribution of *R*-genes in natural populations is not easy to study phenotypically, as resistance to a particular pathogen might result from the presence of one or more different *R*- genes, which is the case for populations of *S. bulbocastanum*. One approach could be to use *Avr*-genes to determine whether a corresponding *R*-gene is present, in our case Avrblb1, which interacts with Rpi-blb1 (Vleeshouwers et al. 2008), Avrblb2 interacting with Rpi-blb2 (Oh et al. 2009) and Pi-Avr2 interacting with Rpi-blb3 (Lokossou et al. 2009). However the *Rpi-blb3* gene has several close relatives, all recognizing the same set of *Avr*-genes (Lokossou et al. 2009). Also carrying out such analysis in a large number of different species is not straight forward. Studying the distribution of *R*-genes with highly specific molecular markers and deduction of the phenotype for positive genotypes is an alternative. Still, one has to keep in mind that the functionality of *R*-genes might be changed by one single nucleotide polymorphism (SNP) or by insertions/deletions. A combination of the two approaches and screening with key *P. infestans* isolates seems a beneficial strategy as that will also allow the identification of other resistance genes towards this devastating disease.

In this paper, we studied the allelic frequencies of the *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* genes in several wild tuber-bearing *Solanum* accessions by performing allele mining with *R*-gene-specific primers. Additionally, Avr expression assays as well as detached leaf assays with key *P. infestans* isolates were utilized to strengthen the evidence for the presence of functional alleles. We relate the occurrence of the genes to the geographical origin of the accessions.

RESULTS

Screening of Solanum section Petota for the presence of Rpi-blb resistance genes

A total of 196 different taxa from *Solanum* section *Petota* were screened for the presence of the *Rpi-blb1* and *Rpi-blb3* genes. The presence of *Rpi-blb2* was not assessed in this wide set of taxa as a

Aardevo Exhibit 1010
Page 0785

previous study had already shown that the distribution of this gene was confined to *S. bulbocastanum* (Wang et al. 2008). We used the plant material described by Jacobs et al (2008) and as far as available at least 5 accessions from each species and 5 individuals per accession (in total 4929 genotypes). One screen was carried out with *Rpi-blb1* recognizing 1/1' primer pair, which is specific for the functional allele of *Rpi-blb1* (Colton *et al.* 2006). PCR fragments were detected in *S. bulbocastanum*, *S. cardiophyllum* ssp. *cardiophyllum* and *S. stoloniferum* (sensu Spooner et al. 2004) only.

The collection was also screened with primers specific for *Rpi-blb3* and 6 species were found to contain putative *Rpi-blb3* homologues: *S. pinnatisectum*, *S. bulbocastanum* (including some subspecies), *S. hjertingii, S. nayaritense, S. brachistotrichum* and *S. stoloniferum*.

Detailed investigation of S. bulbocastanum and related species

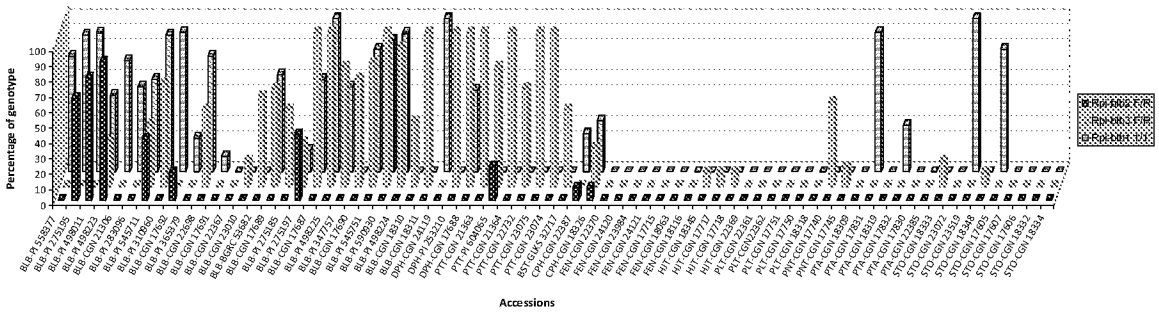
An additional 72 accessions originating from Mexico and Guatemala of the series *Bulbocastana*, *Pinnatisecta* and *Longipedicellata* was evaluated for the presence of *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* genes. For 63 accessions precise coordinates (longitude, latitude data) were available, pointing to 52 different locations (Table S1).

The presence or absence of *Rpi-blb1* was analyzed using the primer combinations Blb1 F/R, detecting the *Rpi-blb1* locus (Wang et al. 2008) and Blb 1/1' (Colton et al. 2006). The RGA1 F/R primer pair served as a positive control for DNA quality as this gene is present in all tuber-bearing *Solanaceae* (Wang et al. 2008).

The primer pair Blb1 F/R amplified fragments of the expected size in 27 *S. bulbocastanum*, 1 *S. brachistotrichum*, 2 *S. cardiophyllum*, and 4 *S. stoloniferum* accessions. The primer pair Blb 1/1' amplified fragments in a total of 29 accessions from 23 different locations and composed of 23 accessions of *S. bulbocastanum*, 2 of *S. cardiophyllum*, and 4 of *S. stoloniferum* (Table S1 and Figure 1A).

Aardevo Exhibit 1010
Page 0786

A.



B.

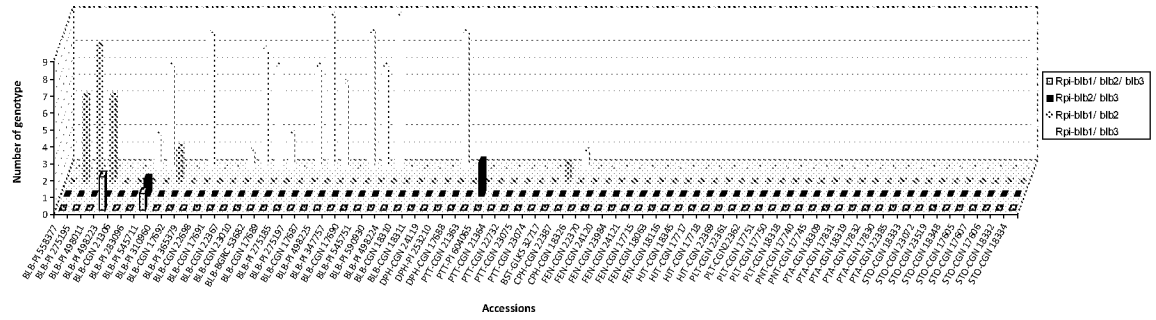


Figure 1:*Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* presence in *S.bulbocastanum* and related species. (A) Percentage of genotype per accessions containing *Rpi-blb* genes fragments. (B) Number of genotypes per accessions containing combinations of *Rpi-blb* genes fragments. Abbreviations: BLB: *S. bulbocastanum*, BST: *S. brachistotrichum*, CPH: *S. cardiophyllum*, DPH: *S. bulbocastanum sbsp dolichophyllum*, FEN: *S. fendleri*, HJT: *S. hjertingii*, PLT: *S. polytrichon*, PNT: *S. pinnatissectum*, PTA: *S. papita*, PTT: *S. bulbocastanum* subsp. *partitum*, STO: *S. stoloniferum.*

Supplementary Table S1: List of the accessions used to identify the frequency of the three *S. bulbocastanum* genes *Rpi-blb1*, *Rpi-blb2*, *Rpi-blb3*. From the left to the right the columns report the plant taxon, the accession number, the country of origin, the location coordinates (latitude-longitude in degrees and minutes); the total number of genotype tested positive by PCR with specific primers to *Rpi-blb1*, *Rpi-blb2*, *Rpi-blb3*, the haplotypes identified after sequencing; the number of genotype per accession which contain the different combination of genes *Rpi-blb1+Rpi-blb2*, *Rpi-blb1+Rpi-blb3*, *Rpi-blb2+Rpi-blb3*, *Rpi-blb1+Rpi-blb2+ Rpi-blb3*.

S1a. *S. bulbocastanum* accessions

Taxon	Accession number	Country	Coordinates		Number of genotypes	Rpi-blb genes identification								Rpi-blb genes Combination			
			lat	lon		RGA1	Blb1 F/R	Blb1 1/1´	Haplotype	Blb2 F/R	Haplotype	Blb3 F/R	Haplotype	blb1/ blb2	blb1/ blb3	blb2/ blb3	blb1/ blb2/ blb3
BLB	PI 558377	MEX	16.16N	96.41W	8	8	7	6	A6, A7	-		-		-	-	-	-
BLB	PI 275195	MEX	17.02N	96.46W	9	9	8	8	A1, A3	6	A	-		**5**	-	-	-
BLB	PI 498011	MEX	17.02N	96.46W	10	10	9	9	A3, C7	8	A	-		**8**	-	-	-
BLB	PI 498223	MEX	17.02N	96.46W	10	10	6	5	A9, A10, C2	9	A	3	A1	**5**	-	-	**2**
BLB	CGN 21306	MEX	17.30N	96.27W	11	11	8	8	A1, A2, A3	-		-		-	-	-	-
BLB	PI 283096	MEX	17.30N	96.52W	9	9	7	5	A4	-		-		-	-	-	-
BLB	PI 545711	MEX	17.33N	99.30W	10	10	6	6	D2, D3	4	A	4	A2		**2**	**1**	**1**
BLB	PI 310960	MEX	17.49N (b)	98.08W	9	9	8	8	H1, I1	-		6	A2	-	**6**	-	-
BLB	CGN 17692	MEX	17.53N	96.33W	11	11	10	10	A2, B1	2	A	-		**2**	-	-	-
BLB	PI 365379	MEX	18.43N	97.19W	9	9	2	2	A8	-		-		-	-	-	-
BLB	CGN 22698	MEX	19.11N	98.13W	16	12	12	12	A1, C2	-		8	A2	-	**8**	-	-
BLB	CGN 17691	MEX	19.13N	98.48W	10	9	5	1	A1, M1	-		-		-	-		-
BLB	CGN 22367	MEX	19.13N	98.48W	10	10	10	-	M1	-		-		-	-	-	-
BLB	CGN 23010	MEX	ND	ND	12	10	8	-	E2, M1	-		2	A2	-	**1**	-	-
BLB	BGRC 53682	MEX	19.15N (a)	98.53W	24	18	11	-	A5, M1	-		14	A2	-	**7**	-	-
BLB	CGN 17689	MEX	19.21N	99.12W	11	11	11	7	A3	-		7	A5	-	**6**	-	-
BLB	PI 275185	MEX	19.21N	99.12W	18	18	10	4	A1, M1	-		9	A2	-	**2**	-	-
BLB	PI 275197	MEX	19.21N	99.12W	7	7	1	1	A3	3	ND	2		-	-	-	-
BLB	CGN 17687	MEX	19.21N (b)	99.12W	10	10	9	6	L1,G1	-		10	A1	-	**6**	-	-
BLB	PI 498225	MEX	19.25N	100.20W	9	9	9	9	A1, M1	-		9	A2		**9**		
BLB	PI 347757	MEX	19.31N	100.15W	9	9	7	5	C3, F1	-		7	A2	-	**5**	-	-
BLB	CGN 17690	MEX	19.50N	101.43W	10	10	3	-	M1	-		7	A1	-	-	-	-
BLB	PI 545751	MEX	20.59N	103.10W	10	10	9	8	B3	-		8	A2		**8**		
BLB	PI 590930	MEX	21.01N	102.59W	7	7	7	6	J1, K1	-		7	A3		**6**		
BLB	PI 498224	MEX	19.24N	100.21W	10	10	10	9	B2	-		9	A2	-	**9**	-	-
BLB	CGN 18310	MEX	ND	ND	12	11	-	-	M1	-		5	B3	-	-	-	-
BLB	CGN 18311		ND	ND	7	7	-	-		-		7	B6	-	-	-	-
DPH	CGN 24119	MEX	18.55N	99.43W	10	10	10	10	C6	-		-		-	-	-	-
DPH	PI 253210	MEX	19.00N (b)	99.06W	5	-	-	-		-		5	A2	-	-	-	-
DPH	CGN 17688	MEX	19.42N	103.31W	15	15	8	8	A6	-		15	A2, A4, A6	-	**8**	-	-
PTT	CGN 21363	GTM	15.09N	90.18W	11	11	-	-	M1	-		11	B2, B8	-	-	-	-
PTT	PI 604065	GTM	15.10N	90.18W	9	9	-	-		2		7	B2	-	-	**2**	-
PTT	CGN 21364	GTM	15.13N	90.18W	11	11	-	-		-		11	B2	-	-	-	-
PTT	CGN 22732	GTM	15.19N	91.31W	11	10	-	-		-		7	B4, B5	-	-	-	-
PTT	CGN 23075	GTM	15.19N	91.33W	11	11	-	-	M1	-		11	B6	-	-	-	-
PTT	CGN 23074	GTM	15.46N	91.30W	12	10	-	-		-		12	A2, B1, B7	-	-	-	-

Aardevo Exhibit 1010
Page 0788

S1b. *S. brachistotrichum*, *S. cardiophyllum*, *S. nayaritense* and polyploid *Solanum* accessions

Taxon	Accession number	Country	Coordinates		Number of genotypes	Rpi-blb genes identification								Rpi-blb genes Combination			
			lat	lon		RGA1	Blb1 F/R	Blb1 1/1´	Haplotype	Blb2 F/R	Haplotype	Blb3 F/R	Haplotype	blb1/ blb2	blb1/ blb3	blb2/ blb3	blb1/ blb2/ blb3
BST	GLKS 2717	MEX	ND	ND	6	1	1	-		-		3	A2	-	-	-	-
CPH	CGN 22387	MEX	ND	ND	12	10	5	3	D1	1	B	-		1	-	-	-
CPH	CGN 18326	MEX	ND	ND	12	10	4	4	D1	1	B	3	A1	-	1	-	-
FEN	CGN 22370	MEX	25.25N	101.00W	10	9	-	-		-		-		-	-	-	-
FEN	CGN 24120	MEX	28.08N	107.30W	10	9	-	-		-		-		-	-	-	-
FEN	CGN 23984	MEX	28.19N	107.21W	10	9	-	-		-		-		-	-	-	-
FEN	CGN 24121	MEX	29.08N	108.08W	10	9	-	-		-		-		-	-	-	-
FEN	CGN 17715	USA	31.26N	110.19W	10	9	-	-		-		-		-	-	-	-
FEN	CGN 18063	USA	31.54N	109.16W	10	9	-	-		-		-		-	-	-	-
FEN	CGN 18116	USA	33.23N	108.36W	11	9	-	-		-		-		-	-	-	-
HJT	CGN 18345	MEX	24.52N	100.13W	11	10	-	-		-		1	A1	-	-	-	-
HJT	CGN 17717	MEX	25.25N	100.51W	11	10	-	-		-		1	A1	-	-	-	-
HJT	CGN 17718	MEX	25.25N	101.00W	11	9	-	-		-		1	A1	-	-	-	-
HJT	CGN 22369	MEX	25.25N	100.51W	10	10	-	-		-		-		-	-	-	-
PLT	CGN 22361	MEX	20.20N	100.00W	10	10	-	-		-		-		-	-	-	-
PLT	CGN 22362	MEX	20.20N	100.00W	10	10	-	-		-		-		-	-	-	-
PLT	CGN 17751	MEX	22.47N	102.35W	5	5	-	-		-		-		-	-	-	-
PLT	CGN 17750	MEX	ND	ND	10	10	-	-		-		-		-	-	-	-
PLT	CGN 18318	MEX	ND	ND	10	10	-	-		-		-		-	-	-	-
PNT	CGN 17740	MEX	20.36N	100.23W	11	-	-	-		-		6	A1	-	-	-	-
PNT	CGN 17745	MEX	20.40N	103.20W	8	-	-	-		-		1	A1	-	-	-	-
PTA	CGN 18309	MEX	23.20N	105.07W	10	10	-	-		-		-		-	-	-	-
PTA	CGN 17831	MEX	23.30N	103.35W	11	10	10	10	C4, C5	-		-		-	-	-	-
PTA	CGN 18319	MEX	24.11N	105.02W	10	10	-	-		-		-		-	-	-	-
PTA	CGN 17832	MEX	ND	ND	10	10	3	3	C4, C6	-		-		-	-	-	-
PTA	CGN 17830	MEX	24.57N	103.54W	10	10	-	-		-		-		-	-	-	-
PTA	CGN 22385	MEX	ND	ND	10	10	-	-		-		-		-	-	-	-
STO	CGN 18333	MEX	19.05N	98.41W	12	11	-	-		-		2	A1	-	-	-	-
STO	CGN 23072	MEX	19.05N	98.41W	10	10	-	-		-		-		-	-	-	-
STO	CGN 23519	MEX	19.07N	98.46W	10	10	10	10	D1	-		-		-	-	-	-
STO	CGN 18348	MEX	19.35N	99.04W	11	10	-	-		-		1	A1	-	-	-	-
STO	CGN 17605	MEX	19.42N	101.07W	10	10	8	8	B3	-		-		-	-	-	-
STO	CGN 17607	MEX	19.42N	101.07W	10	10	-	-		-		-		-	-	-	-
STO	CGN 17606	MEX	ND	ND	10	10	-	-		-		-		-	-	-	-
STO	CGN 18332	MEX	ND	ND	10	10	-	-		-		-		-	-	-	-
STO	CGN 18334	MEX	19.15N (a)	98.53W	10	10	-	-		-		-		-	-	-	-

(a): Coordinates estimated with Google Earth using the CGN route description. (b): Data derived from Heijmans (2007). Abbreviations:ND: not determined BLB: *S. bulbocastanum*, BST: *S. brachistotrichum*, CPH: *S. cardiophyllum*, DPH: *S. bulbocastanum sbsp dolichophyllum*, FEN: *S. fendleri*, HJT: *S. hjertingii*, PLT: *S. polytrichon*, PNT: *S. pinnatissectum*, PTA: *S. papita*, PTT: *S. bulbocastanum* subsp. *partitum*, STO: *S. stoloniferum*.

The primer pair Blb2F/R (Wang et al. 2008) amplified *Rpi-blb2* in 7 accessions of *S. bulbocastanum* and in 2 *S. cardiophyllum* accessions from 6 different locations (Table S1 and Figure 1A).

The primers specific for *Rpi-blb3* amplified distinct fragments in 35 accessions from 28 different locations. Accessions showing amplification originated from 26 *S. bulbocastanum*, 2 *S. pinnatissectum,* 1 *S. brachistotrichum*, 1 *S. cardiophyllum*, 3 *S. hjertingii* and 2 *S. stoloniferum* accessions (Table S1 and Figure 1A).

The *Rpi-blb1* and *Rpi-blb3* gene combinations were found in genotypes from 14 *S. bulbocastanum* accessions originating from 12 different locations. The *Rpi-blb1* and *Rpi-blb2* gene were found together in genotypes from 5 *S. bulbocastanum* accessions from 2 different locations. The *Rpi-blb2* and *Rpi-blb3* fragments were found together in 2 *S. bulbocastanum* accessions from 2 distinct locations (Table S1 and Figure 1B).

The percentages of the individual genotypes of *S. bulbocastanum* containing *Rpi-blb1*, *Rpi-blb2* or *Rpi-blb3* fragments are 36%, 9% and 56%, respectively.

Three genotypes, belonging to PI498223 and PI545711 accessions, harbored all three genes. Only in a small number of accessions all genotypes harbored the same gene; 2 accessions were fixed for *Rpi-blb1* and 10 for *Rpi-blb3*. Interestingly 91% of the genotypes from the subspecies *S. bulbocastanum* subsp. *partitum* contained *Rpi-blb3*.

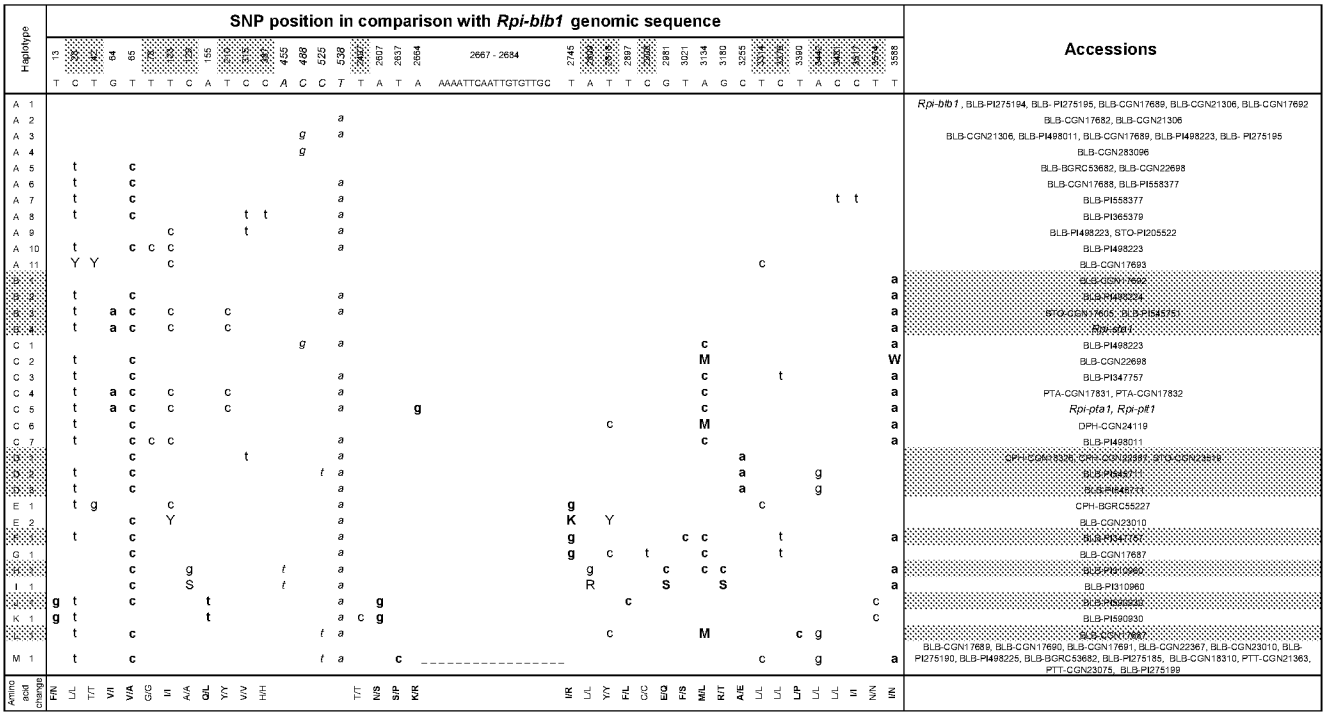
Allelic variation in the Rpi-blb1, Rpi-blb2 and Rpi-blb3 gene fragments

To verify the nature of the amplified fragments and to explore the R-gene diversity at the nucleotide level, part of the gene was sequenced for all the positives accessions. The results are presented in Table 1.

The *Rpi-blb1* amplified sequence spans 1734 base pairs in total, composed of two separate parts: the first part is from the start codon to position 545 obtained with primer pair 517/ 1519. The second part is from position 2404 to the stop codon obtained with primer pairs Blb1F/R, 1531/519 and 1/1'. Sequence alignment revealed a total of 56 SNPs compared to the cloned *Rpi-blb1* gene with 34 SNPs in the coding region, 4 SNPs in an intron part and the deletion of 18 nucleotides which is characteristic for the *Rpi-blb1* non-functional allele.

Aardevo Exhibit 1010
Page 0790

Table 1a: Allelic variation for *Rpi-blb1* in *S. bulbocastanum* accessions and related species.



The *Rpi-blb1* sequence spans 1709 base pairs (bp) in total, composed of two separate parts: (1) from the start codon to position 520 derived from the primer 517/1519. In italic are the located in the intron sequence. (2) from position 2404 to stop codon derived from the primer BLB1F/R and 1521/519.

Table 1b: Allelic variation for *Rpi-blb2* in *S. bulbocastanum* accessions and related species.

Haplotype	SNP position in comparison with *Rpi-blb2* genomic sequence																									Accessions
	2226-2297	2298	2299	2300	2302	2303	2304	2305	2306	2308	2309	2310	2311	2312	2313	2315	2316	2317	2318	2319	2320	2321	2322	2323	2324	2325	2326	
A	TCTGGAAGAAGTGGTGAAAATTTATTTGGATGATTTAATTTCCAGTAGCTTGGTAATTTGTTTCAATGAGAT	A	G	G	G	A	T	T	A	C	C	T	A	C	T	G	C	C	A	A	C	T	T	C	A	T	G	PI 498011, BLB-P498233, BLB
B	'	C	A	C	T	T	G	G	G	T	T	A	A	T	C	A	G	A	T	T	T	T	G	T	A	G	T	CPH-CGN18326, CPH-CGN22387, CPH-BGRC55227

The *Rpi-blb2* sequence spans 715 bp derived from the primers Blb2F/R

Haplotypes code are indicated in the left column. Nucleotide positions are numbered according to the genomic sequences of the genes, beginning at the START codon and including introns in the case of *Rpi-blb1* and *Rpi-blb2*. Amino acid changes are indicated on the bottom line. SNP's part of the intron region are in italic. In light grey are highlighted the position where SNP's did not change the amino-acid. SNP's resulting in non-synonymous amino-acid change are indicated in bold. *Solanum* accessions in which the haplotypes where identified are indicated on the right column.

Nucleotides abbreviations according to the IUB code: K, GT; M,AC; R, AG; S, GC; W,AT; Y, CT.

Table 1c: Allelic variation for *Rpi-blb3* in *S. bulbocastanum* accessions and related species.

Haplotype	SNP position in comparison with *Rpi-blb3* genomic sequence	Accessions
	941 972 1001 1016 1021 1027 1026 1036 1043 1045 1065 1103 1107 1116 1131 1133 1196 1235 1246 1255 1258 1272 1293 1309 1326 1335 1352 1371 1396 1439 1487 1501 1510	
	G T G T G G G A G T A G A G A A T C G C T T G C G C G C C C T G	
A 1		PNT-CGN17745, PNT-CGN17740, *Rpiblb3,* DPH-CGN17688, PTT-CGN23074, BLB-PI545711, BLB-CGN17690, BLB-CGN17687, HJT-CGN22370, HJT-CGN18345, PNT-CGN17742, STO-CGN18348, STO-CGN18333
A 2	a	BLB-CGN22698, BLB-CGN23010, BLB-PI 275185, BLB-BGRC53682, BLB-PI275190, BLB-PI310960, BLB-PI347757, BLB-PI498224, BLB-PI498225, BLB-PI545711, BLB-PI545751, BLB-CGN176888, DPH-PI253210, BST-GLKS32717, PTT-CGN23074, BLB-GLKS31741
A 3	c	BLB-PI590930
A 4	c	DPH-CGN17688
A 5	g	PTT-CGN17689
A 6	g *	DPH-CGN17688
A 7	* a	BLB-BGRC53682
A 8	t	PTT-PI275200
B 1	a t a	PTT-CGN23074
B 2	a a a t a	PTT-CGN21364, BLB-PI604065, PTT-CGN21363
B 3	a a a t a g	BLB-CGN18310
B 4	a a t a	PTT-CGN22732
B 5	a a a t a	PTT-CGN22732
B 6	c a a t a t	BLB-CGN18311, PTT-CGN23075
B 7	c a a t a	PTT-CGN23074
B 8	Y K R s R Y M	PTT-CGN21363
C 1	a a a t t	STO-PI205522
C 2	a t a t t	BST-CPC3822, BST-PI320265
C 3	a c t t	BST-PI320265
C 4	a c t t	NYR-PI545825
C 1	c c a	*R2-like, Rpi-abpt*
E 1	t a t c a t a	*R2*
Amino acid change	R/I S/S R/K V/A A/S V/I M/I M/L S/N L/L E/D K/K G/G S/N fs fs I/N T/S A/A I/L Y/H F/L K/K R/W E/E P/P R/I D/E R/stop T/S A/V F/L V/I	

The *Rpi-blb3* sequence spans 618 bp derived from the primers Blb3F/R

The 34 haplotypes could be classified into 13 groups (A-M) based on the identity of the non-synonymous SNP changes (Table 1a). Each haplotype group contained 1 to 18 accessions. Group A, which is the dominant group, contains haplotypes without non-synonymous SNPs and is only composed of *S. bulbocastanum* accessions and the cloned *Rpi-blb1* gene. Group B additionally contains *S. stoloniferum (Rpi-sto1* like) haplotypes while *S. papita* and *S. polytrichon* (*Rpi-pta1* and *Rpi-plt1* like) are part of group C. Haplotype C2, A11, C6, E2, I1 and L1 display double SNPs which are probably the result of heterozygous genotypes. Haplotype M1 represents the known non functional allele, identified by the 18 bp deletion (Song et al. 2003).

The *Rpi-blb2* amplified sequence spans 715 base pairs from position 2157 to 2872 derived from the primers Blb2F/R (Wang et al. 2008). From the 2 sequences obtained, one is 100% identical to the *Rpi-blb2* sequence and the second one displayed a variable region composed of 72 nucleotide deletion at the beginning of the sequence followed by 26 SNPs in the remaining part (Table 1b). The *Rpi-blb3* amplified sequence spans 618 base pairs from position 918 to 1536. Sequence alignment reveals 33 SNPs displayed in 22 haplotypes containing 1 to 16 accessions (Table 1c). Our classification differentiates 5 groups containing 1 to 34 accessions, according to the type of polymorphism present in the sequence. Group A displays one SNP per haplotype, A1 and A2 being the most commonly present in 12 and 16 accessions, respectively. Haplotype A6 and A7 show one nucleotide deletion leading to a frame shift. Group B haplotypes have 4 SNPs in common clearly defining a polymorphic sequence block. Haplotypes B are exclusively identified in accessions originating from Guatemala (Table S1 and Appendix 1). Haplotype B6 contains a SNP in position 1396 resulting in a stop codon. Group C comprises haplotypes with 4 SNPs in common defining as well a polymorphic sequence block, present in *S. brachistotrichum*, *S. nayaritense* (Table 2) and *S. stoloniferum*. The *R2-like*, *Rpi-abpt* and *R2* alleles are classified in haplotypes D1 and E1.

Aardevo Exhibit 1010
Page 0794

Validation of Late Blight resistance by effector recognition and detached leaf assay

In an attempt to link the presence of *Rpi-blb* haplotypes to Late Blight resistance, a set of 50 plants from our in vitro collection was screened. It included a molecular characterization by PCR-amplification with *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* gene specific primers followed by sequencing the PCR fragment. An additional combination of Rpi-blb3 primer, blb3 F/Ra was used, with Ra being specific to *Rpi-blb3* and its homolog *R2-like* (Lokossou et al. 2009), in order to pick up intermediates of *Rpi-blb3* and *R2-like.*

Phenotypic characterization was carried out through detached leaf assays with six *P. infestans* isolates (Table S2) and agro-infiltration with Pi-effectors interacting with *Rpi-blb1* and *Rpi-blb3*. The chosen *P. infestans* isolates were genetically very different from each other, as it has been shown by simple sequence repeat (SSR) fingerprinting (Champouret et al. 2009), and display different virulence spectra with respect to the *Rpi-blb* genes tested. The *Rpi-blb2* gene confers resistance to the six isolates used and the *Rpi-blb1* and *Rpi-blb3* gene confer the resistance spectrum shown in Table 2. None of the tested plants contained *Rpi-blb2*, as no fragment was amplified with the primers Blb2F/R. Unfortunately, we observed background responses caused by the agro-infiltration of Avrblb2 in all the plants tested (not shown), thus *Rpi-blb2* presence or absence could not be verified by effector recognition in wild *Solanum* species. Results obtained for *Rpi-blb1* and *Rpi-blb3* are presented in Table 2 and show the diversity of possible interactions, classified in 5 categories.

Table 2: Evaluation of *Solanum* genotypes by molecular characterization, effector agro-infection and detached leaf assay. Form the left to the right are indicated in the column the *Solanum* accession taxon; identification number (genotype number in bracket); geographic coordinates (latitude-longitude); results of the specific amplification of *Rpi-blb1* by the primers combination 1/1' and *Rpi-blb3* by blb3F/R and blb3 F/Ra with Ra being specific to *Rpi-blb3* and *R2-like* (Lokossou et al. 2009); the haplotype identified after sequencing of the specific fragment referring to table 1; results of the effector agro-infection with Avr-blb1, Pi-Avr2 and the controls R3a and Pi-Avr3a/R3a together, are indicated by degree of response intensity from (-) meaning no response to (+++) meaning clear hypersentive reaction; results of the detached leaf assay conducted with the isolates PIC99177, PIC 99189, PIC 99183, IPO-C, 90128 and UK7824 scored R (resistant), S (susceptible), Rq (quantitatively resistant), Q (quantitative).

Abreviations: ND: not determined sequences, BLB: *S. bulbocastanum*, BST: *S. brachistotrichum*, CPH: *S. cardiophyllum*, DPH: *S. bulbocastanum* subsp. *dolichophyllum*, FEN: *S. fendleri*, HJT: *S. hjertingii*, NYR: *S. nayaritense*, PLT: *S. polytrichon*, PNT: *S. pinnatisectum*, PTA: *S. papita*, PTT: *S. bulbocastanum* subsp. *partitum*, STO: *S. stoloniferum*. CPH-BGRC55227* contains *Rpi-blb2* haplotype B.

Aardevo Exhibit 1010
Page 0795

Taxon	Accession number	Coordinates		Specific amplification			Sequence		Effector Agro-infiltration				*Phytophtora infestans Detached leaf assay*						Comments
		lat	lon	Rpi-blb1 1/1'	Rpiblb3 F/R	Rpiblb3 F/Ra	Rpiblb1 Haplotype	Rpiblb3	Avr-blb1	Pi-Avr2	Controls R3a	Controls R3a/Avr3a	PIC99177	PIC99189	PIC99183	IPO-C	90128	UK7824	
BLB	CGN17692 (Blb8005-8)	17.53N	96.33W	x		x	A1		+ + +	-	-	+++	S	S	R	R	R	R	Genotype containing Rpi-blb1
DPH	CGN17688 (Blb99-256-3)	19.42N	103.31W		x	x		A1	-	+ + +	-	+++	Q	S	R	S	R	S	Genotype containing Rpi-blb3
HJT	CGN17718	25.25N	100.00W			x		A1	-	+ + +	-	+++	S	S	Rq	S	R	Q	
HJT	CGN22370	25.25N	101.00W		x			A1	-	+ + +	-	+++	S	S	R	S	R	S	
HJT	CGN18345	24.52N	100.13W		x			A1	-	+ + +	-	+++	Q	S	R	S	R	S	
HJT	BGRC32671	ND	ND		x			A1	-	+ + +	-	+++	S	R	Rq	S	R	S	
HJT	CGN17717	25.25N	100.51W		x	x		A1	-	+ + +	-	+++	R	S	R	S	R	S	
STO	CGN18333	19.05N	98.41W		x			A1	-	+ + +	-	+++	S	R	R	S	R	S	
PNT	CGN18331	ND	ND		x	x		A1	+	+	-	+++	R	R	R	R	R	R	Plants with Avr2 recognition and Rpi-blb3 fragment.
BST	CGN17681	ND	ND		x	x		A2	-	+ + +	-	+++	S	S	R	S	Rq	S	
BLB	CGN18310	ND	ND		x			B2	-	+ +	-	+++	R	R	R	Q	R	R	
PTT	CGN21364	15.13N	90.18W		x			B2	-	+	-	+++	R	Q	R	R	R	S	
BST	PI320265	29.08N	106.05W			x		C2	-	+ + +	-	++	S	R	R	S	R	Q	
BST	PI320265	29.08N	106.05W			x		C3	-	+ + +	-	+	S	S	R	S	R	S	
NYR	PI545825	ND	ND		x			C4	-	+ + +	-	+++	Q	R	R	S	R	R	
STO	PI205522	19.07N	98.46W	x	x		A9	C1	+ + +	+ + +	-	+++	Q	R	R	R	R	R	Plant with Avr2 and AvrBlb1 recognition and Rpi-blb1 and Rpi-blb3 fragment.
BLB	CGN17689	19.21N	99.12W	x	x		A 1	A5	+ + +	-	-	+++	R	R	R	R	R	R	Plants with AvrBlb1 recognition and Rpi-blb1 fragment.
BLB	CGN21306	17.30N	96.27W	x	x		A1	nd	+ + +	-	-	+++	R	R	R	R	R	R	
BLB	CGN17692	17.53N	96.33W	x		x	A1		+ + +	-	-	++	R	S	R	R	R	R	
STO	CGN18348	19.35N	99.04W		x			A1	+ + +	-	-	+	R	S	R	R	R	R	Plants with AvrBlb1 recognition and no Rpi-blb1 fragment.
BLB	CGN17693	19.43N	99.47W						+	-	-	++	R	R	R	R	R	R	
STO	CGN18333	19.05N	98.41W		x			A1	-	-	-	++	Q	R	S	S	R	R	Plants with Rpi-blb1 and/or Rpi-blb3 fragment
BLB	BGRC53682	19.15N	98.53W	x	x	x	A5	A2	-	-	-	+++	R	S	R	R	R	R	
PTT	PI275200	15.10N	91.31W		x			A8	-	-	-	+++	Q	Q	Q	S	Q	R	
PTT	CGN23075	15.19N	91.33W		x			B6	-	-	-	+++	R	R	R	R	R	R	
BLB	CGN22698	19.11N	98.13W	x		x	A5		-	-	-	+++	R	R	R	R	R	R	
PNT	GLKS1586	ND	ND			x			-	-	-	+++	R	R	R	R	R	R	
CPH	GLKS99	ND	ND			x				-	-	+++	Q	S	S	S	S	R	
BLB	CGN18310	ND	ND			x			-	-	-	+++	Q	Q	S	S	S	Q	
PTA	CGN17830	24.57N	103.54W			x			-	-	-	+++	R	Q	S	S	S	S	
BLB	CGN23010	ND	ND						-	-	-	+++	R	R	R	R	R	R	
BLB	PI275199	19.29N	98.54W			x			-	-	-	+	R	R	R	R	R	R	
CPH	CGN22387	ND	ND						-	-	-	+	R	R	R	R	R	R	Plants containing different late blight resistance genes other than the tested Rpi-blb genes.
CPH	CGN18325	ND	ND						+	-	-	+++	R	R	R	R	R	R	
CPH	CGN18326	ND	ND						-	-	-	+++	Q	Q	S	R	Q	R	
CPH	CGN18326	ND	ND						-	-	-	+++	Q	R	Q	R	S	R	
CPH	BGRC55227	ND	ND						-	-	-	+++	Q	Q	S	R	S	R	
PTT	CGN23074	15.46N	91.30W		x			B1	-	-	-	++	S	S	S	S	S	S	
PTT	CGN21363	15.09N	90.18W		x			B2	-	-	-	+++	S	S	S	S	S	S	
PTT	CGN23074	15.46N	91.30W		x			B7	-	-	-	+	S	S	S	S	S	S	
BST	PI320265	29.08N	106.05W			x		C2	-	-	-	++	S	S	S	S	S	S	Late Blight suceptible plants
BST	CGN17681	ND	ND						+	-	-	+++	S	S	S	S	S	Q	
FEN	CGN18116	33.23N	108.36W						-	-	-	+++	S	S	S	S	S	S	
BST	CPC3822	ND	ND		x	x		nd	-	+ + +	-	+++	S	S	S	S	S	S	
STO	GLKS0620	ND	ND		x			nd	-	+	-	++	S	S	S	S	S	S	
PNT	CGN17743	ND	ND			x			+	++	++	++	R	R	R	R	R	R	
BLB	CGN22367	19.13N	98.48W			x			-	-	-	-	R	Q	R	R	R	R	
PNT	CGN17740	20.36N	100.23W		x			A1	+	+	++	++	R	R	R	R	R	R	Plants not suitable for agro-infiltration
PNT	CGN17742	21.07N	101.40W		x			A1	-	-	-	-	R	R	R	R	R	R	
BST	GLKS2717	ND	ND						-	+	+	+++	S	S	S	S	Q	S	

The first category comprises plants harboring the *Rpi-blb1* and/or *Rpi-blb3* gene, as judged from the marker analysis. They also interacted with the Avrblb1 and/or Pi-Avr2 elicitor and displayed a resistance spectrum identical or broader than the *S. bulbocastanum* derived *R*-genes. Based on the assay with Avrblb1, 3 *S. bulbocastanum* and 1 *S. stoloniferum* plants contained *Rpi-blb1*. PCR amplification and sequencing strengthened the presence of functional *Rpi-blb1* haplotypes, identified as A1 and A9.Their resistance spectrum matches with that of *S. bulbocastanum* plant (CGN17692), which was confirmed to harbor *Rpi-blb1* (van der Vossen et al. 2003). However, they were also resistant to isolates PIC99189 and PIC99177, known to break *Rpi-blb1* (Champouret et al. 2009), which suggests the presence of additional resistance factors in these genotypes. The *Rpi-blb3* haplotypes A1, B2, C1, C2, C3 and C4 were found in 14 plants from *S. bulbocastanum*, *S. brachistotrichum*, *S. hjertingii*, *S. nayaritense* and *S. stoloniferum* accessions. They all interact with Pi-Avr2 and display an identical or broader resistance spectrum in comparison to the genitor used to isolate *Rpi-blb3,* thus suggesting the presence of a functional *Rpi-blb3* haplotype and additional Late Blight resistance factors.

From plants classified in the second category fragments were amplified with primers specific for the *Rpi-blb1* and/or *Rpi-blb3* gene, but no interaction with Avrblb1 or Pi-Avr2 was observed. The *Rpi-blb* genes amplified might contain mutations preventing the interaction with the tested effectors. Moreover, these plants putatively contain Late Blight resistance genes other than the one tested, as judged from the responses to the isolates (Table 2).

The third category comprises resistant plants lacking *Rpi-blb1* or *Rpi-blb3* as judged from the marker assays and effector interaction. Interestingly, 2 *S. bulbocastanum* and 2 *S. cardiophylum* plants featured complete resistance to the full set of isolates. These plants probably contain late blight resistance genes other than the tested *Rpi-blb* genes. One *S. cardiophyllum* plant (CPH-BGRC55227*) was tested positive for *Rpi-blb2* haplotype B and displays a resistance spectrum not identical to the one of *Rpi-Blb2* containing plant.

Late Blight susceptible plants not containing any functional *R*-gene as judged from the response with the tested isolates, but showing amplification with *Rpi-blb3* primers, are found in category 4. One *S. brachistotrichum* clone contains *Rpi-blb3* haplotype C2 and interacts strongly with Pi-Avr2 but is completely susceptible to the set of *P. infestans* isolates tested. Three accessions from *S. bulbocastanum* subsp. *partitum*, 2 of *S. brachistotrichum* and 1 of *S. stoloniferum* contain *Rpi-blb3* haplotypes B1, B2, B7 and C2 but do not interact with Pi-Avr2 and display a susceptible phenotype when infected with the set of isolates.

Aardevo Exhibit 1010
Page 0797

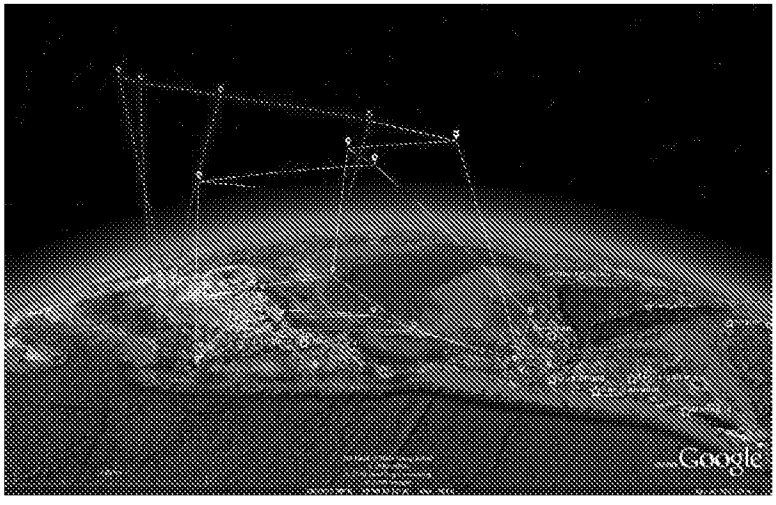
The last category of plants was not suitable for the agro-infiltration. The reactions with the effectors were non specific and resulted in a hypersensitive response to all the tested effectors, or gave no reaction to control *Pi-Avr*/*R*-gene co-infiltration (Table 2). However plants showing resistance to all tested isolates are likely to contain new late blight resistance gene.

Geographical distribution of the Rpi-blb genes

The 3 *Rpi-blb* genes are mainly present in the *S. bulbocastanum* accessions from Central Mexico (Figure 2) and plants not containing the 3 studied *Rpi-blb* genes originate from the northern part of Mexico and the southern part of the USA. Also the allotetraploid species *S. fendleri*, which is found in the north of Mexico, lacks the *Rpi-blb* genes tested.

Solanum stoloniferum from the Durango region contains *Rpi-blb1* while *Rpi-blb3* is present in *S. hjertingii* originating from Monterray, *S. brachistotrichum* from Chihuahua and *S.bulbocastanum* subsp. *partitum* from Guatemala.

In order to see if haplotypes diverge when they originate from more geographically distant areas, the haplotypes were phylogenetically mapped in Google Earth using Supramap (Janies et al. 2007). From the results, it can be seen that the M1 haplotype of *Rpi-blb1* (Appendix 1) is present in Central Mexico as well as in Guatemala and that the less supported clade containing the C and B haplotypes from *S. stoloniferum* is confined to the upper part of Mexico. For *Rpi-blb3*, the haplotypes of group C originate from Central towards Northern Mexico, while haplotypes recognized as group B all are from Guatemala.



Appendix 1 (KMZ file): *Rpi-blb* genes diversity and geographical origin. Phylogenetic and geographical localization of genotypes containing *Rpi-blb* haplotypes. *Rpi-blb3* groups B and C haplotypes and *Rpi-blb1* haplotype M1 are indicated in red, blue and white, respectively. The original kmz file can be provided as and be opened in a Google Earth desktop application. Additionally to the phylogenetic tree, the location of the collection sites of the accessions can be visualized.

Aardevo Exhibit 1010
Page 0798

A.

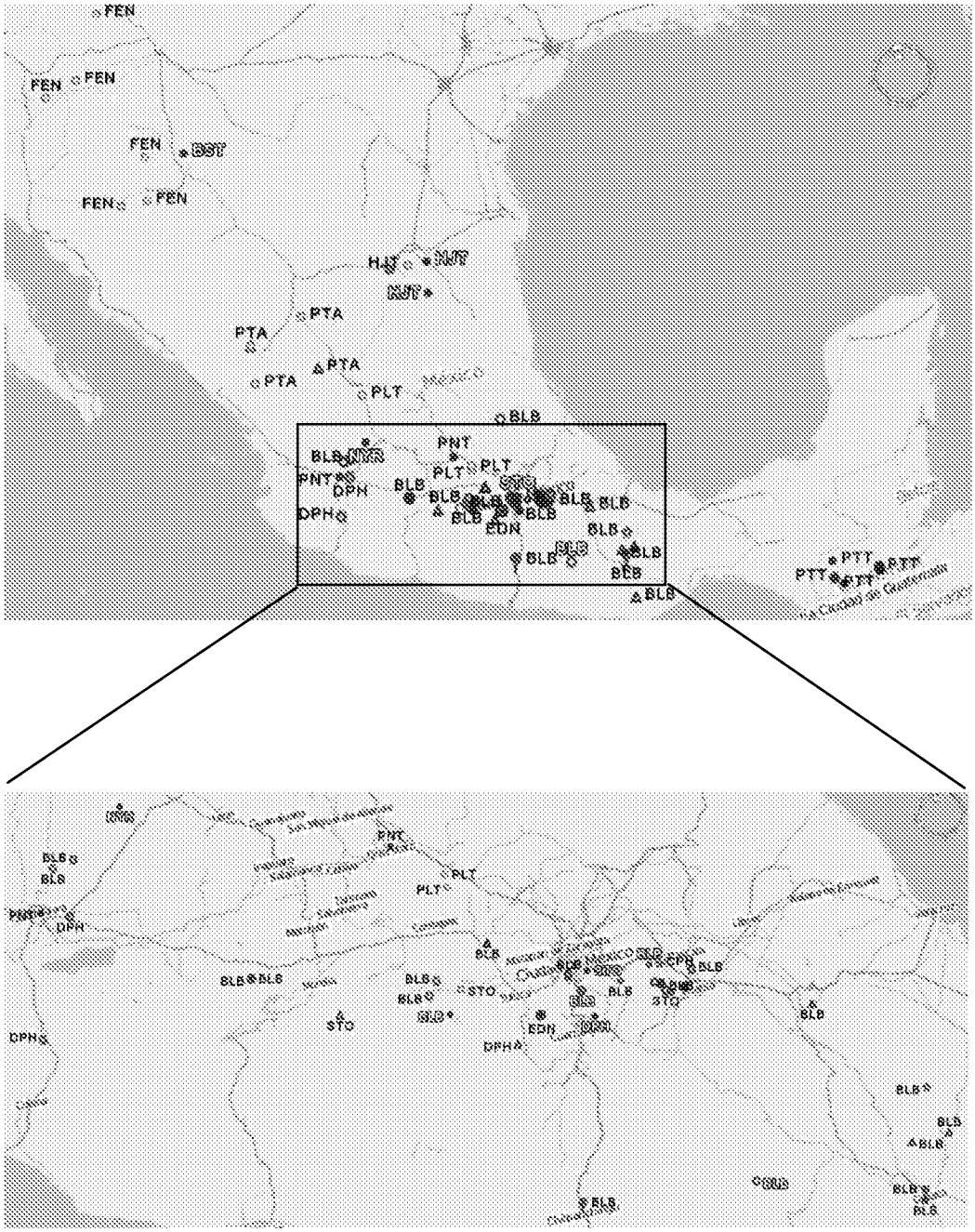


Figure 2: Geographical location of the *Solanum* accessions under study containing only *Rpi-blb1* (blue triangular), only *Rpi-blb3* (red dots), *Rpi-blb1* and *Rpi-blb3* (purple lozenges), *Rpi-blb1* and *Rpi-blb2* (green stars), the three *Rpi-blb* genes (red drops) and the accessions not containing any Rpi-blb gene (yellow dots)

Aardevo Exhibit 1010
Page 0799

DISCUSSION

The use of gene specific primers and gene functionality

Gene specific primers are powerful tools for the evaluation of presence or absence of resistance genes (Arens et al. 2010). Although *R*-genes often have numerous close homologues, in most cases it is possible to develop markers in such a way that a fragment specific to the gene of interest is amplified. Subsequent sequencing of the fragment makes it possible to verify its identity, to differentiate the haplotypes and to discard recognized pseudo-genes thus indicating the presence or absence of most likely functional genes. We validated this approach by phenotyping 50 individual plants using effectors and detached leaf assays. The plants were first characterized for the presence of *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* with markers. In the 50 cases we only observed two cases for *Rpi-blb1* in which the markers predicted the presence of the gene where there was no effector response. This indicates that there is a good but not 100% correlation between markers and phenotype for *Rpi-blb1* (table 2). All plants tested negative when using the gene specific markers for *Rpi-blb2*. Unfortunately, this could not be validated by an effector response due to background that was observed in the agro-infiltration experiment with Avrblb2. For *Rpi-blb3* we found 13 cases in which there was amplification with the gene specific primers but no response to the effector. Also there were 3 cases in which there was a response to the effector but no amplification of the gene specific fragment and one case in which we observed an effector response and no resistance in the DLA assay. An additional primer pair (Rpiblb3 F/Ra) did amplify in some of the cases where there was no amplification with the *Rpi-Blb3* specific primers was found (Table 2), suggesting that similar but slightly different genes are present. This was also clear from the sequence analysis of some of the fragments obtained with the *Rpi-blb3* specific primers (e.g. the B and C haplotypes of *Rpi-blb3*). These results reflect the complexity of *Rpi-blb3* gene family and underline the usefulness of the combined techniques to identify *Rpi-blb3* from the other alleles of the cluster. However, it should be stressed that in most cases where there was a discrepancy observed between marker assays and phenotypic assays this was outside *S. bulbocastanum*.

Discrepancies between molecular and functional assays can have several causes. This can be the case when plants containing an *R*-allele specific fragment are for some reason unable to interact with the Avr-protein. In such cases the *R*-gene fragment found is probably not translated into a protein interacting with the Avr-protein. Hall et al. (2009) studied natural variation of *Atr13* in a set of *Hyaloperonospora arabidopsidis* isolates and showed differential interaction of *Atr13* with *Rpp13* alleles. The *Atr13* alleles are recognized by *Rpp13* alleles and by *R* genes or genes at other loci in *A.*

Aardevo Exhibit 1010
Page 0800

thaliana. Interestingly, the *Rpp13-Rld* allele is unable to recognize *Atr13* alleles, and most probably detects an alternative effector protein in *H. arabidopsidis* isolates. Recognition specificity of different effectors by different alleles has previously also been demonstrated at the *RPM1* locus of *A. thaliana* (Bisgrove et al. 1994; Grant et al. 1995), at the *L* locus in flax (Dodds et al. 2004) and at the *Pto* locus in tomato (Kim et al. 2002; Ronald et al. 1992).

Also, plants may produce an *R*-allele fragment and show recognition of the Avr-protein (in our case for example in CPC3822), but still be susceptible to some of the key isolates. This might occur because of the high concentrations of the AVR-protein in our transient expression assay. A similar case was demonstrated by Schornack et al. (2004) for the Bs4/AvrBs3 interaction. Another explanation could be that the *R*-gene is probably active but unable to initiate a hypersensitive response in this specific phenotypic context. Finally, also plants not containing an *R*-allele specific fragment, but able to cause necrosis in presence of the Avr- protein will give discrepancies. Genotypes without any of these discrepancies and no indication for known *R*-gene presence, or plants with indication for known *R*-genes but with enhanced resistance spectra are good candidates to contain new *R*-genes.

Distribution of Rpi-blb genes in Solanum

The presence of *Rpi-blb1* and *Rpi-blb3* has been evaluated in approximately 1000 *Solanum* accessions. In addition, 72 accessions from *S. bulbocastanum* and related species were tested for *Rpi-blb1, Rpi-blb2* and *Rpi-blb3*. In total 35 *Rpi-blb1* haplotypes were found in the Mexican diploid species *S. bulbocastanum* and *S. cardiophyllum* as well as in the polyploid Longipedicellata *S. stoloniferum* (including *S. papita* and *S. polytrichon*), all geographically located in Central and South Mexico. Although the data have to be treated with caution, similar observations were made for *Rpi-blb3* were 22 haplotypes were found in the Mexican diploids *S. bulbocastanum*, *S. cardiophyllum S. pinnatissectum, S. nayaritense*, and *S. brachistotrichum* , as well as in the polyploid Longipedicellata *S. stoloniferum* and *S. hjertingii*. Interestingly, one *Rpi-blb3* haplotype was exclusively found in *S. bulbocastanum* originating from Guatemala thus showing a clear geographical/ *R*-gene haplotype association. The *Rpi-blb2* gene was only identified in a small number of *S. bulbocastanum* accessions from the Mexican provinces Oaxaca, Guerrero and Toluca. Since no haplotype variation within *Rpi-blb2* was found, this is a strong indication that this *R*-gene is from recent origin, as was suggested by Wang et al. (2008)

Aardevo Exhibit 1010
Page 0801

The results for the 3 genes show that their spread is limited to the diploid Mexican and Longipedicellata group and confined to Central America. The occurrence of similar haplotypes in different diploid species may be explained by the fact that these species are able to form interspecific hybrids, especially *S. bulbocastanum* and *S. cardiophyllum* (Graham 1965). The presence of both *Rpi-blb1* and *Rpi-blb3* in the polyploid Longipedicellata may be explained by the fact that this group of *Solanum* section *Petota* is of allotetraploid origin. It has been shown that *S. hjertingii*, for example, consists of an *S. verrucosum* like and an *S. cardiophyllum* like genome (Pendinen et al. 2008). Wang et al. (2008) suggested that *S. bulbocastanum* may be one of the parental species of the allotetraploid *S. stoloniferum*. The observation that the Late Blight resistance genes evaluated are confined to Central America might be true for the distribution of other *R*-genes as well.

Rpi-blb genes are found in different frequencies in S. bulbocastanum populations

Genebank accessions used in this study consist of plant material that has been generatively multiplied by the genebank prior to usage. In the Netherlands, at CGN, this is done without any *P. infestans* pressure, since protective spraying is applied every week. This might have resulted in slight frequency changes in the presence of the *Rpi-blb* genes compared to the actual situation in Mexico.

We assessed the frequency in which the different genes are present in the *S. bulbocastanum* accessions under study. Individual plants containing all 3 *Rpi-blb* genes have been found at very low frequency (less than 1 %) and in two *S. bulbocastanum* accessions only, probably because of the rarity of *Rpi-blb2*. The *Rpi-blb1* and *Rpi-blb3* genes have been detected together in 27% of the *S. bulbocastanum* plants analyzed (52% of the accessions). Only *S. bulbocastanum* PI498225 is fixed for *Rpi-blb1* and *Rpi-blb3*, suggesting that accumulation of these genes in *S. bulbocastanum* is a rare event.

Pyramiding of resistance genes is suggested to enhance durable resistance under agricultural conditions (Park et al. 2009; Haverkort et al. 2009; Pink 2002) and is also observed in polyploid *Petota* species, like *S. demissum*. Although pyramiding of resistance genes appears to be a common mechanism in polyploids, in diploid species it seems to be rare with respect to *Rpi-blb1, Rpi-blb2* and *Rpi-blb3*. On the other hand, our functional test with the 50 plants showed frequently a broader resistance spectrum than expected with the tested *Rpi-blb* genes, indicating the presence of other, unknown, *Rpi*-genes.

Aardevo Exhibit 1010
Page 0802

Almost all the accessions tested contain putative resistant and susceptible individuals. This might reflect a strongly fluctuating *P. infestans* pressure. The coexistence of resistant and susceptible individuals might also have an ecological meaning; in susceptible genotypes, newly recombined *R*-genes are under full pathogen pressure and functional *R*-genes are allowed to enter the population level.

The susceptible haplotype of *Rpi-blb1,* denoted as M1, is abundantly present and not confined to one specific geographical region, but scattered around in Central America (Appendix 1). The wide distribution of such a non-functional allele could imply that carrying a functional copy of *Rpi-blb1* affects the plants fitness in the absence of *P. infestans*. It is known that *R*-genes can result in serious fitness costs (Tian et al. 2003; McDowell and Simon 2006) and Kramer et al. (2009) showed that the *Rpi-blb1* gene is highly upregulated (37 times) once inoculated with *P. infestans*. Such observations suggest that we deal with a 'net cost of fitness under attack' (Korves and Bergelson 2004). In such cases null alleles might counterbalance potent but costly R-genes (McDowell and Simon 2006). Long-lived co-existence of resistant and susceptible alleles across the range of *Arabidopsis thaliana* ecotypes might be an example, as both alleles frequently occurred together within natural populations (Stahl et al. 1999). Another example would be RPS5 from *A. thaliana* (Henk et al. 1999).

Evolution of Rpi-blb genes

Sequence analysis of the fragments obtained for the *Rpi-blb1* and *Rpi-blb3* genes suggests an evolution through recombination and point mutations. In this respect there are clear differences between the 2 genes. The *Rpi-blb1* is thought to have resulted from a recombination event involving *RGA1-blb* and *RGA3-blb* (van der Vossen et al. 2003) and sequence analysis (Table 1) suggests a further evolution through point mutations, an evolutionary pattern typical for a Type II resistance gene as described for *R*-genes in natural lettuce populations (Kuang et al. 2004).

The situation with *Rpi-blb3* is more complex than with *Rpi-blb1* and most likely related to the complex nature of the cluster in which the gene is present. In this cluster several genes have almost identical sequences (Lokossou et al. 2009). Although all sequence information available has been taken into account for the design of the gene specific primers for *Rpi-blb3* it can not be excluded that some variants may not be discriminated by these primers. In *Solanum* as a whole even more *Rpi* genes have been mapped to the *Rpi-blb3* cluster. Examples are *Rpi-demf1* from *S. demissum* (Hein et al. 2007), *Rpi-Skn1* (Jacobs et al. 2010) and QTL's from *S. microdontum* (Colon et al. 1995; Micheletto et al.

Aardevo Exhibit 1010
Page 0803

2000; Sandbrink et al. 2000). Such resistances might represent *R* genes which are homologues or recombinants sharing ancestry with *Rpi-blb3* as well as *R*-genes that have evolved independently and interact with other effectors.

The presence of conserved SNP's patterns among the haplotypes suggests recombinations between ancestors of *Rpi-blb3*. Such recombinations might have resulted in the *Rpi-blb3* group of B and C haplotypes which are similar to the *R2* gene as suggested by Lokossou et al. (2009).

Recent studies show that the recombination level at *R*-clusters is increased following pathogen infection, suggesting a mechanism that induces temporary genome instability in response to extreme stress, as recently reviewed by Friedman (2007). This instability might well be connected with the high intensity of transcription of some *R*-genes seen just after infection (e.g. Kramer et al. 2009) in which the genome temporary must be accessible for efficient transcription. It is conceivable that, depending on the disease pressure, we have a fast pattern of evolution of *R*-gene clusters dominated by sequence exchange or a slow pattern of evolution dominated by point mutations. Both evolutionary mechanisms appear to have shaped the *Rpi-blb1* and *Rpi-blb3* clusters. Additionally, the distinction between type I and II seems rather artificial; in real life a continuum is seen with type I and II as extreme borders.

The absence of mutation or recombination in *Rpi-blb2* fragment amplified from *S. bulbocastanum* and the low percentage of *Rpi-blb2* containing plants show that this gene emerged recently and didn't evolve yet.

New P. infestans R-genes identified

In our analysis, 76% of Late Blight resistant genotypes can not be explained by only the presence of the *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* genes. Clear examples are *S. bulbocastanum* accession CGN23010 and P1275199, but also genotypes from CGN17689 and CGN21306 contain additional resistance gene(s), because of their resistance spectrum different from *Rpi-blb1*, *Rpi-blb2* or *Rpi-blb3* and thus providing new source(s) for future Late Blight resistance breeding. The *S. stoloniferum* accession PI205522, containing *Rpi-blb1* and *Rpi-blb3* based on the marker assays, contains an additional gene because of the resistance spectrum observed (Table 2). The gene involved is probably a paralog of $R4^{MA}$ (Van Poppel et al. 2009) as it confers resistance to isolate PIC99189 (avirulent on $R4^{MA}$, see supplementary Table 2) and a hypersensitive response with Avr4 effector (van Poppel et al. 2008) was found (unpublished results). It can not be excluded that the recently described gene *Rpi-bt1* (Oosumi et al. 2009), is responsible for some of the resistant phenotypes found in *S. bulbocastanum.*

Aardevo Exhibit 1010
Page 0804

Since we found *Rpi-blb1* and *Rpi-blb3* in species related to *S. bulbocastanum* it would make sense that some of newly identified resistance genes in *S. bulbocastanum* might already have been identified in related species, like the resistances found in *S. cardiophyllum* and *S. pinnatisectum* (Kuhl et al. 2001).

CONCLUSION

Our study provides a clear picture of the distribution of Late Blight resistance genes so far isolated from *S. bulbocastanum.* It shows that *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* have a very limited distribution among species. Few other species outside *S. bulbocastanum* contain the genes underlining the restricted interaction between *S. bulbocastanum* and other species. Several different haplotypes were detected for *Rpi-blb1* and *Rpi-blb3* and for 27 % of them functionality was shown. Our functional screen reveals the presence of more Late Blight resistance genes in *S. bulbocastanum*, confirming that this species is a rich source of Late Blight resistance genes as indicated by Budin (2002).

Allele mining in *Petota* germplasm with primers identifying known *R*-genes, allows the breeders to identify more amenable species for breeding. For example, *S. bulbocastanum* hardly crosses with cultivated potato (Helgeson et al. 1998), and the identification of *Rpi-blb1* alleles in *S. stoloniferum* largely facilitates the introgression breeding of *Rpi-blb1* for commercial usage (Vleeshouwers et al. 2008; Wang et al. 2008).

As observed by us and others (e.g. Budin. (2002)) a hotspot for resistance genes is found were host and pathogen co-evolve. This is also reflected by the putative new *R*-genes identified in this study. Given the high diversity of genes found, some promising *R*-genes might even not be present in the germplasm collections. New extensive collections in hot-spots of co-evolution and subsequent evaluations will undoubtedly provide new sources of resistance that can be used in breeding.

MATERIAL & METHODS

Plant material

The *Solanum* species used are listed in Table S1. They were obtained from the Centre for Genetic Resources of the Netherlands (CGN). DNA samples of 14 *S. bulbocastanum* accessions originating from the United States potato Genebank, in Sturgeon Bay, WI, USA, were kindly supplied

Aardevo Exhibit 1010
Page 0805

by Dr. A. del Rio, Univ. Wisconsin, USA. (referred to as PI accessions in Table 1). When available up to 10 genotypes per accession were used. Individual clones listed in Table 2 were retrieved from the plant material described in Jacobs et al. (2008) for *in vivo* experiments consisting of detached leaf assays and effector agro-infiltrations.

PCR amplification, DNA sequencing and computer analyses

Specific primers used to amplify fragments of the 3 *R* genes studied are listed in Table S3 and Fig.S1. PCR products of randomly selected positive genotypes within an accession were sequenced directly to confirm their identity and to identify single nucleotide polymorphism (SNPs). Sequencing reactions were performed using a dye terminator cycle sequencing reaction kit (Perkin-Elmer, Pt Biosystem, Warrington, UK) and analyzed on an ABI3700 automated sequencer (Applied Biosystem, La Jolla, CA, USA). Multiple sequence alignments were conducted using DNASTAR Lasergen MegAlign version 8.0.2, permitting the distinction of the SNP's in comparison to the cloned *Rpi-blb* gene sequence (*Rpi-blb1* GenBank accession number AY426259.1; *Rpi-blb2* GenBank: DQ122125.1; *Rpi-blb3* GenBank: FJ536326.1). Unique sequences are reported in Table 1a and 1b. Nucleotides are numbered from the first nucleotide of the coding sequence of the cloned gene. The synonymous or silent SNP's not leading to a change in amino acid were differentiated from the non synonymous SNP's.

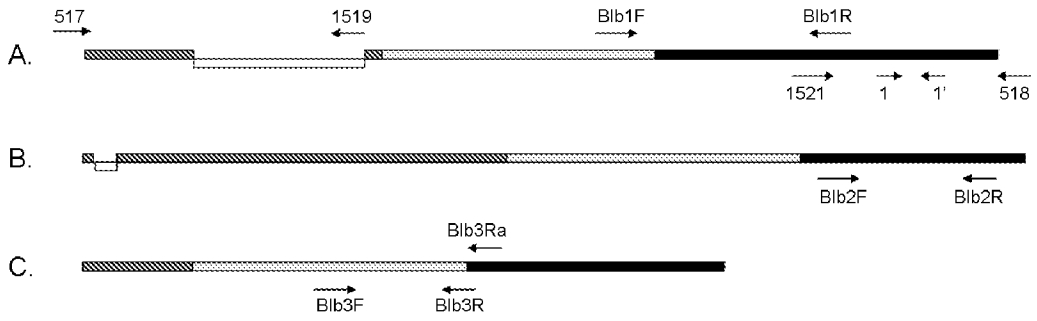


Figure S1: Location of *Rpi-blb* genes specific primers. *Rpi-blb1* (A), *Rpi-blb2* (B) and *Rpi-blb3* (C) nucleotide sequences are from the start to the stop codon and drawn to scale. The regions corresponding to the leucine zipper or the coil coiled, the intron, the NBS domain, and the LRR domain are highlighted in grey, white, light grey and black, respectively. Primer pairs used in this study are 517/1519, Blb1 F/Blb1 R, 1521/518, 1/1', Blb2F/Blb2R, Blb3F/Blb3R and Blb3F/Blb3Ra.

Aardevo Exhibit 1010
Page 0806

Resistance assay

Detached leaf assays were used to determine the resistance phenotypes of the tested genotypes with 8 *Phytophthora infestans* isolates, PIC99177, PIC99189, PIC99183, IPO-C, 90128 and UK7824. Inoculum preparations and inoculations were performed as described by Vleeshouwers et al. (1999). Six days after inoculation, infection symptoms were scored on a scale from 1-8, to discriminate between very susceptible until very resistant; score 1-3 means clear infection with 1 heavily sporulating lesion and 3 ample sporulating lesion thus characterized as susceptible (S). Scores 4 and 5 are not clearly susceptible or resistant, therefore called quantitative (Q). Resistant (R) phenotypes are characterized by the score 6 with hypersensitive lesions larger than 10 mm, score 7 in between 4 and 10 mm and score 8 implicates hypersensitive lesions smaller than 4 mm in diameter.

Effector agro-infiltration on wild species

The Avr-effectors *Avrblb1* (Vleeshouwers et al. 2008) and *Avrblb2* were cloned into vector pK7WG2 and *Pi-Avr2* was cloned into vector pGRAB (Lokossou et al. 2009). The resistance genes *R3a* (Huang et al. 2004) and its cognate effector *Pi-Avr3a* (Bos et al. 2006) were cloned into the vector pGRAB (kindly provided by S.N. Chapman) and used as control for the agro-transformation efficiency. The constructs were transferred into the same *A. tumefaciens* strain AGL1 carrying pVirG. The different constructs cultures were grown in LB medium supplemented with 75 mg/L choramphenicol and 50 mg/L kanamycin and 4 mg/L tetracycline for *Pi-Avr2, Avr3a and R3a*, with 100 mg/L spectinomycin, 75 mg/L chloramphenicol and 4 mg/L tetracycline for *Avrblb1*and *Avrblb2*. Inoculum of the first experiment was prepared using the growth of bacteria in LB media (15 gr/L Agar, 10 gr/L Bacterial Peptone, 10 gr/L NaCl and 5 gr/L Yeast Extract) until stationary phase and subsequently spinned down at 3600 RPM. Bacteria were resuspended in MMA media (10mM Acetosyringone, 10mM MES and 5 gr/L MS salts) In the second experiment the bacteria were precultured in 3 ml LB, where after they were transferred to YEB medium(5 gr/L Beef Extract, 1 gr/L Yeast Extract, 5 gr/L sucrose, 5 gr/L Bacterial Peptone and 2mM MgSO4) and allowed to grow to OD_{600} 0.4-2.0, afterwards they were spinned down at 3600 RPM for 10 min. and subsequently resuspended in MMA medium. Individual constructs were prepared at an OD_{600} of 0.2 and the combination *Pi-Avr3a /R3a* at 0.3 were used to infiltrate 3 to 5 weeks old wild species plants, reaching their 9th leaf stage (Vleeshouwers and Rietman 2009). Specific recognition of the *Avr*-gene by a putative *R* gene present induces hypersensitive cell death response (HR) from 5 days post-infiltration. Response intensity was scored in percentage of

Aardevo Exhibit 1010
Page 0807

infiltrated area showing cell death. Zero to 20% of dark lesion were characterized by a (-), 21 to 35% by (+), 36 to 65% by (++) and 66 to 100% by (+++).

Geographical localization of the accessions

Geographical origin (latitude, longitude) of the accessions was retrieved from the CGN website (www.cgn.wur.nl) and from literature (Rodriguez and Spooner 1997; Hijmans et al. 2007). The desktop application Google Earth (GE) was used to visualize the location of the genotypes. Different tags were associated to genotypes containing *Rpi-blb1*, *Rpi-blb2*, *Rpi-blb3* or their combinations.

We used the internet application SUPRAmap (http://supramap.osu.edu; Janies. 2007) in order to associate the different haplotypes with their geographic distribution. The out-group for the *Rpi-blb1* and *Rpi-blb3* was assigned by performing a phylogenetic analysis in Mega Software (version 4), using the 'minimal evolution' option with standard parameters. The classification described in Table 1a and 1c was used to illustrate the geographical localization of groups B and C (for Table 1c) and the M1 clade from Table 1a is highlighted to illustrate the spread of the M1 allele (see supplementary KMZ file).

ACKNOWLEDGEMENTS

We would like to thank dr. Danial Janies (institute, country) for his kind help and access to the SUPRAmap website. We are grateful, to the Scottish Crop Research Institute (SCRI) and The Sainsbury Laboratory (TSL) for giving us the opportunity to use the effector *Pi-Avr2* and *Avrblb2* respectively; and to Nicolas Champouret (Wageningen UR Plant Breeding) for cloning *Avrblb2* into the expression vector. We acknowledge P.R.J. Birch, G. Kessel and F. Govers for providing *Phytophtora* isolates. Funding for this project was provided by the EU (contract No. FOOD-CT-2005-513959 BIOEXPLOIT) for the Bioexploit project and a grant from the Wageningen University Fund.

Aardevo Exhibit 1010
Page 0808

Supplementary Table S2: Characteristics of the set of *P.infestans* isolates used in this study

Isolate	Year	Geographic origin	Mating type	Obtained from	Virulence profile	Reference
PIC 99177	1999	Metepec, Mexico	A2	Kessel, PRI, WUR	1.2.3.4.7.9*.11	Flier et al. 2002
PIC 99189	1999	Metepec, Mexico	A2	Kessel, PRI, WUR	1.2.5.7.10.11	Flier et al. 2002
PIC 99183	1999	Metepec, Mexico	A2	Kessel, PRI, WUR	1.2.3.4.5.7.8.10.11	Flier et al. 2002
IPO-C	1982	Belgium	A2	Kessel, PRI, WUR	1.2.3.4.5.6.7.10.11	
90128	1990	Geldrop, Netherlands	A2	Govers, Phytopathology WUR	1.3.4.7.8.10.11	Vleeshouwers et al. 1999
UK7824	1978	United Kingdom	A1	Govers, Phytopathology, WUR	1.2.3.6.7	

Supplementary Table S3. Overview of primers used for the screening of the wild potatoes species

Marker	PCR primer (5′ to 3′)	Tma	Fragment lenght	Reference
Blb1 F/R	F: AACCTGTATGGCAGTGGCATG	58 °C	820 bp	Wang et al. 2008
	R: GTCAGAAAAGGGCACTCGTG			
1/1'	F: CACGAGTGCCCTTTTCTGAC	50 °C	213 bp	Colton et al. 2006
	R: ACAATTGAATTTTTAGACTT			
1521/518	F: GAAAGTCTAGAGTTACACTGG	58 °C	704 bp	Wang et al. 2008
	R: CAATCACAATGGCAGGAACC			
517/1519	F: CATTCCAACTAGCCATCTTGG	58 °C	750 bp	Wang et al. 2008
	R: TATTCAGATCGAAAGTACAACG			
RGA1F/R	F: CAGTCACTTTCTTGTTTGCCG	55 °C	845 bp	Wang et al. 2008
	R: CAGTAGTGAAGTCACTGTGTG			
Blb2F/R	F: GGACTGGGTAACGACAATCC	58 °C	773 bp	Wang et al. 2008
	R: AGCACGAGTTCCCCTAATGC			
Blb3F/R	F: TGTCGCTGAAAGAGTAGGCC	50 °C	618 bp	
	R: TATGGAGTGGCTTCTTGAAC			
	Ra: TCCTGAGGTTTATATGACTCATC		757 bp	

Aardevo Exhibit 1010
Page 0809

Chapter 4

Phytohthora infestans resistance gene *Rpi-mcd1.1* from the Argentinean *Solanum microdontum* is functionally distinct from other *R2* homologues of Mexican origin

---

Anoma A. Lokossou, Vivianne G. A. A. Vleeshouwers, Edwin A. G. van der Vossen, Richard G. F. Visser, Evert Jacobsen

ABSTRACT

Wild *Solanum* species are an important reservoir of disease resistance (*R*) genes, which are under constant evolutionary pressure amongst others via recombination events between alleles. The major Late Blight (MLB) resistance locus on linkage group IV harbors *R2* homologues isolated from *Solanum bulbocastanum*, *S. demissum*, *S. edinense*, *S. hjertingii* and *S. schenckii* that all originate from Mexico. Here we report about the cloning of the NBS-LRR gene *Rpi-mcd1.1* which belongs to the same MLB locus in *S. microdontum* originating from Argentina. *R2* allele mining in the resistant parent RH90-038-21 resulted in 27 homologues among which *Rpi-mcd1.1* was identified. *Rpi-mcd1.1* shares 91% nucleotide identity with *R2* and polymorphic nucleotides are mainly located in the LRR region. *Rpi-mcd1.1* is a unique member of the *R2* family since it provides a resistance that is functionally different from the resistance conferred by the Mexican *R2* gene family. The analysis of recombinant genotypes and the amino acid sequence both reveal multiple recombination events which recently occurred inside the *Rpi-mcd1.1* resistance locus. Phenotypically, *Rpi-mcd1.1* was found to confer qualitative resistance to some *Phytophthora infestans* strains, but quantitative resistance to some other, more aggressive strains. Further characterizations of parents and RH94-076 progeny permitted the identification of two additional *R* genes segregating in the population, namely *Rpi-mcd1.2* also originating from *S. microdontum,* and *Rpi-phu2* originating from *S. phureja.*

Aardevo Exhibit 1010
Page 0811

INTRODUCTION

Potato is an important staple food originating from South America and is grown in more than 100 countries, under temperate, subtropical and tropical conditions. About 200 wild potato species, partly domesticated by generations of local farmers, have been collected in South America by different expeditions in the last decades (Hawkes and Hjerting 1969). In the rest of the world, all potato cultivars are derived from the wild species *Solanum tuberosum*. Potato monoculture and the lack of genetic diversity helped the emergence and dominance of late blight disease caused by the oomycete *Phytophthora infestans* (*Pi*).

In general the plant defense system is triggered upon pathogen effector recognition by the plant's resistance genes resulting in cell death of the cells attacked (McHale et al. 2006) and consequently decreasing or even blocking the pathogen's growth. So far, the cloned late blight resistance (*R*) genes belong to the NBS-LRR gene family and *P.infestans* avirulence (*PiAVR*) effectors to the RXLR family. Key domains necessary for R/AVR interactions have been identified in the nucleotide binding site (NBS) and the leucine rich repeat (LRR) domains (Catanzariti et al. 2010; Collier and Moffett 2009; Ellis et al. 2007; Jones and Dangl 2006; Rairdan and Moffett 2006).

The attempt to combat the disease by introgression of *Solanum demissum* resistance genes (R-genes) into susceptible cultivars failed because of the rapid emergence of virulent *P. infestans* isolates, thus resulting in the so-called "broken R-genes". To control the disease, the potato polyculture as applied in the Toluca valley of Mexico and in the Andes inspired modern potato breeders to R-gene pyramiding in cultivated potatoes. Pyramiding is pictured in mixing individual *R-gene* containing cultivars or creating a cultivar containing stacked R-genes. In the attempt to assemble this molecular weapon, additionally to the *Solanum demissum* R-genes, many R-genes were identified and isolated from wild *Solanum* species comprising *S. bulbocastanum* hosting *Rpi-blb1*, *Rpi-blb2*, *Rpi-blb3*, *Rpi-bt1* (Lokossou et al. 2009; Oosumi et al. 2009; van der Vossen et al. 2003; van der Vossen et al. 2005), *S. stoloniferum* harboring *Rpi-blb1* allelic versions *Rpi-sto1* and *Rpi-pta1* (Wang et al. 2008) *; S. venturii* containing *Rpi-vnt1.1, Rp-vnti1.2* and *Rpi-vnt1.3* (Foster et al. 2009; Pel et al. 2009).

R2 allele mining using homologous primers successfully permitted the cloning of *Rpi-abpt*, *R2like*(Lokossou et al. 2009; Park et al. 2005a; Park et al. 2005b; Park et al. 2005c) and *Rpi-snk1-1*, *Rpi-snk1-2*, *Rpi-edn1-1*, *Rpi-hjt1-1*, *Rpi-hjt1-2* and *Rpi-hjt1-3* (Champouret 2010; Jacobs et al. 2010), originating from *S. demissum*, *S. schenckii* , *S. edinense* and *S. hjertingii*. A focused allele mining study using *Rpi-blb3* specific primers in *Solanum* germplasm identified *S. pinnatisectum*, *S. bulbocastanum* (including some subspecies), *S. hjertingii, S. nayaritense, S. brachistotrichum, S. cardiophyllum* and *S.*

Aardevo Exhibit 1010
Page 0813

stoloniferum as containing putative functional *Rpi-blb3* homologues (Chapter 3). All *Rpi-blb3*-like containing *Solanum* species are geographically located in Mexico and Guatemala. The functional *R* genes that confer resistance to *P. infestans* among them, all interact with the cognate *P. infestans* avirulence gene *PiAvr2* (RXLRfam7) (Champouret 2010; Lokossou et al. 2009). Another strategy to control late blight is to identify and introgress chromosome loci harboring genes responsible for quantitative resistance traits. Quantitative resistance loci (QRL) have been largely studied and are believed to provide a more stable durable resistance (Gebhardt 1994; Leonards-Schippers et al. 1994; Oberhagemann et al. 1999), so called horizontal resistance in contrast to the vertical resistance provided by *R* genes. Late blight QRL have been identified in many wild *Solanum* species including the Argentinean wild diploid species *S. microdontum* (Colon et al. 1995; Micheletto et al. 2000; Sandbrink et al. 2000). Interestingly, several of the identified PiQRL mapped to the short arm of linkage group IV (Bradshaw et al. 2004; Bradshaw et al. 2006; Leonards-Schippers et al. 1994; Sandbrink et al. 2000), on the *R2* locus (Hein et al. 2009; Hein et al. 2007). Recent studies determined the quantitative resistance of *S. microdontum* accession BGRC 18302-34 (CGN 21342) to be conferred by *Rpi-mcd1,* which was mapped to the *Rpi-blb3* locus at linkage group IV. *Rpi-mcd1* was characterized as a weak-effect resistance gene and expected to be a member of the NBS-LRR gene family (Tan et al. 2008). Since *Rpi-mcd1* was mapped with a complex race IPO-C that is virulent on known members of *R2* cluster, we were intrigued by the possibility to identify a functionally distinct R gene at the *R2* locus.

In our study, we investigated the late blight QRL *Rpi-mcd1* identified in *S. microdontum*. We characterized the resistance spectrum of the population that segregates for *Rpi-mcd1* (Tan et al. 2008) with genetically diverse *P. infestans* isolates permitting the identification of at least three segregating genes. Allelic mining strategies resulted in the identification of *Rpi-mcd1.1*which was functional in a transient complementation assay with *Nicotiana benthamiana.*

RESULTS

Characterization of the parental and progeny resistance spectra

To functionally characterize the resistance of *Rpi-mcd1*, detached leaves of the parental clones RH90-038-21 and RH88-025-50 and their RH94-076 progeny were inoculated with *P. infestans* isolates IPO-C, IPO-0 and PIC99177. In addition, Blb99-256-3, 707TG11-1, AM3778-16 and

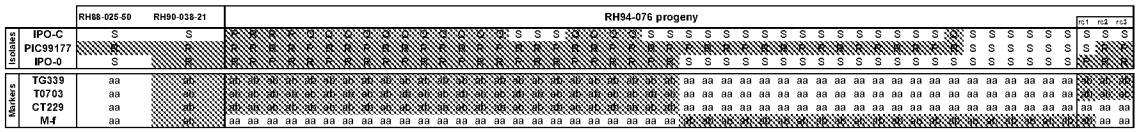
Aardevo Exhibit 1010
Page 0814

Cebeco44158-4 from which *Rpi-blb3*, *Rpi-abpt*, *R2-like* or *R2* were isolated, respectively, were included as controls (Table 1 and Fig 1). As expected for typical *R2*or *Rpi-blb3* activity, Blb99-256-3, 707TG11-1, AM3778-16 and Cebeco44158-4 were resistant to IPO-0 and susceptible to IPO-C and PIC99177 (Fig 1). Both clones RH90-038-21 and RH88-025-50 were resistant to PIC99177, with clear cell death lesions at the inoculation spot. However the population segregated into 41 resistant and 7 susceptible genotypes, which suggests that the resistance to PIC99177 is based on 2 (or more) *R* genes originating from both parents. When inoculated with IPO-0, RH90-038-21 was moderately resistant displaying small to large lesions with no or weak sporulation, starting at day 7, whereas RH88-025-50 was susceptible. The resistance segregated in a 1:1 manner in the RH94-76 population, with 27 resistant and 21 susceptible genotypes, suggesting monogenic inheritance of a single dominant *R* gene. In the first experiment, the highly aggressive isolate IPO-C generally caused susceptible phenotypes with large sporulating lesions (Table 1), although previous studies detected *Rpi-mcd1* with this isolate under field conditions. In order to have a better comparison of the resistance phenotypes, we challenged the RH94-076 population plants with the isolates IPO-C and PIC99177, and applied both isolates to the same leaf (Fig.2). In this second experiment, three resistance patterns were observed; tested plants were resistant to PIC99177 and IPO-C, or only to PIC99177, or susceptible to both isolates. None of the tested plants was simultaneously resistant to IPO-C and susceptible to PIC99177. Half of the RH94-76 population displayed clear hypersensitive response (HR)-like cell death lesions at 5 days post inoculation with isolate IPO-C, followed by a low degree of sporulation at 7 dpi, and this was interpreted as quantitative resistance. More than three quarters of the progeny displayed clear hypersensitive response lesions as the parental clones upon PIC99177 inoculation (Table 1). Additionally, there appeared to be a correlation between the resistance to PIC99177 and IPO-C. Champouret et al (2009) described PIC99177 as relatively weak isolate, which can detect a "QRL" or weak R genes in detached leaf assays (DLA). In contrast, for the much more aggressive IPO-C isolate, DLA is considered less appropriate to detect QRL or weak R genes . In line with this, the resistance to IPO-C was mapped under field conditions (Tan et al. 2008). We decided that for our mapping studies using DLA, PIC99177 is an appropriate isolate to use. In conclusion, the genotypes from RH94-76 population which are resistant to the isolates PIC99177, IPO-0 and quantitatively resistant to IPO-C contain the targeted resistance gene *Rpi-mcd1*.

Markers bordering the late blight resistant locus on linkage group IV, TG 339, T0703 and CT 229 (Tan et al. 2008, Park et al. 2005b) fully co-segregate with resistance to IPO-0 PIC99177 and IPO-C with the exception of 1/3 of the PIC99177 resistant offspring in which the markers were not detected (Table 1). This is in accordance with the resistance phenotype of the parental clones RH90-038-21 and RH88-025-50, confirming the presence of two different genes conferring resistance to PIC99177.

Aardevo Exhibit 1010
Page 0815

Table 1: Overview of the resistance screening of the parental clones RH88-025-50 and RH90-038-21 and their progeny, the cross population RH94-076. Plants were challenged with three *P. infestans* isolates, IPO-C, IPO-0 and PIC99177. The plants genomic DNA was tested for the presence of the markers TG339, T0703, CT229 and Mf. Abbreviations: S: susceptible; R: resistant.



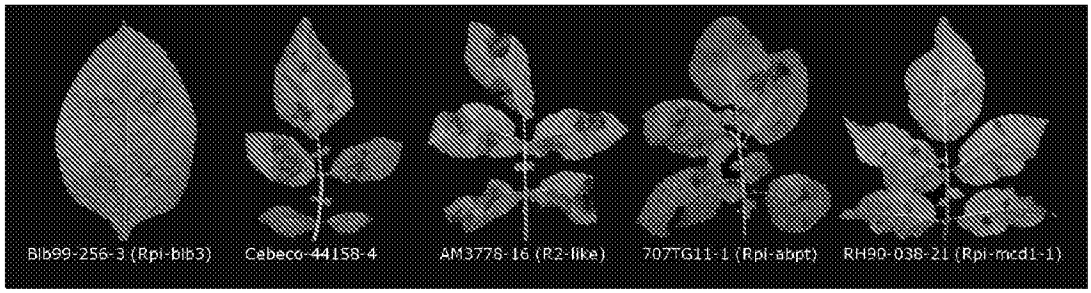


Figure 1: Typical disease phenotypes 8 days after inoculation with a sporangiospore suspension of Phytophtora infestans isolate PIC99177. The genotypes carrying the resistance genes *Rpi-blb3* (Blb99-256-3), *R2* (R2-differential), *R2-like* (AM3778-16), *Rpi-abpt* (707TG11-1) couldn't stop the pathogen proliferation. While the genotype carrying *Rpi-mcd 1-1*(RH90-038-21) showed clear hypersensitive response (HR) thus stopping *Phytophtora* growth.

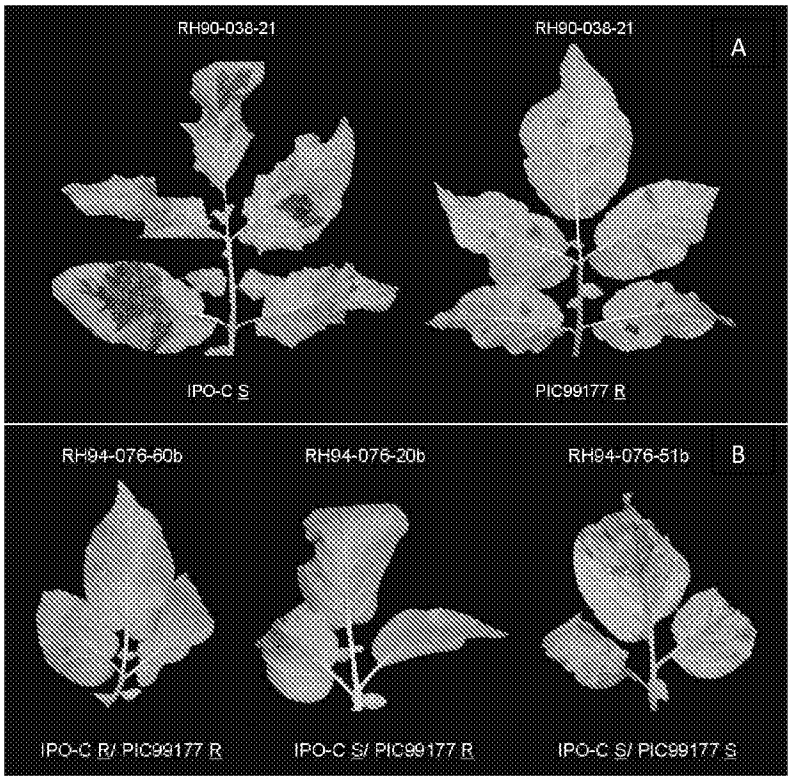


Figure 2: (A) Phenotype of the resistant parent RH90-038-21 with clear susceptibility to IPO-C and resistance to PIC99177 and (B) different possible phenotypes of RH94-076 individuals at seven days post inoculation with *P. infestans* isolates IPO-C and Pi99177. The isolate IPO-C has been inoculated on the left part of individual leaves, and PIC99177 on the right part.

Aardevo Exhibit 1010
Page 0816

The parental clones are resistant to the isolate PIC99177 and are derived from *S. microdontum* for RH90-038-21 and from *S. phureja* for RH88-025-50. We deduced that these genotypes carry different independently inheriting genes which confer resistance to PIC99177 that we named *Rpi-mcd1.1* and *Rpi-phu2*, respectively.

In addition, three recombinant genotypes were identified (Table 1). Two recombinant genotypes have resulted from one recombination event which occurred between the marker CT229 and the resistance locus. Remarkably, the existence of the third recombinant genotype can only be explained by the existence of 2 R genes in the locus, *Rpi-mcd1.1* and *Rpi-mcd1.2* conferring resistance to PIC99177 and IPO-0, respectively. The recombination event occurred between the two resistance genes, with *Rpi-mcd1.1* located next to the marker CT229 and *Rpi-mcd1.2* next to the markers TG339 and T0703.

Cloning of Rpi-mcd1.1

Rpi-mcd1.1 belongs to the major late blight resistance locus on LG IV that also harbors *Rpi-blb3* (Tan et al. 2008). In view of the conserved markers CT229 and TG339 we hypothesized that *Rpi-mcd1.1* and *Rpi-blb3* were homologues and thus attempted to clone the gene through an allele mining strategy. The same primers used to amplify the *Rpi-abpt, R2* and *R2-like* (Lokossou et al 2009) candidates were used to amplify full-length *R2GH* from the potato clone RH90-038-21. Amplicons of the expected size were cloned into pDONR221 and fully sequenced. In total, 27 unique sequences were obtained from RH90-038-21 featuring 88 % to 92% nucleotide (nt) identity with *Rpi-blb3* and 89% to 94% nt identity with *R2*.

Phylogenetic analyses using UPGMA algorithm method resulted in the classification of the *R2GH* sequences in three clades (Fig.3). Specific primers differentiating between and within the clades of homologues (Table S2 and Fig 3) were tested on RH94-076 population to search for a correlation with the resistant phenotype observed. The primer pairs M-a and M-d could differentiate the clades I and IV, respectively, but there was no correlation with the resistance phenotype. M-f primer pair is specific to clade II and interestingly, M-f and M-h primer pairs amplified specific fragments which segregate in the progeny of RH94-076 in repulsion phase when comparing to the resistant phenotype (Table 1). This result means that the candidate *R2GH1-18*, *R2GH1-19*, *R2GH1-23*, *pseudoR2GH1-3, pseudoR2GH1-5* and *-pseudoR2GH1-9* originate from the *S. tuberosum* genotype SH 76-128-1857. (Tan et al.2008). Since these candidates are not from *S. microdontum* descent they were discarded from further analyses.

Aardevo Exhibit 1010
Page 0817

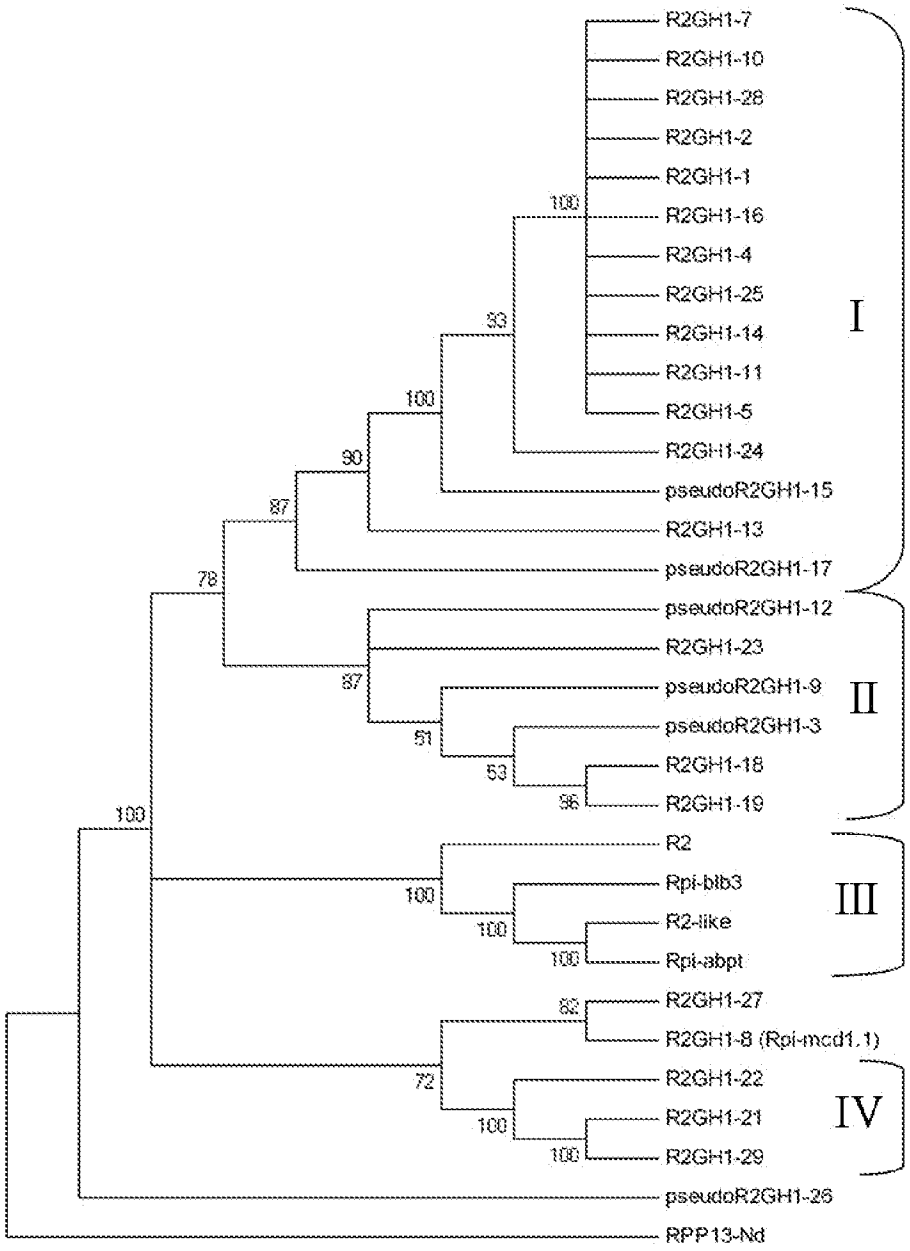


Figure 3: Phylogenetic tree at the nucleotide level of 27 *R2GH* candidates including *Rpi-mcd1-1*and four resistance genes *Rpi-blb3*, *Rpi-abpt*, *R2* and *R2-like* belonging to the same locus on LG IV. RPP13 had been used as an out-group. The clade's numbers are indicated on the right side. UPGMA algorithm with bootstrap analysis of 500 replicates. Numbers above the branches are bootstrap values in percentages.

Aardevo Exhibit 1010
Page 0818

Additionally, nucleotide and amino acid sequence analyses identified six pseudo-genes harboring early stop codons in the open reading frame which were also discarded from further studies Finally, the remaining eleven *R2GHs* which are coding for unique amino acid sequences were considered candidates for *Rpi-mcd1.1*.

To study which of the eleven *R2GHs* confers late blight resistance, they were targeted for functional analysis. Candidates were transiently expressed in *N. benthamiana* leaves and further inoculated in DLA with the avirulent *P. infestans* isolates PIC99177 and IPO-C. Phenotypes were observed at seven days post inoculation (Fig 4). No traces of sporulation were observed for both isolates on *N. benthamiana* leaves transformed with *R2GH1-8*, whereas the leaves expressing the other constructs showed large sporulating lesions (Fig S1). *P. infestans* isolate PIC99177 displayed clearly smaller lesion at the inoculation point in comparison to IPO-C, which may be explained by the higher aggression level of the latter strain (Champouret et al. 2009). In conclusion, scoring of the tested leaves indicated that *R2GH1-8* conferred strong resistance to PIC99177 and was therefore designated *Rpi-mcd1.1*.

To confirm complementation by *Rpi-mcd1.1* in potato, stable transformants of potato Desiree were generated. Detached leaves of mature transgenic potato plants were challenged with *P. infestans* isolate PIC99177 and IPO-0. As expected, stable transformed plants containing *Rpi-mcd1.1* were resistant to PIC99177, but susceptible to IPO-0 (data not shown), indirectly confirming the existence of the second R-gene in the locus of the parental plant RH90-038-21, which was named *Rpi-mcd1.2*.

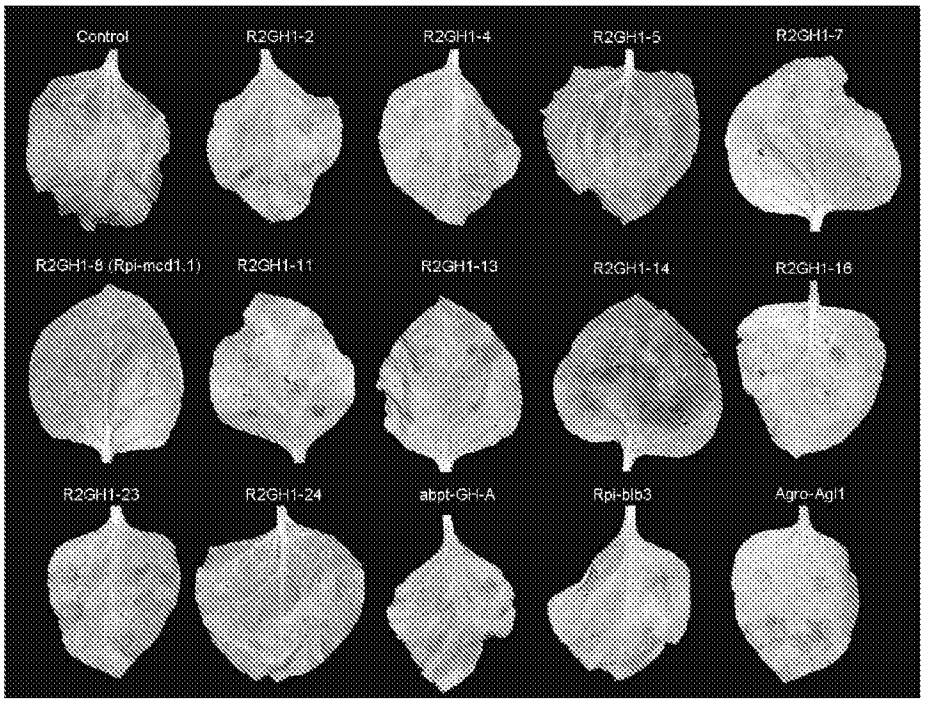


Figure 4: *N. benthamiana* infiltrated leaves with *R2GH*-candidates, the resistance gene *Rpi-blb3* or an empty *Agrobacterium* (Agl1) displaying different resistance phenotypes when inoculated with *P. infestans* isolates IPO-C (left part of the leaf) and PIC99177 (right part of the leaf).

Aardevo Exhibit 1010
Page 0819

Molecular structure and recognition specificity

Alignment of the nucleotide sequences coding for complete ORFs, and subsequent analysis of informative polymorphic sites (IPS), i.e. sites where two or more genes carry the same nucleotide (Parniske et al. 1997), reveals a region of 1200 nucleotides residing between kinase 2 and LRR6 (Fig 5) where recombination is increased. Three constant nucleotide structures are present in the *R2GH* pool represented in the clustal W alignment (Fig.5) by *R2GH1-1*, *R2GH1-18* and *R2GH1-21* belonging to clades I, II and IV, respectively (Fig 3). Recombination between these constant structures gave rise to seven different homologues, i.e. *R2GH1-8, R2GH1-22, R2GH1-13, R2GH1-19, R2GH1-23, R2GH1-24* and *R2GH1-27.* Interestingly, *R2GH1-19* and *R2GH1-24* appear to be complementary recombinant genes which arose from a double recombination event in the region of 379 nucleotides between nt positions 1259 and 1638 (Fig 5). *Rpi-mcd1.1* (*R2GH1-8)* appears to be a combination of sequences of the three constant structures plus one unknown gene between the nt positions 1043 and 1259 (Fig 5 and Fig 6). These clear blocks of sequences from different members of *R2GH*s pool indicate that sequence exchange events have been involved in the rise of resistance gene *Rpi-mcd1.1.*

To study the nature of specificity conferred by *Rpi-mcd1.1*, the nucleotide sequences of R2GHs were first aligned with *R2/ Rpiblb3* homologues. The *R2GHs* contain ORFs of 2538-2541 nucleotides that encode proteins of 845-846 amino acids harboring the same signature sequences as R2 homologues. Clustal W amino acid sequence alignment of *Rpi-mcd1.1* together with the three constant structures described above *R2GH1-1, R2GH1-18* and *R2GH1-21*, and with *R2* reveals identical LZ-NBS region with *R2* delimited by the same break point described in the structural analyses of *R2* homologues by Lokossou et al (2009) (Fig.6). *Rpi-mcd1.1* LRR domain differs from *R2* LRR in the polymorphic amino acids which are mainly localized in the xxLxLxxx repeats indicating a putative Pi-Avr differential recognition.

To study *Rpi-mcd1.1* specificity at the functional level, the R-AVR interaction was reconstructed in *N. benthamiana.* The avirulence protein PiAvr2 that is known to evoke cell death responses when co-expressed with R protein of the R2 family was tested for response to Rpi-mcd1.1. *Agrobacterium* strains expressing PiAvr2 were co-infiltrated together with *Agrobacterium* strains expressing Rpi-mcd1.1 in *N. benthamiana* leaves. Co-infiltration of Pivr2 together with R2 or Rpi-blb3 constructs were used as a positive control and single R-gene or PiAvr2 gene infiltration were used as negative controls.

Aardevo Exhibit 1010
Page 0820

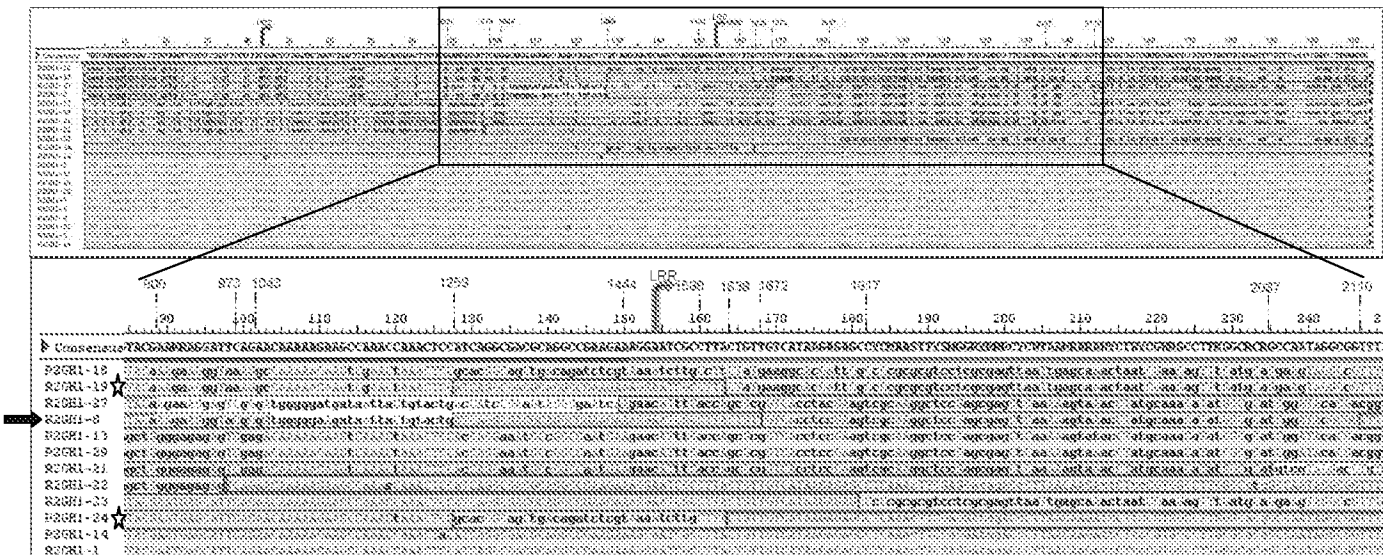
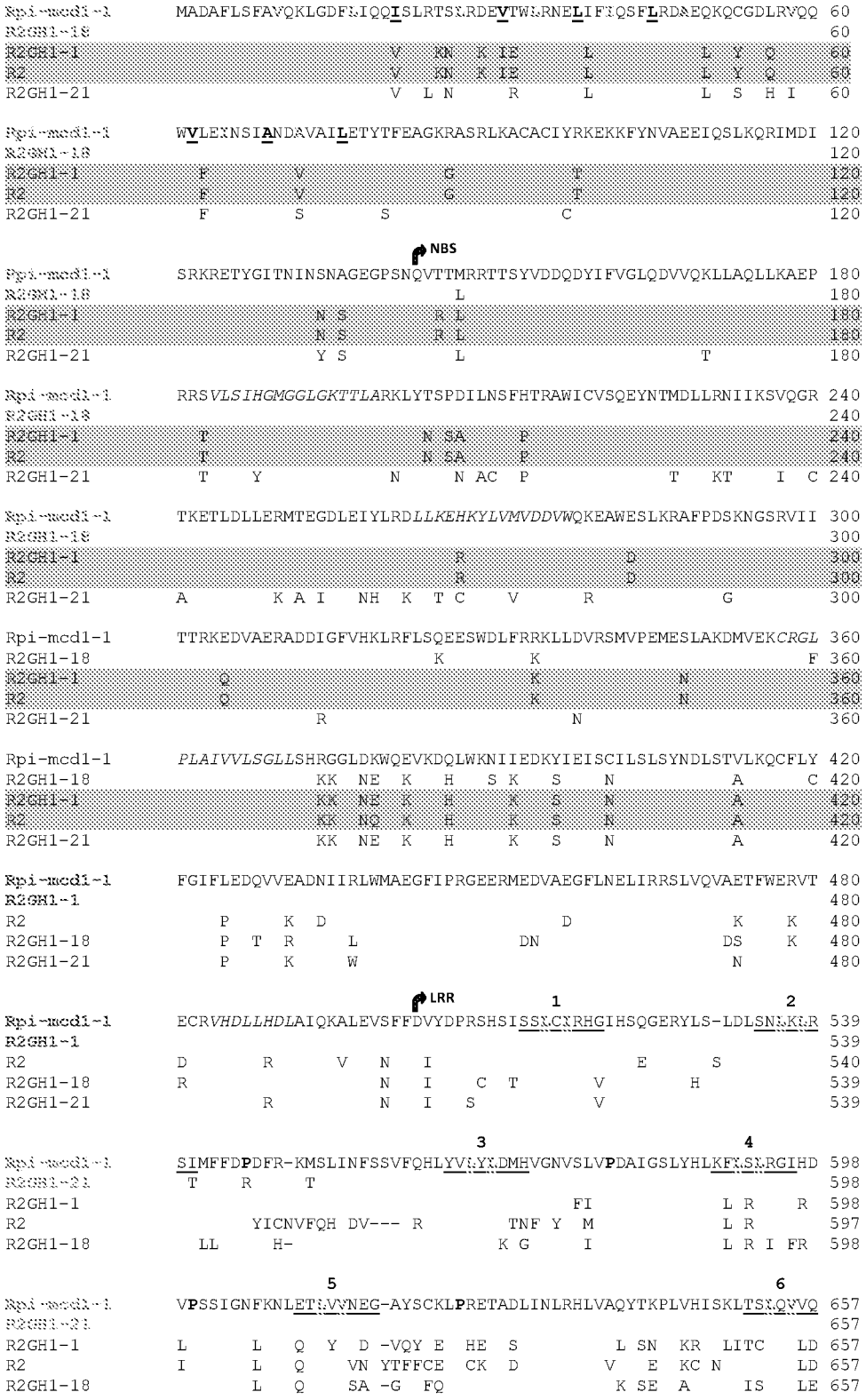


Figure 5: Modular structure analyses of *R2GH* candidates. Sequence of the 314 IPS (Informative Polymorphic Sites) which appear in blue, from the alignment of 21 *R2GH* candidates spanning complete ORFs. The arrow indicates the functional candidate *R2GH1-8* (*Rpi-mcd1-1*). Highlighted in red, green or purple are the sequence blocks referring to R2GH1-18, R2GH1-21 and R2GH1-1, respectively. On the top are indicated the breaking points between the different blocks referring to the original sequence numbering. The figure lights up 3 recombination regions: between [800-1043 bp], [1444-1917 bp], [2037- 2110], and one recombination point at [1259 bp]. *Rpi-mcd-1-1* modular structure is depicted in detail in figure 7. The stars indicate the complementary recombinant *R2GH1-19* and *R2GH1-24*. The beginning of the NBS and LRR regions are indicated by arrows.



Aardevo Exhibit 1010
Page 0822

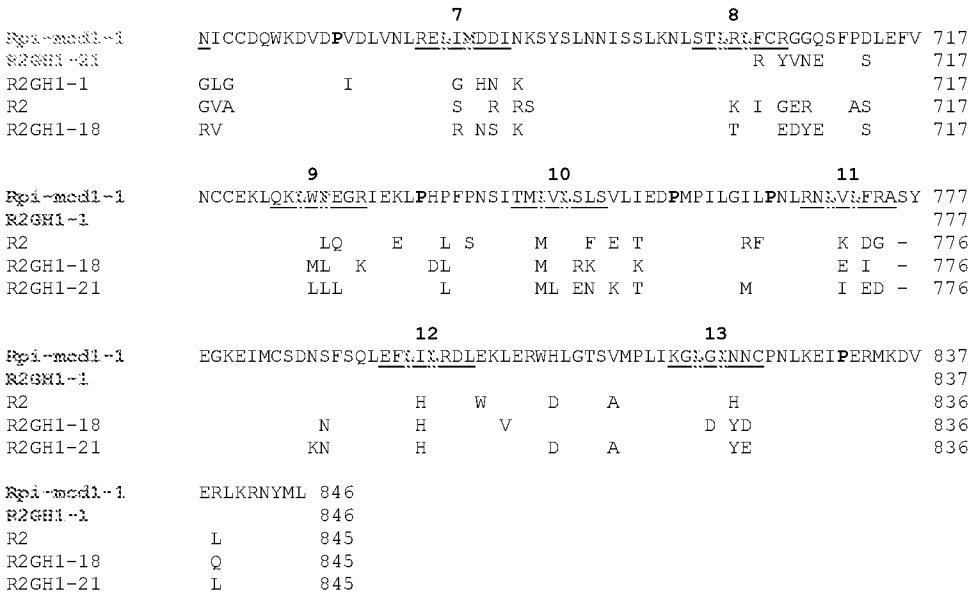


Figure 6: Structure and functional analyzes. ClustalW comparing Rpi-mcd1-1 to R2GH1-1, R2GH1-18, R2GH1-21 and R2.The sequence names appear in red, green or purple color when Rpi-mcd1-1 sequence is identical to R2GH1-18, R2GH1-21 or R2GH1-1, respectively. Highlighted in grey are the identical block sequences between R2GH1-1 and R2. The NBS and LRR regions are indicated by an arrow. Conserved domains present in the NBS region are in italic. LRR repeats are underlined and numbered. The amino acid L, I, F, A, M and V from the leucine zipper heptads and the LRR repeats appear in bold red

Specific recognition of *AVR* gene by the *R* gene induces hypersensitive cell death response (HR) from 5 days post-infiltration. In contrast to R2, the Rpi-mcd1.1coinfiltrated with PiAvr2 did not show any cell death (data not shown). This is in line with the disease tests with *P. infestans* isolates that are virulent on plants expressing R2 but avirulent on plants expressing Rpi-mcd1.1, and indicates that *Rpi-mcd1.1* is interacting with other effectors than previously described *R2* homologues from Mexican origin.

DISCUSSION

In this study, we cloned and elucidated the function of one resistance gene, *Rpi-mcd1.1*, originating from *Solanum microdontum* accession BGRC 18302-34 (CGN 21342) collected in northern Argentina. This *Rpi*-gene provides qualitative resistance to some *P. infestans* isolates and a typical hypersensitive reaction was observed after infection with the late blight isolate PIC99177 and IPO-0. To other isolates e.g. IPO-C, a quantitative resistance was observed, in line with previous studies (Sandbrink et al. 2000; Tan et al. 2008). Resistance assays and screening of the closest known markers of linkage group IV in

Aardevo Exhibit 1010
Page 0823

the population RH94-076 indicated the presence of at least three *R*-genes, *Rpi-mcd1.1* and *Rpi-mcd1.2* conferring resistance to the *Pi* isolates PIC99177 and IPO-0, respectively, and *Rpi-phu2* conferring resistance to PIC99177. *Rpi-mcd1.1* and *Rpi-mcd1.2* originate from *S. microdontum* and are located on linkage group IV. *Rpi-mcd1.1* was cloned by *R2/ Rpi-blb3* adapted allele mining and conferred resistance to PIC99177 when transiently expressed in *N. benthamiana* leaves and in stable transformants of potato cultivar. Stable transformants confirmed the functionality of *Rpi-mcd1.1* by complementing the susceptibility of the cultivar Desiree when challenged with the isolate PIC99177. No complementation was evaluated against the isolate IPO-0, confirming that the resistance against this isolate comes from a different gene.

Correlation between quantitative and qualitative resistance

Rpi-mcd1.1 is characterized as a "weak" resistance gene by Tan et al (2008) which is in line with the description of PIC99177 by Champouret et al (2009) as a "weaker" isolate than IPO-C or IPO-0. The differences in degree of aggressiveness of these isolates permitted the mapping of *Rpi-mcd1.1* in the field and via detached leaf assay. Additionally, we hypothesize that the quantitative resistance to IPO-C evaluated in the population RH94-076 is also due to different resistance factors including *Rpi-mcd1.1, Rpi-mcd1.2 and Rpi-phu2.* The last one being also characterised as a QRL when the population RH94-076 was challenged to the isolate IPO-C (Dr. C. Celis Gamboa, Wageningen UR Plant Breeding, unpublished results).

Transfer of genes or common ancestor?

The presence of *Rpi-blb3* homologues in *S. microdontum* emphasises the existence of a common ancestor with *S. bulbocastanum* from Mexico. According to Hawkes classification (2004), both belong to the *Petota* section, series *Bulbocastana* for *S. bulbocastanum* and series *Tuberosa* for *S. microdontum*. Using AFLP markers, (Kardolus et al. 1998) unexpectedly found that *S. microdontum* was associated with ser. *Megistacroloba* rather than with ser. *Tuberosa.* Recent phylogenetic studies using AFLP molecular markers classified *S. microdontum* as a distinct genetic unit or species (Jacobs et al. 2008). These results highlight the difficulty to properly classify *S. microdontum* and find phylogenetic links with other *Solanum* species. Our findings shed light on a clear link between the Mexican polyploid *S. demissum* and the Argentinean diploid *S. microdontum*. *S. bulbocastanum* (*Rpi-blb3*), *S. demissum* (*R2*) and *S. microdontum* (*Rpi-mcd1.1*) appear to have a common ancestor but

Aardevo Exhibit 1010
Page 0824

different evolutionary forces dependant on their geographical location and the exposure to local *P. infestans* isolates may have shaped functionally distinct R genes for specificity to Avr-genes. *S. bulbocastanum* (*Rpi-blb3*) and *S. demissum* (*R2*) evolved in Mexico and are specifically resistant to P.infestans isolates expressing the effectors *PiAvr2*. In contrast, *S. microdontum* (*Rpi-mcd1.1*) originates from Argentina and probably specifically recognizes different effectors than PiAvr2. Perhaps, an R-AVR pair other than R2-PiAvr2 has driven a co-evolutionary arms race of the Argentinean *Solanum* with local *P. infestans* isolates in the Andes. We hypothesize that the *P. infestans* populations in Mexico and Argentina may have evolved independently, and co-evolutionary forces might have been targeted at different R-AVR pairs. The presence of the effector Pi-Avr2 in isolates might have maintain the LRR structures of *Rpi-blb3* or functional *R2* homologues in Mexico. A survey of the Argentinean late blight population is not yet available in order to relate the presence of Avr-mcd1 with the maintenance of *Rpi-mcd1.1*. in *S. microdontum*.

Intragenic recombination resulted in new recognition specificity of the MLB resistant cluster located on linkage group IV

Rpi-mcd1.1 is so far the only and thus a unique member of the *R2/ Rpi-blb3* cluster providing resistance to the late blight isolate PIC99177. Significant excess of amino acid polymorphism resides in the LRR domain when compared to the one of *Rpi-blb3*, indicating that *Rpi-mcd1.1* probably interacts with another PIC99177-specific effector(s). Similar results were obtained in *RPP13* (*Hyaloperonospora arabidopsidis* resistance gene) allele mining in a diverse set of *Arabidopsis thaliana* accessions (Rose et al. 2004). *RPP13* exhibits the highest reported level of sequence diversity among known R-genes. Hall et al (2009) demonstrated the recognition of the effector ATR13 was mediated by alleles belonging to just a single clade, as it had been reported for *Rpi-blb3*, *R2*, *R2-like* and *Rpi-abpt* (Lokossou et al 2009). Additionally RPP13 alleles in other clades have evolved the ability to detect other *H. arabidopsidis* ATR protein(s) as it might be the case for *Rpi-mcd1.1*. Although certain resistance gene clusters like the one of *Rpi-vnt1* (Foster et al. 2009; Pel 2010; Pel et al. 2009), *Rpi-blb1* (van der Vossen et al. 2003; Vleeshouwers et al. 2008) and *Rpi-blb3* (Champouret 2010; Lokossou et al. 2009), tend to yield only similar genes, it is still worthwhile to further study them in geographically and phylogenetically diverse *Solanum* to find new R genes with distinctive specificity.

Molecular structure studies showed that *Rpi-mcd1.1* has in common five segments with at least three genes present in the gene pool, underlining the role of genetic recombination inside the *R-gene* cluster, resulting in the generation of novel resistance specificities (Hammond-Kosack and Kanyuka 2007;

Aardevo Exhibit 1010
Page 0825

Kuang et al. 2004; Meyers et al. 1999; Michelmore and Meyers 1998). Despite the absence of data about the genetic position of each *R2GH* gene, the presence of recombinants in the gene pool implies the close proximity of these genes from each other.*R2/ Rpi-blb3* homologues originating from *S. tuberosum* were identified among the candidate genes isolated from the hybrid *S. microdontum/ S. tuberosum* clone RH90-038-21. Additionally, the presence of a *R2GH* fragment identified as being from the *S. tuberosum* parent, inside *Rpi-mcd1.1* and the identification of complementary recombinant genes demonstrate the occurrence of multiple recent recombination events inside the *Rpi-mcd1.1* resistance locus. The profusion of alleles identified in different studies, 27 alleles in this study, 24 alleles described in Lokossou et al (2009) and 17 alleles isolated by N. Champouret (2010) represent a natural powerful reservoir available for new recombinations and the birth of new resistance alleles in the MLB located on linkage group IV.

MATERIAL AND METHODS

Plant material and late blight isolates

In this study we used the parental clones RH90-038-21, RH88-025-50, and fifty-one new RH94-076 plants from the cross earlier described in Tan et al (2008). The clone RH90-038-21 and the RH94-76 population contain essentially a susceptible *S. tuberosum* genetic background resulting in a germplasm with about 25% *S. microdontum* introgression for the parental clone and less than 12.5% for the progeny (Tan et al. 2008). The clone RH88-025-50 is a hybrid of the *S. phureja* genotype DB-207 and the *S. tuberosum* genotype SH76-128-1865. The characteristics and origin of *P. infestans* isolates IPO-0, IPO-C and PIC99177 are indicated in supplementary Table S1.

Table S1: Race specificity of the different isolates used in this study

Isolate	Year	Geographic origin	Mating type	Obtained from	Virulence profile	Reference
IPO-0	n.d	Unknown	n.d	Kessel, PRI, WUR	0.3b. 4. 7. 10. 11	
IPO-C*	1982	Belgium	A2	Kessel, PRI, WUR	1.2.3.4.5.6.7.10.11	
PIC 99177	1999	Metepec, Mexico	A2	Kessel, PRI, WUR	1.2.3.4.7.9**.11	Flier et al. 2002

* : also known as IPO-82001

** : not conclusive

Aardevo Exhibit 1010
Page 0826

Resistance assay

Detached leaf assays were performed as described by (Vleeshouwers et al. 1999). Six days after inoculation, phenotype responses were determined. Leaves showing no symptoms or a localized necrosis at the site of inoculation were scored as resistant and those with clear sporulating lesions (visible by naked eye) as susceptible.

Allele mining

The *Rpi-blb3* allele mining primers Blb3-start and Blb3-end described in Lokossou et al (2009) were used to amplify *Rpi-blb3* homologous ORFs present in the parental resistant genotype RH90-038-21. The *R2GHs* were cloned into the binary vector pKGW-MG between the *Rpi-blb3* promoter and terminator sequences of 2,723 and 883 nucleotides, which were cloned into pDONR P4-P1R and pDONR P2R-P3. The binary clones were subsequently transferred to *Agrobacterium* strain AGL1 and tested in a transient complementation assay in *N. benthamiana,* (Lokossou et al 2009).

Transient complementation in N. benthamiana

Infiltration with *Agrobacterium* strains carrying R2GH were performed in *N. benthamiana.* After the configuration of the constructs were confirmed by PCR, the final expression constructs were then transferred to *A. tumefaciens* strain AGL1 (Lazo et al. 1991) containing the helper plasmid pVirG and pSoup (van der Fits et al. 2000). Recombinant AGL1 *A. tumefaciens* cultures were grown in LB medium (10 g/L bacteriological peptone, 10 g/L NaCl and 5 g/L yeast extract in MQ water) supplemented with 5 mg/L tetracycline , 100 mg/L carbeniciline, 75 mg/L choramphenicol and 100 mg/L spectinomycin. Recombinant *A. tumefaciens* cultures were grown in LB medium supplemented with 100 mg/L spectinomycin. After one or two days a calculated amount of culture (according to OD 0.5 at 600 nm) was transferred to YEB medium (5 g/L beef extract, 5 g/L bacteriological peptone, 5 g/L sucrose, 1 g/L yeast extract, 2 ml/L 1 M $MgSO_4$ in MQ water) supplemented with spectinomycin. After 1 day overnight cells were centrifuged at 3500 rpm and re-suspended in MMA medium (20 g/L sucrose, 5 g/L MS salts and 1.95 g/L MES) supplemented with 1 ml 200 mM acetosyringone to a final OD of 0.2 and infiltrated into leaves of 4 weeks old plants with a 3ml syringe. Three leaves from four plants were infiltrated. After two days, leaves were detached, and spot-inoculated with *P. infestans* strains IPO-C and PIC99177. Seven phenotypes were distinguishable under the binocular and

Aardevo Exhibit 1010
Page 0827

were scored from 0 to 6 reflecting the resistance level provided by the tested R2GH to the leaf (Fig S1). Scores of the 24 leaves per construct were computed and the average was calculated.

Agrobacterium-mediated co-expression in *N. benthamiana*

The selected clone *R2GH1-8* was co-expressed with the avirulence effector *Pi-Avr2* cloned in pGRAB vector (kindly supplied by Dr. P. Birch, SCRI. Scotland. UK). Pi-Avr2 clone was transferred to *A. tumefaciens* AGL1 cultures and grown in LB medium supplemented with 30 mg/L choramphenicol and 50 mg/L kanamycin. *Rpi-blb3* was used as a control. The same protocol as the transient complementation was used for the sub-cultures. PiAVR *A. tumefaciens* cultures and R2GH1-8 *A. tumefaciens* cultures were mixed at a final OD600 of 0.1 for each and infiltrated into leaves from 5 to 7 week old plants.

Wild type plant Pi-Avr infiltration was done as described in Vleeshouwers and Rietman (2009). In order to verify that the resistance pathway works, co-infiltration of *Rpi-blb3* and *Pi-Avr2* was used as control.

Transformation of susceptible potato variety

The constructs pKGW-R2GH were transferred in AGL1 (Lazo et al. 1991). After verifying their stability in *Agrobacterium* these clones were transformed to the susceptible potato variety Desiree. Overnight cultures of the transformed *A. tumefaciens* strain were used to transform internodal cuttings from *in vitro* grown plants (Heilersig et al. 2006). A total of 100 to 200 explants were used for each transformation

DNA sequencing, primers design and computer analysis

Sequencing reactions were performed using a dye terminator cycle sequencing reaction kit (Perkin-Elmer, Pt Biosystem, Warrington, UK), M13 universal forward and reverse primers, and an ABI377 automated sequencer (Applied Biosystem, La Jolla, CA, USA). Sequence contigs were assembled using the STADEN sequence and analysis program (Dear and Staden 1991). The binary constructs were sequenced using a primer walking strategy (700 bp by 700 bp). Multiple sequence alignments were conducted using ClustalW2 program accessible from EMBL-EBI web page. Alignments were

Aardevo Exhibit 1010
Page 0828

produced using DNASTAR Lasergen Seqman version 8.0.2. The "CLC main workbench 5" program, version 5.0.2 was used to design the specific primers and MEGA4 to produce the phylogenetic trees.

ACKNOWLEDGEMENTS

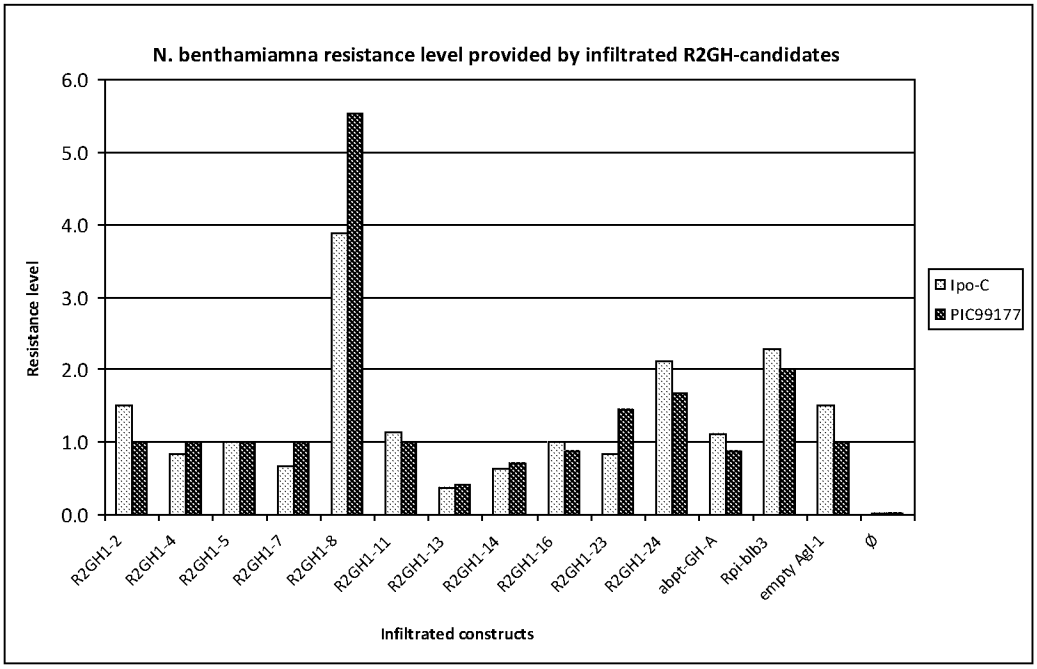
We are grateful, to the Scottish Crop Research Institute (SCRI) and The Sainsbury Laboratory (TSL) for giving us the opportunity to use the effector *Pi-Avr2*. We acknowledge P.R.J. Birch, G. Kessel and F. Govers for providing *Phytophtora* isolates. We would like to thank J. Vossen, M. Arens, H. Rietman and H. Lee for their technical help. Funding for this project was provided by a grant from the Wageningen University Fund.

Table S2: List of primers used in this study

Marker	Primers	sequence (5'----> 3')	Region	Tm C°	Fragment Length (bp)	Specific to
M-a	mcd-Fa	GAGTTCCTTATTCTTSGTGA	2389-2408	61	146	Clade I
	mcd-Ra	TTCAACCGCTCCACGTC	2518-2534			
M-d	mcd-Fd	GAGGATGTCGCTGAAAGAG	922-940	61	1520	Clage III
	mcd-Rd	GTGCCTAAATCCCATCTTTCT	2421-2441			
M-f	mcd-Ff	AAAAACCTTAGCACTCTCAC	2098-2117	61	336	Clade II
	mcd-Rf	ATGCCATCTTTCTACCTTC	2415-2433			
M-h	mcd-Fh	CCAAGAAAGCACTCCAAA	1528-1545	50	469	pseudoR2GH-5b
	mcd-Rh	CACAACCAACCCAATTAAGA	1977-1996			

Primers	sequence (5'----> 3')	Enzyme	Tm C°	Reference
TG339-F	GCTGAACGCTATGAGGAGATG	*Mnl*I	58	Tan et al .2008
TG339-R	TGAGGTTATCACGCAGAAGTTG			
CT229-F	TTGTGAGTGGTGAACTACGGGC	*Hpy*CH4IV	58	Park et al . 2005b
CT229-R	CGGCAATGGTTATGGGAACG			
T0703-F	CCAGTAAGAACAAGCCGATT	*Bme*1390I	54	Tan et al .2008
T0703-R	ATCACCAATTACGCGATCTA			

Aardevo Exhibit 1010
Page 0829



0	Heavy sporulation
1	Lesion + heavy sporulation on dead tissue
2	Lesion + moderate sporulation on dead tissue
3	Large lesion and weak sporulation
4	Lesion + weak sporulation on dead tissue
5	Large lesion but no sporulation
6	hypersensitive response

Figure S1: *N. benthamiana* resistance level to Pi isolates IPO-C and PIC99177, provided by infiltrated *R2GH*-candidates and the controls *abpt-GH-A* and *Rpi-blb3* known not to complement susceptibility of *N. benthamiana* to both isolates. Score from 1 to 6 characterize the different phenotypes observed; 1 being susceptible and 6 highly resistant.

Aardevo Exhibit 1010
Page 0830

Chapter 5

Exploration of sequence polymorphism in *R2* gene homologues permitted the identification of a single amino acid which triggers the potato/ *P. infestans* interaction R2/PiAVR2

Anoma A. Lokossou, Edwin A. G. van der Vossen, Richard G. F. Visser, Evert Jacobsen

ABSTRACT

The major late blight resistance cluster on linkage group IV has been extensively investigated in various *Solanum* species leading to the identification and/ or isolation of an abundance of late blight (LB) resistance allelic variants. These genes share high percentage identity and define a new *R* gene family characterized by the already known functional *Rpi-blb3*, *Rpi-abpt*, *R2 and R2-like* alleles (Lokossou et al. 2009). Functional LB resistance homologues were identified by susceptible phenotype complementation and/ or effector recognition using the transient co-expression system in *N. benthamiana*. Resistance against a set of *Phytophthora infestans* isolates was coupled with the recognition of the RXLR effector PiAVR2. In our study, we used the sequence information provided by the alleles to identify and functionally characterize the repeats in the LRR domain which are necessary for PiAVR2 recognition. Domain swaps between *Rpi-abpt* or *R2* which interact with PiAVR2 and R2GH-2, which is non functional, permitted the identification of the critical LRR repeats 11, 12 and 13 involved in recognition. Site directed mutagenesis on *Rpi-abpt* pointed to one solvent exposed amino acid in LRR13 crucial for PiAVR2 recognition. These results are an indication of evolutionary differentiation between both partners because of recognition failure by the pathogen.

Aardevo Exhibit 1010
Page 0831

INTRODUCTION

The pathosystem formed by the oomycete *Phytophthora infestans* and its hosts among *Solanaceae* is under intensive investigation since the Irish potato famine in 1845. Classical breeding strategies first succeeded in combating Late Blight (LB) by introgression of dominant resistance genes from the wild specie *Solanum demissum* into cultivated potato (Malcolmson 1969; Malcolmson and Black 1966; Müller and Black 1952). The set of eleven *R* genes (*R1* to *R11*) were exploited in monoculture, which besides providing an efficient crop protection, was exerting a strong selection pressure on the pathogen resulting in its rapid adaptation and consequently the defeat of those *R* genes (Fry 2008; McDonald and Linde 2002). A need for new resistance genes appeared and more wild *Solanum* species were investigated for their LB resistance potential. In the last decade, many *R*-genes were identified and isolated from wild *Solanum* species comprising *S. demissum* harboring *R1* (Ballvora et al.), *S. bulbocastanum* hosting *Rpi-blb1*, *Rpi-blb2*, *Rpi-blb3*, *Rpi-bt1* (Lokossou et al. 2009; Oosumi et al. 2009; van der Vossen et al. 2005; van der Vossen et al. 2003), *S.stoloniferum* and *S. papita* harbouring *Rpi-blb1* allelic versions *Rpi-sto1* and *Rpi-pta1* (Wang et al. 2008); *S. venturii* containing *Rpi-vnt1-1* and *Rpi-vnt1-3* (Foster et al. 2009; Pel et al. 2009).

An explanation of the rapid adaptability of *P.infestans* was found in the recent sequencing of its genome which appears to be large and complex with about 74% of repetitive DNA. Annotation of the genome identifies many gene families encoding for effector proteins that are involved in causing pathogenicity. Additionally, comparison with the sequenced *Phytophthora sojae* and *Phytophthora ramorum* showed rapid turnover and extensive expansion of these effector genes (Haas et al. 2009). *P.infestans* effector proteins are classified in two groups, the effectors secreted in the cytoplasm or the apoplasm. The RXLR effectors have been identified among the cytoplasmic secreted proteins and are characterized by an arginine-X-leucine-arginine motif at the N-terminus of the protein that defines a domain required for delivery inside plant cells followed by an acidic region (Jiang et al. 2008; Morgan and Kamoun 2007; Whisson et al. 2007; Win et al. 2007).

Avirulent RXLR effectors (AVR) are detected by the potato plant upon *P.infestans* infection resulting in a hypersensitive reaction stopping the growth of the pathogen (McHale et al. 2006). AVR protein detection is possible in a direct way or indirectly by the resistance genes harboring key domains for pathogen recognition, the nucleotide binding site (NBS) and the leucine rich repeat (LRR) domains (Catanzariti et al. 2010; Collier and Moffett 2009; Ellis et al. 2007; Jones and Dangl 2006; Rairdan and Moffett 2006). Although, the N terminal domain of NBS-LRR protein has been demonstrated to

Aardevo Exhibit 1010
Page 0833

play a crucial role in AVR protein recognition (Moffett et al. 2009), multiple studies showed that the LRR domain is the region of the protein that confers recognition specificity (Hulbert et al. 2001; Meyers et al. 1998; Mondragon Palomino and Meyers 2002; Noel et al. 1999). In silico secondary structure of the NBS-LRR protein shows that the LRR repeats with the motif xxLxLxx form a β-strand/β-turn structure in which the solvent x residues are exposed and available for interactions with potential ligands (Kobe and Deisenhofer 1995). These solvent exposed residues are hyper variable and subject to diversifying selection, suggesting that these residues have evolved to detect variation in pathogen-derived ligands (Michelmore and Meyers 1998).

So far six matching pairs of *PiAVR* and potato *R*-gene have been identified, *PiAVR3a/R3a* (Armstrong et al. 2005), *AVR4/ R4* (van Poppel et al. 2008), *AVR-blb1/Rpi-blb1* (Vleeshouwers et al. 2008), *AVR-blb2/ Rpi-blb2* (Oh et al. 2009), *PiAVR2 /R2* (Lokossou et al.) and *AVRvent1 /Rpi-vent1-1* (Pel 2010). Detailed investigation of PiAVR/R interaction was so far orientated to the effector side to identify the crucial domain necessary for the activation of the hypersensitive reaction. The C terminal domains were found indispensable for PiAVR3a and AVR4 recognition (Bos et al. 2006); (van Poppel et al. 2009). Remarkably, PiAVR2 is so far recognized by 10 R genes, *Rpi-blb3*, *Rpi-abpt*, *R2*, *R2-like* (Lokossou et al.) *Rpi-snk1-1*, *Rpi-snk1-2*, *Rpi-edn1-1*, *Rpi-hjt1-1*, *Rpi-hjt1-2* and *Rpi-hjt1-3* (Champouret 2010).

In this study, we explore the information provided by the alleles generated in different *Rpi-blb3* allele mining strategies and use the transient co-expression system in *N. benthamiana* to identify and functionally characterize the repeats in the LRR domain which are necessary for PiAVR2 recognition. Domain swaps between R genes interacting or not with PiAVR2 permitted the identification of a region spanning three LRR repeats of the cloned *Rpi* genes that determine the *R2* resistance specificity. Site directed mutagenesis pinpointed one solvent exposed amino acid to be crucial for PiAVR2 recognition.

RESULTS

Allelic variation

A total of 75 full length amino acid sequences identified on linkage group IV were retrieved from the public nucleotide sequence database and this thesis. These alleles originated from *Rpi-blb3* or *R2* allele mining in *S. bulbocastanum*, *S. microdontum*, *S. demissum*, *S. schenckii*, *S. edinense*, and *S.*

Aardevo Exhibit 1010
Page 0834

hjertingii. A blast research on the public nucleotide sequence database resulted in the identification of three BAC clones from the *Solanum tuberosum* diploid genotype RH89-039-16 and two BAC clones from tomato. All BAC clones are mapped on linkage group IV. Annotation of the BAC sequences for *Rpi-blb3* like genes resulted in 5 genes present on the *S. tuberosum* BACs and 4 genes in the tomato BACs, spanning full length ORF. The corresponding protein sequences are classified in a phylogenetic tree into 12 clades (Fig 1). Functional resistance genes interacting with PiAVR2 share 95 to 100% identity with each other, and are bulked in clade X. The functional gene *Rpi-mcd1-1* which doesn't interact with PiAVR2, belongs to clade VI. The genes isolated from wild *Solanum* species which are non functional and do not interact with PiAVR2 share 88 to 99% identity with the functional genes belonging to clade X. The genes identified in *S. tuberosum* and tomato BAC clones share 78 to 89% identity with *Rpi-blb3* and are bulked in clade XII.

Interestingly, three non functional genes, *R2GH 2*, *R2GH-D3* and *R2GH-65*, share 95 to 99% amino acid identity with *Rpi-abpt* or *R2*. In an attempt to identify the causes of non functionality, nucleotide sequences were aligned by clustal W and informative polymorphic sites were identified in comparison to *R2* (Fig 2). R2GH-65 is almost identical to R2 in the exception of a region between the nucleotide positions 693 and 1338, comprising the kinase 2 and glpl domain, which confirm that disruption of the NB-ARC conserved motifs alters the functionality of the gene. The same explanation for non functionality can be given for the polymorphism found in the leucine zipper and NB-ARC domains of the R2GH-6 sequence.

Identification of the LRRs of R2 required for PiAVR2 specific interaction

Remarkably, the R2-GH-2 nucleotide sequence is almost identical to *Rpi-abpt* except in a region comprising LRR11 to LRR13 (Fig 2). Comparison of the amino acid sequences confirms that the majority of the R2GH-2-specific amino acids are concentrated in the putative solvent exposed residues of LRR11-LRR13 (Fig. 3), suggesting that the corresponding LRRs in the functional genes play an important role in determining *R2* specificity.

To analyse the role of LRR11-LRR13 in the interaction with PiAVR2, chimeras were constructed between *R2* and the non-functional *R2GH-2* allele. The unique but conserved restriction sites *Spe*I (located between LRR6 and LRR7), *Bsu*316 (located between LRR11 and LRR12) and *Bsg*I (located between LRR12 and LRR13) were used to swap 5' and 3' ends of the two homologues (Figs. 3 and 4). The constructs were cloned under *Rpi-blb3* regulatory elements and tested by agro-infiltration together with PiAVR2.

Aardevo Exhibit 1010
Page 0835

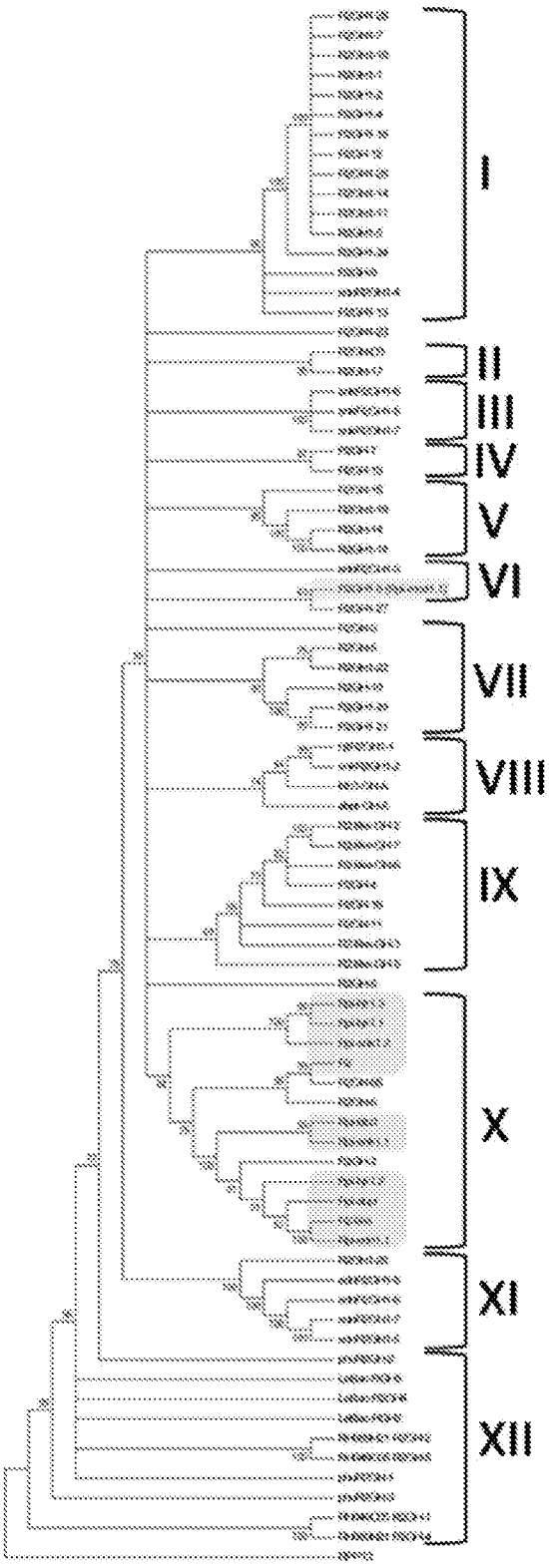


Figure 1: Phylogenetic tree of 75 amino acid sequences of Rpi-blb3 like genes. Functional genes interacting with PiAVR2 are highlighted in blue; the one not interacting with PiAVR2 is highlighted in green. The clade numbers are indicated on the right side. RPP13 had been used as an out-group. UPGMA algorithm with bootstrap analysis of 500 replicates. Numbers above the branches are bootstrap values percentages.

Aardevo Exhibit 1010
Page 0836

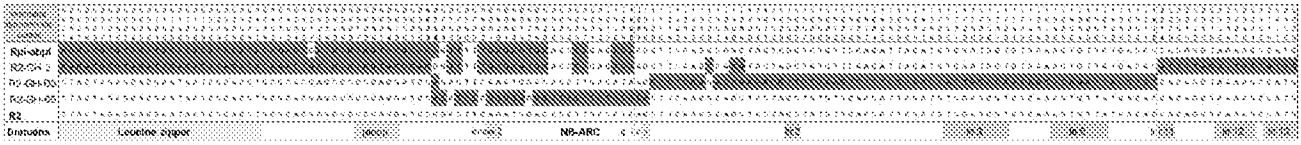


Figure 2: Nucleotide polymorphism analysis of *R2* and 4 homologues sharing informative polymorphic or homologous sequences. In bold are the genes *R2* and *Rpi-abpt* which interact with *PiAVR2*. Only informative polymorphic sites are shown, which reduces the sequence size from 2444 bp to 156 bp. Highlighted in green are the positions polymorphic to *R2* sequence. The vertical number at the top of each row indicates the corresponding nucleotide position in the full consensus sequence, the limits of the recombination regions positioned on the bottom of the figure are indicated in bold. The positions of the different domains characterizing LZ-NBS-LRR genes are indicated down to the sequences.

Table 1: List of primers used for the site-directed mutagenesis

Primer	Sequence (in 5'----> 3' order)	Mismatches	% GC	Tm
lrr11-misLV-F	GATGGTTCTGAGTTT**A**TCAG**T**ACTGACAGAAGATCCGATGC	2	44	78
lrr11-misLV-R	GCATCGGATCTTCTGTCAGTACTGATAAACTCAGAACCATC			
inter-lrr11-12-misIL-F	GAAGATCCGATGCCTATTTTGGGAA**TA**TT**G**CCAAACCTAAGGAATCTC	3	42	78
inter-lrr11-12-missIL-R	GAGATTCCTTAGGTTTGGCAATATTCCCAAAATAGGCATCGGATCTTC			
lrr12-misV-F	GGTTTCCAAACCTAAGGAATCTC**GTT**TTAGATGGAGCTTACGAAGG	3	43	78
lrr12-misV-R	CCTTCGTAAGCTCCATCTAAAACGAGATTCCTTAGGTTTGGAAACC			
lrr12-misK-F	GAAGGTTTCCAAACCTAAGGAATCTC**AAA**TTAGATGGAGCTTACGAAGG	3	41	78
lrr12-misK-R	CCTTCGTAAGCTCCATCTAATTTGAGATTCCTTAGGTTTGGAAACCTTC			
lrr13-misI-F	CTTCAGTCAACTAGAGTTCCTT**AT**TCTTCGTGATCTTTGGAAGC	2	41	78
lrr13-misI-R	GCTTCCAAAGATCACGAAGAATAAGGAACTCTAGTTGACTGAAG			
lrr13-misE-F	GTTCCTTCATCTTCGTGATCTT**GA**GAAGCTAGAAAGATGGG	2	44	78
lrr13-misE-R	CCCATCTTTCTAGCTTCTCAAGATCACGAAGATGAAGGAAC			

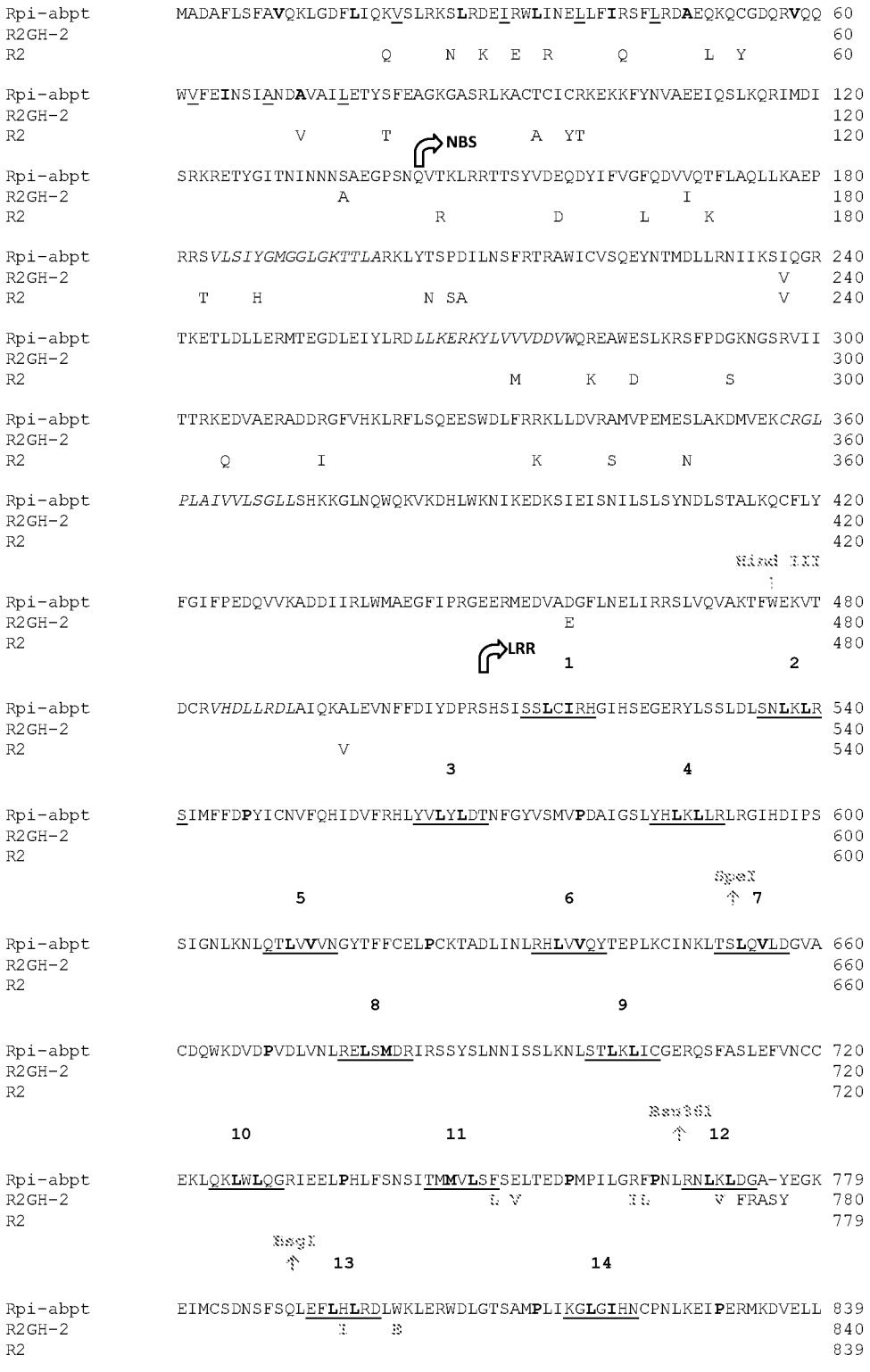
The chimeric constructs R2GH2-SpeI-R2 and R2GH2-Bsu316-R2 were the only constructs able to induce an HR when co-infiltrated with PiAVR2. However, we observed a weaker response with R2GH2-Bsu316-R2 compared to R2GH2-SpeI-R2, suggesting that LRR11, LRR12 and LRR13 are all three involved in the interaction or recognition of PiAVR2. We can also conclude that the two amino acid differences in the NBS domain do not influence the interaction with PiAVR2 (Figs. 3 and 4) while the four amino acids polymorphic between R2 and R2GH-2 in LRR11 influence the recognition but do not suppress the response. However, presence of the R2-specific LRR11, LRR12 and LRR13 alone is not sufficient for R2 specificity, as illustrated by the non-functional homolog R2GH-65, which contains the R2 specific LRR11-LRR13 sequence but differs from the functional genes in the region harbouring NB-ARC conserved motifs. Additionally, R2GH-D3 contains the R2 specific LRR11-LRR13 sequence and differs from R2 in the region harbouring LRR3-LRR10 (Fig 2). The latter observation illustrates the crucial role that putative intra-molecular interactions within the LRR domain and possibly between the LRR domain and the LZ or NBS domain play in determining recognition by the functional Blb3GHs.

R2GH2-Bsu-R2 recognized PiAVR-2 but induced a weaker response than R2GH2-Spe-R2. Our observation indicates that the 4 polymorphic amino-acids in R2GH-2-LRR11 negatively influence but don't completely suppress PiAVR2 recognition. R2GH2-Spe-R2 recognizes PiAVR2 indicating that the 2 SNPs present in the NBS domain of R2GH-2 are not loss of function SNPs. R2-Spe-R2GH2 and Abpt-Spe-R2GH2 are non-functional, indicating that the repeats 11, 12 and 13 are crucial in PiAVR2 recognition. These results are supported by the protein structure prediction of Rpi-abpt and R2GH-2 which show a clear difference of helix, strand and coil structures in the LRR11-LRR13 region (Fig 5).

On the opposite page:

Figure 3: Identification of regions and amino acids crucial for PiAVR2 recognition. Clustal W comparing the amino acid sequences of the functional genes Rpi-abpt and R2 to the non functional gene R2GH-2. The NBS and LRR regions are indicated by an arrow. The unique restriction sites used in domain swapping experiment are indicated in red. Conserved domains present in the NBS region are in italic. LRR repeats are underlined and numbered. The amino acid L, I, F, A, M and V from the leucine zipper heptads and the LRR repeats appear in bold red.

Aardevo Exhibit 1010
Page 0838



Aardevo Exhibit 1010
Page 0839

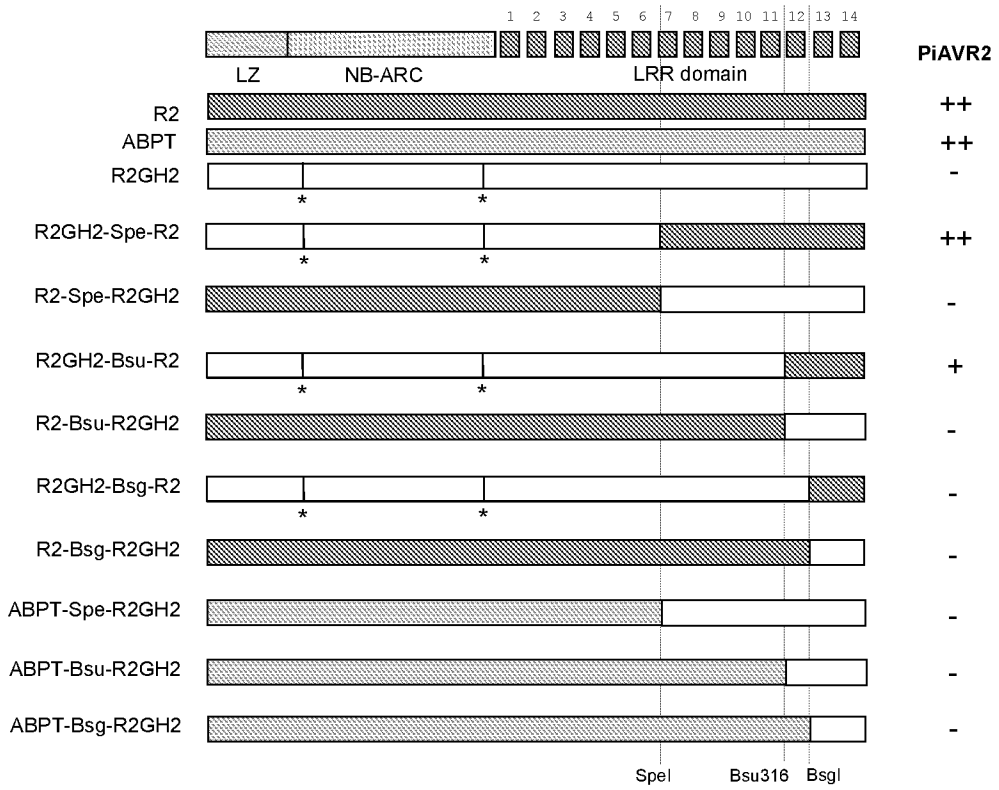


Figure 4: Domain swaps between the functional gene *R2* or *Rpi-abpt* and the non-functional gene R2GH-2. On the top are indicated the different domains (LZ: leucine zipper, NB-ARC and LRR domain with its 14 repeats). Below are indicated the position of the restriction sites used to swap the domains. (*) indicates single nucleotide polymorphism present in R2GH- sequence .in comparison with *Rpi-abpt*. Infiltrated leaves were scored 5 days post infiltration, white or black infiltration spots were scored as (++), yellow pale spots were scored as (+)and no visible cell death region were scored as (-).

Pinpointing of a single amino acid important for PiAvr2 recognition

R2GH-2 is almost identical to Rpi-abpt with the exception of 11 amino acids residing in LRR11, 12 and 13. These amino acids differ from R2GH-2 and Rpi-abpt sequences by their chemical propriety (Fig 3). In an attempt to determine which amino acid(s) in the non functional R2GH2 are critical for the avirulence protein recognition Pi-AVR2, a PCR-based site-directed mutagenesis strategy was used to convert the Rpi-abpt residue individually into the corresponding R2GH-2 residue (Fig 6). Five constructs were made which target seven amino acids individually or in combinations. The construct mut-abpt-1 displays L and V amino acid instead of F and E in the LRR11, mut-abpt-2 has the amino acids I and L instead of R and F also in LRR11, mut-abpt-3 contains the amino acid V instead of K in LRR repeat 12, mut-abpt-4 spans the amino acid I instead of H in LRR13, mut-abpt-5 has the amino acid E instead of W also in LRR13.

Aardevo Exhibit 1010
Page 0840

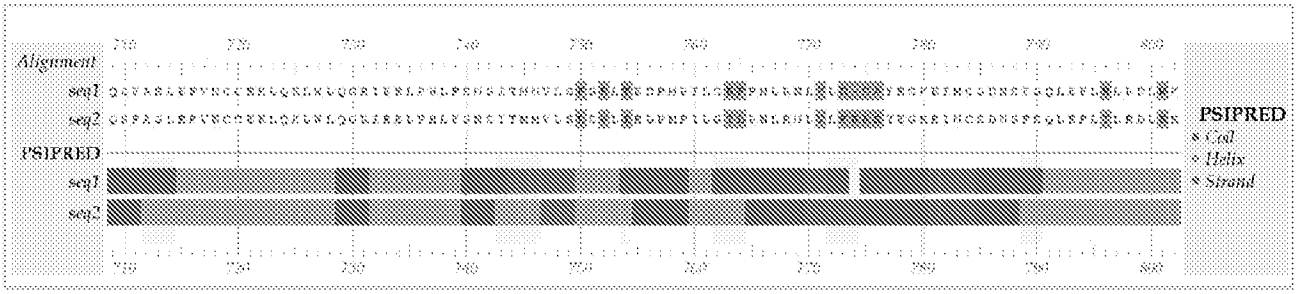


Figure 5: Comparison of the secondary structure prediction of Rpi-abpt (seq1) and R2GH-2 (seq2) in the LRR9 to LRR13 region. Polymorphic amino acids and secondary structure are highlighted in yellow. The figure is a combination of the positional features and differential prediction outputs obtained after analyses of the protein sequences by EPipe 0.922 Server from the center for biological sequences analysis (CBS).

The four constructs mut-abpt- 1, 2, 3 and 5 showed variable intensity of cell death regions. The construct mut-abpt-1 and mut-abpt-2 interact in a less intense reaction with PiAVR2 than the positive control Rpi-abpt. This observation is in correlation with the weak reaction obtained when the chimeric construct R2GH2-Bsu-R2 is co-infiltrated with PiAVR2. Mut-abpt-1 and mut-abpt-2 harbour a double mutation while R2GH2-Bsu-R2 harbours the combination of those double mutations. The constructs mut-abpt-3 and mut-abpt-5 showed a weaker response than mut-abpt-1 and mut-abpt-2. The construct mut-abpt-4 co-infiltrated with PiAVR2 did not induce a hypersensitive response in the *N. benthamiana* leaves. These observations reflect the impact which could have the mutation of strategic amino acids in the structural conformation of Rpi-abpt protein. The mutated amino acids in the constructs mut-abpt-1,2,3 and 5 are identified as residues under strong positive selection pressure (Champouret 2010) with a high posterior probability (p>99%) when the model M8 of the PAML method as applied. The amino acid H in LRR13 displays a posterior probability of 75%. These observations indicate the malleability degree of certain residues like the amino acids K and W belonging to LRR12 and 13, and the crucial importance of others like the amino acid H in LRR13 for *Rpi-abpt* recognition of PiAVR2.

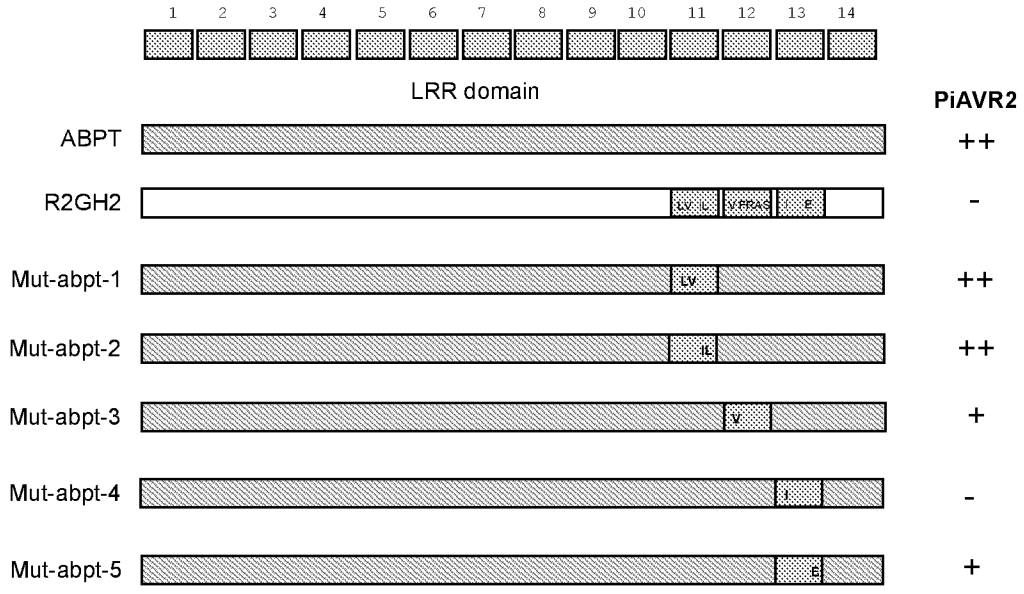


Figure 6: Site-directed mutated constructs tested by co-infiltration with PiAVR2. On the top are indicated the LRR domain with its 14 repeats. Polymorphic amino acids are indicated in comparison with the Rpi-abpt amino acid sequence. Infiltrated leaves were scored 5 days post infiltration, white or black infiltration spots were scored as (++), yellow pale spots were scored as (+) and no visible cell death region were scored as (-).

Aardevo Exhibit 1010
Page 0842

DISCUSSION

The major late blight resistance cluster on linkage group IV harbors a profusion of alleles sharing high percentage identity. These alleles define a new R gene family already identified by the cloning of *Rpi-blb3*, *Rpi-abpt*, *and R2-like* (Lokossou et al. 2009). Additionally, N. Champouret (2010) cloned *Rpi-edn*, *Rpi-hjt* and *Rpi-snk* alleles by R2 allele mining. These genes provide resistance against late blight by recognizing the *Phytophthora infestans* RXLR effector PiAVR2. Sequence analyses showed identical C terminal regions starting at the LRR repeat 3 (Lokossou et al. 2009; Champouret 2010). Three non functional R2GHs share high sequence identity with R2 and Rpi-abpt, giving us the opportunity to investigate PiAVR2 recognition specific region(s).

PiAVR2 recognition specificity resides in LRR11, LRR12 and LRR13.

Domain swap experiments have provided direct evidence that LRRs play a major role in determining resistance specificity (Thomas et al. 1997). In depth studies of the *P* and *P2* genes at the *P* locus in flax (Dodds et al. 2001), the *Mi* locus in tomato (Hwang et al. 2000; Hwang and Williamson 2003), and the *Pi2* and *Piz-t* at the *Pi* locus in rice (Zhou et al. 2006) identified essential amino acids in the LRR domain involved in the recognition of the AVR(s) or the functions of the genes. In depth investigation of the tomato Cf-9 disease resistance protein demonstrated that the major specificity-determining amino acids reside at hypervariable solvent exposed positions (Wulff et al. 2009). Here we demonstrate that LRR11, LRR12 and LRR13 are important for the interaction of R2 with PiAVR2. Further more seven amino acids were pinpointed in site directed mutagenesis experiments resulting in the identification of one of the crucial amino acids determining Rpi-abpt and its homologues specificity. The identified Histidine-795 is located in a solvent exposed position of LRR13 (xxLHLxx). Mutation of this amino acid into an Isoleucine as in R2GH-2 resulted in non recognition of PiAVR2. Our results support the positive selection identified in LRR 11, 12 and 13 by N. Champouret (2010) when analyzing 17 *R2* variants using the PAML method. The Histitine-795 had a posterior probability value lower than the other mutated residues reflecting its reduced potential to mutate and maintain the recognition of PiAVR2. This indicates the R protein potential of adaptation to PiAVR conformational change. Modification in the R protein structure might occur in a slow process of searching for the perfect match by trial and error, thus resulting in the profusion of alleles indentified in our allele mining strategy

Aardevo Exhibit 1010
Page 0843

Type I and II evolution at the MLB locus on linkage group IV

Sequence analyzes of the 24 *Rpi-blb3/R2* and 27 *Rpi-mcd1-1* homologues (Chapter 2 and 4) reveal a modular structure of the genes which evolution was dominated by sequence exchange, an evolutionary pattern typical for a Type I resistance gene as described for *R*-genes in natural lettuce populations (Kuang et al.). Investigation of the 17 *R2* variants isolated by N. Champouret et al (2010) shows residues in the LRR domain under positive selection thus submitted to single mutation like Type II resistance genes (Kuang et al.), with the aim to counter act to *P. infestans* escape. In our study, we demonstrated that a single mutation on a solvent exposed residue can lead to the lack of recognition of the cognate effector. This could also be interpreted as an adaptation of the gene to recognize an evolved version of the effector, or by the presence in the germplasm of R genes ready to protect the plant against a sudden change in the effector structure. This simultaneous evolution in host and pathogen for survival is also reflected in *Rpi-blb3* allele mining in *S.bulbocastanum* and related species (Chapter 3).

Non functionality or differential P. infestans resistance spectrum or differential pathogen recognition

Fifteen % of the full length homologous proteins reproduce the expected LB resistance spectrum. Thus, the other homologues remain with undetermined functionality. The resistance spectrum covered by *Rpi-mcd1-1* is different from the R2 spectrum and proves the existence of R2 homologues with different recognition specificity (Chapter 4). The demonstrated synteny between potato and tomato (Bonierbale et al. 1988; Gebhardt et al. 1991), and the identification of multiple pathogen resistance genes at one locus open the debate of the existence of homologues genes conferring resistance to other pathogens than *P. infestans* among the *Rpi-blb3/R2* homologue gene collection. Such cluster diversity has been found at the Mi-1/ Rpi-blb2 locus on LG VI which shares 82% protein sequence identity (van der Vossen et al.). *Mi-1* originates from tomato and confers resistance to three species of root knot nematodes (*Meloidogyne* spp.) as well as to the potato aphid *Macrosiphum euphorbiae* (Milligan et al. 1998; Rossi et al. 1998; Vos et al. 1998), and to both B and Q biotypes of whitefly *Bemisia tabaci* (Nombela et al. 2003). *Rpi-blb2* originated from potato and conferred resistance to late blight. *Tm2*, which provides resistance to tomato against the *Tomato mosaic virus,* shares 75% amino acid identity with the late blight resistance gene *Rpi-vent*, both are located on LG IX (Lanfermeijer et al. 2003; Pel et al. 2009). The tomato gene *I2* and potato gene *R3a* which are on LG XI share 88% identity and provide resistance against the fungi *Fusarium oxysporum* and *P. infestans*, respectively (Huang et al. 2005; Ori et al. 1997; Simons et al. 1998). Resistance against the

Aardevo Exhibit 1010
Page 0844

potato virus X and the potato cyst nematode *Globodera pallida* are provided by *Rx1* and *Gpa2*, respectively. Both are located on LG V and share an overall homology of over 88% amino-acid identity (Bendahmane et al. 1999; Rouppe van der Voort et al. 1999; van der Vossen et al. 2000). A way to reveal the putative functionality of the *R2/Rpi-blb3* homologues would be to create a library of potato transformants harboring the different alleles and test them with a differential set of *P. infestans* and other pathogens.

MATERIEL ANS METHODS

Domain swaps

pDONR221 constructs harboring *R2*, *Rpi-abpt* and *R2GH-2* were digested according to the manufacturers instructions with the restriction enzymes *Spe*I (Fermentas, Germany), *Bsu*316 (Fermentas, Germany) or *Bsg*I (New England Biolabs, The Netherlands). Digested products were separated by agarose electrophoresis and isolated using the QIAQUICK Gel Extraction Kit from Qiagen (The Netherlands). Different combinations of digested fragments were used in ligation reactions with the T4 DNA Ligase (Fermentas, Germany). Chimeric constructs were verified by comparing restriction digestion patterns obtained after digestion with *Bsu*316 and *Nhe*I (Fermentas, Germany). Additional verification was done through sequencing of the 5'-end, 3'-end and chimeric region of the inserts.

Site direct mutagenesis

Site directed mutations on *Rpi-abpt* in pDONR221 vector were generated by PCR with PfuUltra ® High-Fidelity DNA polymerase (Stratagene) and specific primers containing the desired mutations (Table S1).The PCR product were digested with the enzyme Dpn I according to the manufacturer's instruction. The resulted digestion mix was used to transform XL1-Blue super competent cells (Stratagene QuikChange ® II Site-Directed Mutagenesis kit).

The occurrence of the mutation was verified by sequencing each construct.

Aardevo Exhibit 1010
Page 0845

Cloning and testing

Confirmed chimeric and site directed mutated constructs were transferred to the destination vector pKGW-MG (kindly provided by Andreas Untergasser) in between *Rpi-blb3* regulatory elements via a multiple LR reaction with the *Rpi-blb3* promotor in pDONR-P4P1R, the *Rpi-blb3* terminator in pDONR-P2RP3, the ORF of interest in pDONR221, and the binary destination, using the protocol of (Untergasser 2006). The final expression constructs were then transferred to *A. tumefaciens* AGL1 (Lazo et al. 1991) containing the helper plasmid pVirG and pSoup (van der Fits et al. 2000). Each construct was tested by co-infiltration with the *P.infestans* avirulence protein Pi-Avr2 in *N. benthamiana* (as described by Lokossou et al.2009). *pKGW-Rpi-abpt* construct co-infiltrated with Pi-Avr2 was used as positive control. Negative controls were the infiltration with the R-gene or the AVR-gene construct alone. The chimeras R2GH2-Spe-R2, R2-Spe-R2GH2, R2GH2-Bsu-R2, R2-Bsu-R2gh2, R2GH2-Bsg-R2, R2-Bsg-R2gh2 and Abpt-Spe-R2GH2, and the site directed mutants mut-abpt-1 to 5 were functionally tested by co-infiltration with PiAVR2. Infiltrated leaves were scored 5 days post infiltration, white or black infiltration spots were scored as (++), yellow pale spots were scored as (+) and no visible cell death region were scored as (-).

DNA sequencing and computer analyses

Sequencing reactions were performed using a dye terminator cycle sequencing reaction kit (Perkin-Elmer, Pt Biosystem, Warrington, UK). Sequence contigs were assembled using the STADEN sequence and analysis program (Dear and Staden 1991). Alignment and phylogenetic trees were produced using "CLC main workbench 5" program, version 5.0.2. Gene structures were predicted using FGENESH++ (Softberry). Multiple sequence alignments were conducted using CLUSTALX 1.81 (Thompson et al. 1997). The search for genes homologous to *Rpi-blb3* was carried out using the Basic Local Alignment Search Tool (BLAST). Secondary structure domains were identified using the beta version of EPipe 0.922 Server from the center for biological sequences analysis (CBS) (BioSapiens Network of Excellence et al. 2008; (Jones 1999); (Tress et al. 2007).

ACKNOWLEDGEMENTS

We are grateful, to the Scottish Crop Research Institute (SCRI) and The Sainsbury Laboratory (TSL) for giving us the opportunity to use the effector *Pi-Avr2*. Funding for this project was provided by a grant from the Wageningen University Fund

Aardevo Exhibit 1010
Page 0846

Chapter 6

General discussion

Aardevo Exhibit 1010
Page 0847

The research described in this thesis comprises the isolation of five late blight resistance genes clustering in the major late blight (MLB) locus positioned on potato linkage group (LG)IV. A combination of plant and pathogen investigation techniques permitted the dissection of the MLB locus in terms of functionality, molecular interaction, allelic variation, diversification and evolution. This study could serve as an example for future investigation of resistance gene clusters.

A model for resistance gene cluster studies

We used different techniques in our research, starting with classical map based cloning to isolate the genomic sequence of *Rpi-blb3*, including its regulatory elements. Allelic diversity investigation was done by employing an allele mining strategy permitting the isolation of *Rpi-abpt*, *R2* and *R2-like* (**Chapter 2)**, and leading to the additional identification of 22 *Rpi-blb3* haplotypes among *S. bulbocastanum* and related species (**Chapter 3)**. Functionality of new R genes was best demonstrated in stable transformation experiments with suitable (susceptible) potato cultivars. Allelic versions of a specific R gene could better be tested in an alternative functional test, like described in **Chapter 2,** which is a transient expression of R genes in *N benthamiana* leaves followed by inoculation with *P. infestans* isolates. This method was successfully deployed in **Chapter 4** for the identification of *Rpi-mcd1.1*. Detection of known R genes in *Solanum* species under study in **Chapter 3** was possible using a socalled effectoromics approach, where a hypersensitive response, obtained upon agro infiltration of the avirulence gene in the leaves, gave an accurate diagnostic reaction in the presence of a cognate functional R gene. Finally, co-infiltration of R and AVR gene pairs in *N. benthamiana* leaves allowed the accurate identification of critical amino acid(s) for the determination of a particular R2/PiAVR2 molecular interaction **(Chapter 5)**.

Tracing back the origin of the R2GHs

The major late blight resistance cluster on LG IV harbors a profusion of alleles sharing high percentages of identity. These alleles define a new R gene family, first identified by cloning of *Rpi-blb3*, *Rpi-abpt*, *R2* and *R2-like* (**Chapter 2**). *Rpi-blb3* and *R2* originate from *S. bulbocastanum* and *S. demissum*, respectively. Other allele mining studies revealed an identical sequence of *R2-like* in *S. edinense* (Champouret 2010), thus shedding light on its origin. However, the indistinguishable

Aardevo Exhibit 1010
Page 0849

sequence of *Rpi-abpt* was isolated from a *S. tuberosum*/ *S. demissum* clone (Mahdi Mortazavian Wageningen UR Plant Breeding personal communication) adding to the complexity of the origin of this gene that we previously had suspected to be derived from one of the crossing parents used for creation of the quadruple species hybrid ABPT, *S. acaule*, *S. bulbocastanum, S. phureja* or *S. tuberosum*.

In Chapter 5 we report about all the *R2GHs* coding for full length protein sequences. The different alleles isolated in this research and in N. Champouret's (2010) study have been identified in *S. bulbocastanum, S. demissum, S. microdontum*, *S. schenckii*, *S. edinense* and *S. hjertingii*. Interestingly, the *R2GHs* recovered from the clone *S. tuberosum*/ *S. demissum* (Cebeco44158-4) described in **Chapter 2** and the *R2GHs* retrieved from the clone *S. tuberosum*/ *S. microdontum* (RH90-038-21) described in **Chapter 4** ended up in the same phylogenetic clades (**Chapter 5;** Fig.1) and shared a high percentage of amino acid identity. The alleles that the two clones possess in common are seemingly originating from *S. tuberosum.* In addition, the R2GHs identified in the *S. tuberosum* clone RH89-039-16 shared the same phylogenetic clade with R2GHs discovered in tomato. Also, a focused allele mining study using *Rpi-blb3* specific primers in *Solanum* germplasm identified *Rpi-blb3* haplotypes in *S. pinnatisectum*, *S. bulbocastanum* (including some subspecies), *S. hjertingii, S. nayaritense, S. brachistotrichum, S. cardiophyllum* and *S. stoloniferum* **(Chapter 3).**

Using diverse *Solanum* classification systems (Hawkes 1994; Jacobs et al. 2008; Spooner et al. 1991), we failed in our attempt to find phylogenic relationship between the various *Solanum* species harboring *R2GHs* and to deduce the ancestral origin of the MLB residing on linkage group IV.

Intergenic recombination events and mutations, directed by positive selection, shaped the resistance locus

Sequence analyses of the genes isolated by *R2* or *Rpi-blb3* adapted allele mining showed the modular structure of this LZ-NBS LRR R gene family. We identified clear sequence exchanges between the different members of the cluster, nicely exemplified by the structure of *R2* which displayed a different N-terminal sequence when compared to other members of the MLB also interacting with the same avirulence effector PiAVR2. In addition, *R2GHs* identified by N.

Aardevo Exhibit 1010
Page 0850

Champouret (2010) showed residues in the LRR domain under positive selection. Sequence exchanges and point mutations directed by positive selection appeared to have contributed to the birth of different functional resistance genes identified in this investigation. The same phenomenon is observed in linkage group V, where multiple intergenic recombinations resulted in the unique *RB/Rpiblb1* (Song et al. 2003; van der Vossen et al. 2003). Following the same evolution pattern as *R2GHs*, only point mutations were detected in the *Rpi-blb1* haplotypes identified in **Chapter 3**.

A previous study demonstrates the rare occurrence of meiotic recombination per chromosome arm in potato (Park et al. 2007). In addition, the presence of identical *R2GH* sequences in different species reflects the conservation of the functional gene during the evolution of *Solanum*. We presume from this observation that the evolution process which led to the actual functional resistance genes studied here, preceded the divergence of the *Solanum* species from an apparent common ancestor.

Co-evolution between Phytophthora infestans and Solanum is permanent

P. infestans originated from the Toluca valley, in the central highlands of Mexico where it evolved together with wild *Solanum* species. Mating type A1 and A2 were first localized in Mexico, while only mating type A1 was present in Europe . In the 1980s the A2 mating type appeared, followed by a dramatic increase of genetic variation in *P. infestans* suggested to be caused by sexual reproduction (Drenth et al. 1994; Fry et al. 1992; Goodwin and Drenth 1997). This led to the rapid defeat of *Solanum demissum* R genes introgressed into breeding material. *Rpi-blb3*, *Rpi-abpt*, *R2* and *R2 like* which have been identified in **Chapter 2**, together with *Rpi-snk1-1*, *Rpi-snk1-2*, *Rpi-edn1-1*, *Rpi-hjt1-1*, *Rpi-hjt1-2* and *Rpi-hjt1-3* (Champouret 2010), which originated from Mexican species and all interact with the avirulence effector PiAVR2. In **Chapter 3** we discovered a new *Rpi-blb3* haplotype which did not interact with PiAVR2 and geographically is located in Guatemala. In addition in **Chapter 4**, *Rpi-mcd1-1* which originated from Argentina also did not interact with PiAVR2. These examples demonstrate the adaptation of the *R2/Rpi-blb3* cluster to the local population of the pathogen and reflect the co-evolution occurring between *Solanum* and *P.infestans*. Since a rapid evolution of *P. infestans* is witnessed under the presence of the two mating types, it is expected to observe a similar fast evolution of R genes present in wild *Solanum*.

Aardevo Exhibit 1010
Page 0851

Molecular mechanism underlying quantitative resistance

Among the *Solanum* species known to exhibit quantitative resistance, *Solanum microdontum* is well described for its quantitative resistance gene locus (PiQRL), localized on linkage group IV (Sandbrink et al. 2000; Tan et al. 2008). Cytological characterisation of partial resistance is explained by Vleeshouwers et al. (2000) as a less effective hypersensitive response which is possibly caused by an inadequate or delayed recognition of effectors by weak R genes. Additionally, Champouret et al. (2009) categorized *P.infestans* isolates in use for R-gene characterisation, according to their aggressiveness level. **Chapter 4** depicts the dissection of the quantitative resistance map in a population derived from *S. microdontum.* Leaves of susceptible cultivars transformed with *Rpi-mcd1-1* react with a clear hypersensitive response when inoculated with PIC99177 which displays a very low level of aggression, presuming an underlying "weak" R/ Avr gene pair interaction. Taken together, this information contributes to a better understanding of some, previously called, partial resistance factors and gives an example of a suitable way to characterise these partial resistance factors which now appear to be weak R genes.

Future research and breeding advises

Exploring the synteny existing among Solanaceae

Comparison of genomic positions of phenotypically defined disease resistance genes and R gene homologues in two solanaceous crop genera, *Solanum* (potato and tomato) and *Capsicum* (pepper) highlight syntenous resistance loci. These positions harbor genes or QTL providing resistance to various pathogens. For example, the potato linkage group IV which anchors the major MLB studied in this research is syntenous to the tomato chromosome IV loci hosting the *Hero* gene conferring resistance to *G. rostochiensis*. In pepper, the genomic equivalent position is on chromosome V with *phyt1* QRL conferring resistance to *P. capsici* (Grube et al. 2000a). Only 15% of the R2GHs described in **Chapter 5** reproduce the expected late blight resistance spectrum. The remaining homologues stay with undetermined functionality but the demonstrated synteny between the well studied crops tomato, potato, pepper (Grube et al. 2000b) and the less well studied eggplant (Doganlar et al. 2002) opens speculation to the putative resistance provided by some alleles against more different pathogens than *P. infestans*.

Aardevo Exhibit 1010
Page 0852

Broad spectrum and durable resistance

The preference of breeders in introgression of resistance loci providing field resistance, and their believe in a different mechanism underlying quantitative resistance is not strongly supported by the findings in this thesis. In **Chapter 3**, we showed that most of the *Solanum* species displaying large resistance spectrums, often host multiple resistance genes. **Chapter 4** revealed that a classical R gene is in fact encoding the previously identified PiQRL on linkage group IV. This new knowledge about the nature and the distribution of R genes in wild *Solanum* species suggests that achievement of durable resistance can be possible by stacking resistance genes with different resistance spectra and which target functionally different Pi avirulence genes.

P. infestans effector forecast

Taking actual efficient decision support systems (Skelsey et al. 2009) as an example and making use of the current knowledge gathered about *P. infestans*, a complementary forecast approach is proposed. Harvest of *P. infestans* spores present in the target potato cultivation region, followed by the characterization of the avirulence effector content would identify the weakness of the pathogen (Zhu et al.2010). Cultivars containing cognate R genes would be recommended for next season planting. Ongoing studies in different laboratories are conducted to understand the natural evolution of the R genes and the Avr genes, in their natural environment. Using this knowledge, better predictions about the level and the duration of resistance will be possible in the future.

Aardevo Exhibit 1010
Page 0853

LITTERATURE CITED

Allen, R. L., Meitz J. C, Baumber R. E., Hall S. A., Lee S. C., Rose L. E and Beynon J. L. 2008. Natural variation reveals key amino acids in a downy mildew effector that alters recognition specificity by an Arabidopsis resistance gene. Mol. Plant Pathol.9: 511-523

Allen, R. L., Bittner Eddy, P. D., Grenville Briggs, L. J., Meitz, J. C., Rehmany, A. P., Rose, L. E., and Beynon, J. L. 2004. Host-parasite coevolutionary conflict between Arabidopsi*s* and downy mildew. Science 306:1957-1960.

Arens, P., Mansilla, C., Deinum, D., Cavellini, L., Moretti, A., Rolland, S., van der Schoot, H., Calvache, D., Ponz, F., Collonnier, C., Mathis, R., Smilde, D., Caranta, C., and Vosman, B. 2010. Development and evaluation of robust molecular markers linked to disease resistance in tomato for distinctness, uniformity and stability testing. Theor. Appl. Genet. doi 10.1007/s00122-009-1183-2

Armstrong, M. R., Whisson, S. C., Pritchard, L., Bos, J. I. B., Venter, E., Avrova, A. O., Rehmany, A. P., Bo¨hme, U., Brooks, K., Cherevach, I., Hamlin, N., White, B., Fraser, A., Lord, A., Quail, M. A., Churcher, C., Hall, N., Berriman, M., Huang, S., Kamoun, S., Beynon, J. L., and Birch, P. R. J. 2005. An ancestral oomycete locus contains late blight avirulence gene *Avr3a*, encoding a protein that is recognized in the host cytoplasm. PNAS 102:7766-7771

Bai, Y. L., van der Hulst, R., Bonnema, G., Marcel, T. C., Meijer-Dekens, F., Niks, R. E., and Lindhout, P. 2005. Tomato defense to *Oidium neolycopersici*: Dominant *Ol* genes confer isolate-dependent resistance via a different mechanism than recessive *ol-2*. Mol. Plant-Microbe Interact. 18:354-362.

Ballvora, A., Ercolano, M. R., Weiss, J., Meksem, K., Bormann, C. A., Oberhagemann, P., Salamini, F., and Gebhardt, C. 2002. The R1 gene for potato resistance to late blight (Phytophthora infestans) belongs to the leucine zipper/NBS/LRR class of plant resistance genes. Plant J 30:361-71

Belkhadir, Y., Nimchuk, Z., Hubert, D. A., Mackey, D., and Dangl, J. L. 2004. Arabidopsis RIN4 negatively regulates disease resistance mediated by RPS2 and RPM1 downstream or independent of the NDR1 signal modulator and is not required for the virulence functions of bacterial type III effectors AvrRpt2 or AvrRpm1. Plant Cell 16:2822-2835.

Bendahmane, A., Kanyuka, K., and Baulcombe, D. C. 1999. The *Rx* gene from potato controls separate virus resistance and cell death responses. Plant Cell 11:781-791.

Bergelson, J., Dwyer, G., and Emerson, J. J. 2001. Models and data on plant-enemy co evolution. Annu. Rev. Genet. 35:469-499.

Birch, P. R. J., Boevink, P. C., Gilroy, E. M., Hein, I., Pritchard, L., and Whisson, S. C. 2008a. Oomycete RXLR effectors: delivery, functional redundancy and durable disease resistance. Curr Opin in Plant Biol. 11:373-379.

Birch, P. R. J., Whisson, S., Boevink, P., Armstrong, M., Gilroy, E., Pritchard, L., Grouffaud, S., Sandanandom, A., Taylor, R., Kamoun, S., and Bos, J. 2008b. Translocated oomycete effectors

that target the plant immune system. Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology 150:S176-S177.

Birch, P. R. J., Rehmany, A. P., Pritchard, L., Kamoun, S., and Beynon, J. L. 2006. Trafficking arms: oomycete effectors enter host plant cells. Trends Microbiol. 14:8-11.

Bisgrove, S. R., Simonich, M. T., Smith, N. M., Sattler, A., and Innes, R. W. 1994. A disease resistance gene in *Arabidopsis* with specificity for 2 different pathogen avirulence genes. Plant Cell 6:927-933.

Bisognin, D. A., Douches, D. S., Buszka, L., Bryan, G., and Wang, D. 2005. Mapping Late Blight Resistance in Solanum microdontum Bitter. Crop Sci 45:340-345.

Bittner-Eddy, P. D., Crute, I. R., Holub, E. B., and Beynon, J. L. 2000. *RPP13* is a simple locus in *Arabidopsis thaliana* for alleles that specify downy mildew resistance to different avirulence determinants in *Peronospora parasitica*. Plant Journal 21:177-188.

Black, L. M. 1970. Potato yellow dwarf virus. Assoc. Appl. Biologists, Wellesbourne, UK No. 35.

Bonierbale, M. W., Plaisted, R. L., and Tanksley, S. D. 1988. RFLP Maps Based on a Common Set of Clones Reveal Modes of Chromosomal Evolution in Potato and Tomato. Genetics 120:1095-1103.

Bos, J. I. B., Kanneganti, T.-D., Young, C., Cakir, C., Huitema, E., Win, J., Armstrong, M., Birch, P. R. J., and Kamoun, S. 2006. The C-terminal half of *Phytophthora infestans* RXLR effector AVR3a is sufficient to trigger R3a-mediated hypersensitivity and suppress INF1-induced cell death in Nicotiana benthamiana. Plant Journal in press.

Bradshaw, J. E., Bryan, G. J., Lees, A. K., McLean, K., and Solomon Blackburn, R. M. 2006. Mapping the *R10* and *R11* genes for resistance to late blight (*Phytophthora infestans*) present in the potato (*Solanum tuberosum*) R-gene differentials of Black. Theoretical and applied genetics 112:744-751.

Bradshaw, J., Bryan, G., Hackett, C., McLean, K., Pande, B., Stewart, H., and Waugh, R. 2004. Dissection and analysis of quantitative disease resistance in tetraploid potato. Euphytica 137:13-18.

Brouwer, D. J., Jones, E. S., and St Clair, D. A. 2004. QTL analysis of quantitative resistance to *Phytophthora infestans* (late blight) in tomato and comparisons with potato. Genome 47:475-492.

Brown, C. R. 1990. Modern Evolution of the Cultivated Potato Gene Pool. In Vayda, M.E. and Park, W.D. The Molecular and Cellular Biology of the Potato Wallingford. CAB international. Wallingford:1-12.

Budin, K. Z. 2002. Genetic foci of *Solanum* species, *Petota* Dumort, resistant to *Phytophthora infestans* (Mont.) De Bary. Genet. Res. Crop Evol. 49:229-235.

Caicedo, A. L. 2008. Geographic diversity cline of *R* gene homologs in wild populations of *Solanum pimpinellifolium* (Solanaceae). Am. J. of Bot. 95:393-398.

Catanzariti, A.-M., Dodds, P. N., Ve, T., Kobe, B., Ellis, J. G., and Staskawicz, B. J. 2010. The AvrM Effector from Flax Rust Has a Structured C-Terminal Domain and Interacts Directly with the M Resistance Protein. Molecular Plant-Microbe Interactions 23:49-57.

Aardevo Exhibit 1010
Page 0855

Celebi-Toprak, F., Slack, S. A., and Jahn, M. M. 2002. A new gene, *Ny$_{tbr}$*, for hypersensitivity to potato virus Y from *Solanum tuberosum* maps to chromosome IV. Theor. Appl. Genet. 104:669-674.

Champouret, N. 2010. Functional Genomics of *Phytophthora infestans* Effectors and *Solanum* Resistance Genes. PhD Thesis, Wageningen UR, Plant Breeding.

Champouret, N., Bouwmeester, K., Rietman, H., van der Lee, T., Maliepaard, C., Heupink, A., van de Vondervoort, P. J. I., Jacobsen, E., Visser, R. G. F., van der vossen, E. A. G., Govers, F., and Vleeshouwers, V. G. A. A. 2009. *Phytophthora infestans* isolates lacking class I IpiO variants are virulent on *Rpi-blb1* potato. Mol. Plant-Microbe interact.22:1535-1545

Collier, S. M., and Moffett, P. 2009. NB-LRRs work a "bait and switch" on pathogens. Trends in Plant Science 14:521-529.

Colon, L. T., Jansen, R. C., and Budding, D. J. 1995. Partial resistance to Late Blight (*Phytophtora infestans*) in hybrid progenies of 4 south-american *Solanum* species crossed with diploid *Solanum tuberosum* Theor. Appl. Genet. 90:691-698.

Colton, L. M., Groza, H. I., Wielgus, S. M., and Jiang, J. M. 2006. Marker-assisted selection for the broad-spectrum potato late blight resistance conferred by gene *RB* derived from a wild potato species. Crop Science 46:589-594.

Damasceno, C. M. B., Bishop, J. G., Ripoll, D. R., Win, J., Kamoun, S., and Rose, J. K. C. 2008. Structure of the Glucanase Inhibitor Protein (GIP) Family from Phytophthora Species Suggests Coevolution with Plant Endo-Î²-1,3-Glucanases. Molecular Plant-Microbe Interactions 21:820-830.

Danan, S. 2009. Diversité structurale des locus de résistance a Phytophthora infestans chez la pomme de terre et syntenie chez les Solanacées. Thèse doctorale (INRA d'Avignon et Centre international d'études supérieures en sciences agronomique SupAgr Momtpellier).

Dickinson, M., Jones, D., and Jones, J. 1993. Close linkage between the *Cf-2/Cf-5* and *Mi* resistance loci in tomato. Mol. Plant-Microbe Interact. 6:341-347.

de Vetten, N., Wolters, A. M., Raemakers, K., van der Meer, I., ter Stege, R., Heeres, E., Heeres, P., and Visser, R. 2003. A transformation method for obtaining marker-free plants of a cross-pollinating and vegetatively propagated crop. Nature biotechnology 21:439-442.

Dear, S., and Staden, R. 1991. A sequence assembly and editing program for efficient management of large projects. Nucleic Acids Res. 19:3907-3911.

Dodds, P. N., Lawrence, G. J., Catanzariti, A. M., Ayliffe, M. A., and Ellis, J. G. 2004. The Melampsora lini *AvrL567* avirulence genes are expressed in haustoria and their products are recognized inside plant cells. Plant Cell 16:755-768.

Dodds, P. N., Lawrence, G. J., and Ellis, J. G. 2001. Contrasting modes of evolution acting on the complex *N* locus for rust resistance in flax. The Plant Journal 27:439-453.

Doganlar, S., Frary, A., Daunay, M. C., Lester, R. N., and Tanksley, S. D. 2002. Conservation of gene function in the Solanaceae as revealed by comparative mapping of domestication traits in eggplant. Genetics . Aug 161:1713-1726.

Dou, D., Kale, S. D., Wang, X., Chen, Y., Wang, Q., Wang, X., Jiang, R. H. Y., Arredondo, F. D., Anderson, R. G., Thakur, P. B., McDowell, J. M., Wang, Y., and Tyler, B. M. 2008. Conserved

Aardevo Exhibit 1010
Page 0856

C-Terminal Motifs Required for Avirulence and Suppression of Cell Death by Phytophthora sojae effector Avr1b. Plant Cell 20:1118-1133.

Drenth, A., Tas, I. C. Q., and Govers, F. 1994. DNA fingerprinting uncovers a new sexually reproducing population of *Phytophthora infestans* in the Netherlands. European Journal of Plant Pathology 100:97-107.

Dumas, B., Bottin, A., Gaulin, E., and Esquerré-Tugayé, M.-T. 2008. Cellulose-binding domains: cellulose associated-defensive sensing partners? TRENDS in plant science 13:160-164.

Ellis, J. G., Lawrence, G. J., and Dodds, P. N. 2007. Further analysis of gene-for-gene disease resistance specificity in flax. Molecular Plant Pathology 8:103-109.

Ellis, J. G., Lawrence, G. J., Luck, J. E., and Dodds, P. N. 1999. Identification of regions in alleles of the flax rust resistance gene *L* that determine differences in gene-for-gene specificity. Plant Cell 11:495-506.

EPipe 1.0: a pipeline for discovery of functional differences in proteins originating from alternative splicing, pan-genome variation or protein family evolution. In preparation by several groups from the BioSapiens Network of Excellence, 2008.

Ernst, K., Kumar, A., Kriseleit, D., Kloos, D. U., Phillips, M. S., and Ganal, M. W. 2002. The broad-spectrum potato cyst nematode resistance gene (*Hero*) from tomato is the only member of a large gene family of NBS-LRR genes with an unusual amino acid repeat in the LRR region. Plant J. 31:127-136.

Ewing, E. E., and Simko 2000. Genetic mapping from field tests of qualitative and quantitative resistance to Phytophthora infestans in a population derived from Solanum tuberosum and Solanum berthaultii. Molecular breeding 6:25-36.

Flor, H. H. 1971. Current status of the gene-for-gene concept. Annu. Rev. Phytopathol. 78:275-298.

Foster, S. J., Park, T.-H., Pel, M., Brigneti, G., Śliwka, J., Jagger, L., van der Vossen, E., and Jones, J. D. G. 2009. Rpi-vnt1.1, a Tm-22 Homolog from Solanum venturii, Confers Resistance to Potato Late Blight. Molecular Plant-Microbe Interactions 22:589-600.

Friedman, A. R., and Baker, B. J. 2007. The evolution of resistance genes in multi-protein plant resistance systems. Curr. Opin. Genetics Dev. 17:493-499.

Fry, W. 2008. *Phytophthora infestans*: the plant (and *R* gene) destroyer. Molecular plant pathology 9:385-402.

Fry, W. E., Goodwin, S. B., Matuszak, J. M., Spielman, L. J., Milgroom, M. G., and Drenth, A. 1992. Population genetics and intercontinental migrations of *Phytophthora infestans*. Annual Review of Phytopathology 30:107-130.

Gaulin, E., Drame, N., Lafitte, C., Torto Alalibo, T., Martinez, Y., Ameline Torregrosa, C., Khatib, M., Mazarguil, H., Villalba Mateos, F., Kamoun, S., Mazars, C., Dumas, B., Bottin, A., Esquerre Tugaye, M. T., and Rickauer, M. 2006. Cellulose binding domains of a Phytophthora cell wall protein are novel pathogen-associated molecular patterns. Plant cell 18:1766-1777.

Gebhardt, C., and Valkonen, J. P. 2001. Organization of genes controlling disease resistance in the potato genome. Annu. Rev. Phytopathol. 39:79-102.

Aardevo Exhibit 1010
Page 0857

Gebhardt, C. 1994. RFLP mapping in potato of qualitative and quantitative genetic loci conferring resistance to potato pathogens. American Potato Journal 71:339-345.

Gebhardt, C., Ritter, E., Barone, A., Debener, T., Walkemeier, B., Schachtschnabel, U., Kaufmann, H., Thompson, R. D., Bonierbale, M. W., Ganal, M. W., Tanksley, S. D., and Salamini, F. 1991. RFLP maps of potato and their alignment with the homologous tomato genome. Theoretical and Applied Genetics 83:49-57

Goodwin, S. B., and Drenth, A. 1997. Origin of the A2 mating type of *Phytophthora infestans* outside Mexico. Phytopathology 87:992-999.

Graham, K. M. 1965. Experimental hybridization in certain diploid mexican solanum species. Euphytica 14:113-119.

Grant, M. R., Godiard, L., Straube, E., Ashfield, T., Lewald, J., Sattler, A., Innes, R. W., and Dangl, J. L. 1995. Structure of the *Arabidopsis Rpm1* gene enabling dual-specificity disease resistance. Science 269:843-846.

Grube, R. C., Blauth, J. R., Arnedo, M. S., Caranta, C., and Jahn, M. K. 2000. Identification and comparative mapping of a dominant potyvirus resistance gene cluster in Capsicum. Theor. Appl. Genet. 101:852-859.

Ghislain, M., and Trognitz 2001. Genetic loci associated with field resistance to late blight in offspring of Solanum phureja and S. tuberosum grown under short-day conditions. Theoretical and applied genetics 103:433-442.

Hall, S. A., Allen, R. L., Baumber, R. E., Baxter, L. A., Fisher, K., Bittner-Eddy, P. D., Rose, L. E., Holub, E. B., and Beynon, J. 2009. Maintenance of genetic variation in plants and pathogens involves complex networks of gene-for-gene interactions. Mol. Plant Pathol. 10:449 - 457.

Hammond-Kosack, K. E., and Kanyuka, K. 2007. Resistance genes (R genes) in plants. Encyclopedia of Life Sciences.

Hanson, P., Sitathani, K., Sadashiva, A., Yang, R.., Graham, E., and Ledesma, D. 2007. Performance of Solanum habrochaites LA1777 introgression line hybrids for marketable tomato fruit yield in Asia. Euphytica 158:167-178.

Haas, B. J., Kamoun, S., Zody, M. C., Jiang, R. H. Y., Handsaker, R. E., Cano, L. M., Grabherr, M., Kodira, C. D., Raffaele, S., Torto-Alalibo, T., Bozkurt, T. O., Ah-Fong, A. M. V., Alvarado, L., Anderson, V. L., Armstrong, M. R., Avrova, A., Baxter, L., Beynon, J., Boevink, P. C., Bollmann, S. R., Bos, J. I. B., Bulone, V., Cai, G., Cakir, C., Carrington, J. C., Chawner, M., Conti, L., Costanzo, S., Ewan, R., Fahlgren, N., Fischbach, M. A., Fugelstad, J., Gilroy, E. M., Gnerre, S., Green, P. J., Grenville-Briggs, L. J., Griffith, J., Grunwald, N. J., Horn, K., Horner, N. R., Hu, C.-H., Huitema, E., Jeong, D.-H., Jones, A. M. E., Jones, J. D. G., Jones, R. W., Karlsson, E. K., Kunjeti, S. G., Lamour, K., Liu, Z., Ma, L., MacLean, D., Chibucos, M. C., McDonald, H., McWalters, J., Meijer, H. J. G., Morgan, W., Morris, P. F., Munro, C. A., Oâ€™Neill, K., Ospina-Giraldo, M., Pinzon, A., Pritchard, L., Ramsahoye, B., Ren, Q., Restrepo, S., Roy, S., Sadanandom, A., Savidor, A., Schornack, S., Schwartz, D. C., Schumann, U. D., Schwessinger, B., Seyer, L., Sharpe, T., Silvar, C., Song, J., Studholme, D. J., Sykes, S., Thines, M., van de Vondervoort, P. J. I., Phuntumart, V., Wawra, S., Weide, R., Win, J., Young, C., Zhou, S., Fry, W., Meyers, B. C., van West, P., Ristaino, J., Govers, F., Birch, P. R. J., Whisson, S. C., Judelson, H. S., and Nusbaum, C. 2009. Genome sequence and analysis of the Irish potato famine pathogen Phytophthora infestans. Nature 461:393-398.

Aardevo Exhibit 1010
Page 0858

Haverkort, A. J., Struik, P. C., Visser, R. G. F., and Jacobsen, E. 2009. Applied biotechnology to combat late blight in potato caused by *Phytophthora infestans*. Potato Res. 52:249-264.

Hawkes, J. G. 1994. Origins of cultivated potatoes and species relationships. Pages 3-42. In: Potato Genetics. Bradshaw and MacKay, eds.

Hawkes, J. G. 1978. History of the potato. In Harris, P.M. The potato crop: the scientific basis for improvement Ed. P. M. harris. Chapman and Hall. London:1-69.

Hawkes, J. G., and Hjerting, J. P. 1969. The Potatoes of Argentina, Brazil, Paraguay and Uruguay Oxford University Press.

Heilersig, H. J. B., Loonen, A., Bergervoet, M., Wolters, A. M. A., and Visser, R. G. F. 2006. Post-transcriptional gene silencing of *GBSSI* in potato: Effects of size and sequence of the inverted repeats. Plant Mol. Biol. 60:647-662.

Hein, I., Birch, P., Danan, S., Lefebvre, V., Achieng Odeny, D., Gebhardt, C., Trognitz, F., and Bryan, G. 2009a. Progress in Mapping and Cloning Qualitative and Quantitative Resistance Against Phytophthora infestans in Potato and Its Wild Relatives. Potato Research 52:215-227.

Hein, I., Gilroy, E. M., Armstrong, M. R., and Birch, P. R. J. 2009b. The zig-zag-zig in oomycete–plant interactions. Molecular and plant pathology 10:547-562.

Hein, I., McLean, K., Chalhoub, B., and Bryan, G. J. 2007. Generation and Screening of a BAC Library from a Diploid Potato Clone to Unravel Durable Late Blight Resistance on Linkage Group IV. *International Journal of Plant Genomics* Vol 2007 Article ID 51421:5 pages doi:10.1155/2007/51421.

Helgeson, J. P., Pohlman, J. D., Austin, S., Haberlach, G. T., Wielgus, S. M., Ronis, D., Zambolim, L., Tooley, P., McGrath, J. M., James, R. V., and Stevenson, W. R. 1998. Somatic hybrids between *Solanum bulbocastanum* and potato: a new source of resistance to late blight. Theor. Appl. Genet. 96:738-742.

Henk, A. D., Warren, R. F., and Innes, R. W. 1999. A New Ac-Like Transposon of Arabidopsis Is Associated With a Deletion of the RPS5 Disease Resistance Gene. Genetics 151:1581-1589.

Hermsen, J. G. T., and Ramanna, M. S. 1973. Double-bridge hybrids of *Solanum bulbocastanum* and cultivars of *Solanum tuberosum*. Euphytica 22:457-466.

Hijmans, R. J., Gavrilenko, T., Stephenson, S., Bamberg, J., Salas, A., and Spooner, D. M. 2007. Geographical and environmental range expansion through polyploidy in wild potatoes (*Solanum* section *Petota*). Global Ecol. Biogeography 16:485-495.

Holub, E. B. 2001. The arms race is ancient history in Arabidopsis, the wildflower. Nat. Rev. Genet. 2:516-527.

Huang, S. 2005. Discovery and characterization of the major late blight resistance complex in potato: genomic structure, functional diversity, and implications. PhD thesis. Wageningen University.

Huang, S., van der Vossen, E. A. G., Kuang, H., Vleeshouwers, V. G. A. A., Zhang, N., Borm, T. J. A., van Eck, H. J., Baker, B., Jacobsen, E., and Visser, R. G. F. 2005a. Comparative genomics enabled the isolation of the *R3a* late blight resistance gene in potato. The plant journal 42:251-261.

Aardevo Exhibit 1010
Page 0859

Huang, S., Vleeshouwers, V. G. A. A., Visser, R. G. F., and Jacobsen, E. 2005b. An accurate in vitro assay for high-throughput disease testing to *Phytophthora infestans* in potato. Plant Disease 89:1263-1267.

Huang, S., Vleeshouwers, V. G. A. A., Werij, J. S., Hutten, R. C. B., Eck, H. J. v., Visser, R. G. F., and Jacobsen, E. 2004. The R3 resistance to Phytophthora infestans in potato is conferred by two closely linked R genes with distinct specificities. Molecular Plant Microbe Interactions 17:428-435.

Hwang, C. F., and Williamson, V. M. 2003. Leucine-rich repeat-mediated intramolecular interactions in nematode recognition and cell death signaling by the tomato resistance protein Mi. Plant journal 34:585-593.

Hwang, C. F., Bhakta, A. V., Truesdell, G. M., Pudlo, W. M., and Williamson, V. M. 2000. Evidence for a role of the N terminus and leucine-rich repeat region of the Mi gene product in regulation of localized cell death. Plant Cell 12:1319-1329.

Hulbert, S. H., Webb, C. A., Smith, S. M., and Sun, Q. 2001. Resistance gene complexes: Evolution and Utilization. Annual Review of Phytopathology 39:285-312.

Jacobs, M., Vosman, B., Vleeshouwers, V., Visser, R., Henken, B., and van den Berg, R. 2010. A novel approach to locate Phytophthora infestans resistance genes on the potato genetic map. Theo. Appl.Genet. DOI 10.1007/s00122-009-1199-7

Jacobs, M. M. J., van den Berg, R. G., Vleeshouwers, V. G. A. A., Visser, M., Mank, R., Sengers, M., Hoekstra, R., and Vosman, B. 2008. AFLP analysis reveals a lack of phylogenetic structure within *Solanum* section *Petota*. BMC Evol. Biol. 18479504 8:145

Jacobsen, E., and Schouten, H. J. 2007. Cisgenesis strongly improves introgression breeding and induced translocation breeding of plants. Trends in Biotechnology 25:219-223.

Janies, D., Hill, A. W., Guralnick, R., Habib, F., Waltari, E., and Wheeler, W. C. 2007. Genomic Analysis and Geographic Visualization of the Spread of Avian Influenza (H5N1). Syst. Biol. 56:321-329.

Jiang, R. H. Y., Tripathy, S., Govers, F., and Tyler, B. M. 2008. RXLR effector reservoir in two Phytophthora species is dominated by a single rapidly evolving superfamily with more than 700 members. Proceedings of the National Academy of Sciences 105:4874-4879.

Jones, J. D. G., and Dangl, J. L. 2006. The plant immune system. Nature 444:323-329.

Jones, D. T. 1999. Protein secondary structure prediction based on position-specific scoring matrices. Journal of Molecular Biology 292:195-202.

Kamoun, S. 2006. A Catalogue of the Effector Secretome of Plant Pathogenic Oomycetes. Annu. Rev. Phytopathol. 44:41-60.

Kamoun, S., van West, P., Vleeshouwers, V. G. A. A., de Groot, K. E., and Govers, F. 1998. Resistance of *Nicotiana benthamiana* to *Phytophthora infestans* is mediated by the recognition of the elicitor protein INF1. Plant Cell 10:1413-1425.

Kardolus, J. P., Eck, H. J., and Berg, R. G. 1998. The potential of AFLPs in biosystematics: A first application inSolanum taxonomy(Solanaceae). Plant Systematics and Evolution 210:87-103.

Aardevo Exhibit 1010
Page 0860

Kim, Y. J., Lin, N. C., and Martin, G. B. 2002. Two distinct *Pseudomonas* effector proteins interact with the Pto kinase and activate plant immunity. Cell 109:589-598.

Kobe, B., and Deisenhofer, J. 1995. Proteins with leucine-rich repeats. Current Opinion in Structural Biology 5:409-416.

Korves, T., and Bergelson, J. 2004. A Novel Cost of R Gene Resistance in the Presence of Disease. Amer. Nat. 163:489-504.

Kramer, L. C., Choudoir, M. J., Wielgus, S. M., Bhaskar, P. B., and Jiang, J. 2009. Correlation Between Transcript Abundance of the RB Gene and the Level of the RB-Mediated Late Blight Resistance in Potato. Mol. Plant-Microbe Interact. 22:447-455.

Kruijt, M., Kip, D. J., Joosten, M. H. A. J., Brandwagt, B. F., and de Wit, P. J. G. M. 2005. The *Cf-4* and *Cf-9* resistance genes against *Cladosporium fulvum* are conserved in wild tomato species. Mol. Plant-Microbe Interact. 18:1011-1021.

Kuang, H., Woo, S-S., Meyers, B. C., Nevo, E. and Michelmore, R. W. 2004. Multiple genetic processes result in heterogeneous rates of evolution within the major cluster disease resistance genes in lettuce. Plant Cell 16:2870-2894.

Kuhl, J. C., and Hanneman 2001. Characterization and mapping of Rpi1, a late-blight resistance locus from diploid (1EBN) Mexican Solanum pinnatisectum. Molecular genetics and genomics 265:977-985.

Lanfermeijer, F. C., Dijkhuis, J., Sturre, M. J. G., de Haan, P., and Hille, J. 2003. Cloning and characterization of the durable tomato mosaic virus resistance gene Tm-22 from Lycopersicon esculentum. Plant Molecular Biology 52:1039-1051.

Lazo, G. R., Stein, P. A., and Ludwig, R. A. 1991. A DNA Transformation-Competent Arabidopsis Genomic Library in Agrobacterium. Nat. Biotech. 9:963-967.

Leonards-Schippers, C., Gieffers, W., Schafer Pregl, R., Ritter, E., Knapp, S. J., Salamini, F., and Gebhardt, C. 1994. Quantitative resistance to *Phytophthora infestans* in potato: a case study for QTL mapping in an allogamous plant species. Genetics 137:67-77.

Leonards-Schippers, C., Gieffers, W., Salamini, F., and Gebhardt, C. 1992. The *R1* gene conferring race-specific resistance to *Phytophthora infestans* in potato is located on potato chromosome V. Molecular and General Genetics 233:278-283.

Li, X., Eck, H. J. v., Rouppe van der Voort, J. N. A. M., Huigen, D. J., Stam, P., and Jacobsen, E. 1998. Autotetraploids and genetic mapping using common AFLP markers: the *R2* allele conferring resistance to *Phytophthora infestans* mapped on potato chromosome 4. Theoretical and applied genetics 96:1121-1128.

Lokossou, A. A., Park, T. H., van Arkel, G., Arens, M., Ruyter-Spira, C., Morales, J., Whisson, S. C., Birch, P. R. J., Visser, R. G. F., Jacobsen, E., and van der Vossen, E. A. G. 2009. Exploiting knowledge of *R/Avr* genes to rapidly clone a new LZ-NBS-LRR family of late blight resistance genes from potato linkage group IV. Mol. Plant-Microbe Interact. 22:630-41.

Luck, J. E., Lawrence, G. J., Dodds, P. N., Shepherd, K. W., and Ellis, J. G. 2000. Regions outside of the leucine-rich repeats of flax rust resistance proteins play a role in specificity determination. Plant Cell 12:1367-1377.

Aardevo Exhibit 1010
Page 0861

Malcolmson, J. 1969. Factors involved in resistance to blight (*Phytophthora infestans* (Mont.) de Bary) in potatoes and assessment of resistance using detached leaves. Annals of Applied Biology 64:461-468.

Malcolmson, J. F., and Black, W. 1966. New *R* genes in *Solanum demissum* Lindl. and their complementary races of *Phytophthora infestans* (Mont.) de Bary. Euphytica 15:199-203.

Mauricio, R., Stahl, E. A., Korves, T., Tian, D. C., Kreitman, M., and Bergelson, J. 2003. Natural selection for polymorphism in the disease resistance gene *Rps2* of *Arabidopsis thaliana*. Genetics 163:735-746.

McDonald, B. A., and Linde, C. 2002. Pathogen population genetics, evolutionary potential, and durable resistance. Annual Review Of Phytopathology 40:349-379.

McDowell, J. M., and Simon, S. A. 2006. Recent insights into R gene evolution. Mol. Plant Pathol. 7:437-448.

McGillivary, J. H., and Clemente, L. J. 1956. Effect of tomato size on solids content. Proc. Am. Soc. Hprt. Sci. 68: 466-469.

McHale, L., Tan, X., Koehl, P., and Michelmore, R. 2006. Plant NBS-LRR proteins: adaptable guards. Genome Biology 7:212.

Meyers, B. C., Dickerman, A. W., Michelmore, R. W., Sivaramakrishnan, S., Sobral, B. W., and Young, N. D. 1999. Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. Plant J. 20:317-332.

Meyer, R. C., Milbourne, D., Hackett, C. A., Bradshaw, J. E., McNichol, J. W., and Waugh, R. 1998a. Linkage analysis in tetraploid potato and association of markers with quantitative resistance to late blight (Phytophthora infestans). Molecular and General Genetics MGG 259:150-160.

Meyers, B. C., Chin, D. B., Shen, K. A., Sivaramakrishnan, S., Lavelle, D. O., Zhang, Z., and Michelmore, R. W. 1998b. The major resistance gene cluster in lettuce is highly duplicated and spans several megabases. Plant Cell 10:1817-1832.

Micheletto, S., Boland, R., and Huarte, M. 2000. Argentinian wild diploid Solanum species as sources of quantitative late blight resistance. Theor. Appl.Genet. 101:902-906.

Michelmore, R., and Meyers, B. 1998. Clusters of resistance genes in plants evolve by divergent selection and a birth-and-death process. Genome Res 8:1113 - 1130.

Milligan, S. B., Bodeau, J., Yaghoobi, J., Kaloshian, I., Zabel, P., and Williamson, V. M. 1998. The root knot nematode resistance gene *Mi* from tomato is a member of the leucine zipper, nucleotide binding, leucine-rich repeat family of plant genes. Plant Cell 10:1307-1319.

Moffett, P., Gad, L., and John, P. C. 2009. Mechanisms of Recognition in Dominant R Gene Mediated Resistance. Pages 1-33, 228-229. In: Advances in Virus Research. Academic Press.

Mondragon Palomino, M., and Meyers 2002. Patterns of positive selection in the complete NBS-LRR gene family of Arabidopsis thaliana. Genome research 12:1305-1315.

Morgan, W., and Kamoun, S. 2007. RXLR effectors of plant pathogenic oomycetes. Current opinion in microbiology 10:332-338.

Aardevo Exhibit 1010
Page 0862

Müller, K. O., and Black, W. 1952. Potato breeding for resistance to blight and virus diseases during the last hundred years. Zeitschrift für Pflanzenzüchtung 31:305-318.

Niederhauser, J., and Mills, W. 1953. Resistance of *Solanum* species to *Phytophthora infestans* in Mexico. Phytopathology 43:57.

Noel, L., Moores, T. L., van der Biezen, E. A., Parniske, M., Daniels, M. J., Parker, J. E., and Jones, J. D. G. 1999. Pronounced intraspecific haplotype divergence at the *RPP5* complex disease resistance locus of *Arabidopsis*. Plant Cell 11:2099-2111.

Nombela, G., Williamson, V. M., and MuÃ±iz, M. 2003. The Root-Knot Nematode Resistance Gene Mi-1.2 of Tomato Is Responsible for Resistance Against the Whitefly Bemisia tabaci. Molecular Plant-Microbe Interactions 16:645-649.

Oberhagemann, P., Chatot-Balandras, C., Schäfer-Pregl, R., Wegener, D., Palomino, C., Salamini, F., Bonnel, E., and Gebhardt, C. 1999. A genetic analysis of quantitative resistance to late blight in potato: towards marker-assisted selection. Molecular Breeding 5:399-415.

Oh, S.-K., Young, C., Lee, M., Oliva, R., Bozkurt, T. O., Cano, L. M., Win, J., Bos, J. I. B., Liu, H.-Y., van Damme, M., Morgan, W., Choi, D., Van der Vossen, E. A. G., Vleeshouwers, V. G. A. A., and Kamoun, S. 2009. In Planta Expression Screens of Phytophthora infestans RXLR Effectors Reveal Diverse Phenotypes, Including Activation of the Solanum bulbocastanum Disease Resistance Protein Rpi-blb2. Plant Cell 21:2928-2947.

Oosumi, T., Rockhold, D., Maccree, M., Deahl, K., McCue, K., and Belknap, W. 2009. Gene Rpi-bt1 from Solanum bulbocastanum Confers Resistance to Late Blight in Transgenic Potatoes. American journal of potato research 86:456-465.

Ori, N., Eshed, Y., Paran, I., Presting, G., Aviv, D., Tanksley, S., Zamir, D., and Fluhr, R. 1997. The I2C family from the wilt disease resistance locus I2 belongs to the nucleotide binding, leucine-rich repeat superfamily of plant resistance genes. Plant Cell 9:521-32.

Park, T., Gros, J., Sikkema, A., Vleeshouwers, V. G. A. A., Muskens, M., Allefs, S., Jacobsen, E., Visser, R. G. F., and Vossen, E. A. G. v. d. 2005a. The late blight resistance locus *Rpi-blb3* from *Solanum bulbocastanum* belongs to a major late blight R gene cluster on chromosome 4 of potato. Molecular Plant Microbe Interactions 18:722-729.

Park, T. H., Vleeshouwers, V. G. A. A., Huigen, D. J., van der Vossen, E. A. G., van Eck, H. J., and Visser, R. G. F. 2005b. Characterization and high-resolution mapping of a late blight resistance locus similar to *R2* in potato. Theoretical and applied genetics 111:591-597.

Park, T. H., Vleeshouwers, V. G. A. A., Hutten, R. C. B., Eck, H. J. v., Vossen, E. v. d., Jacobsen, E., and Visser, R. G. F. 2005c. High-resolution mapping and analysis of the resistance locus *Rpi-abpt* against *Phytophthora infestans* in potato. Molecular Breeding 16:33-43.

Park, T., Vleeshouwers, V. G. A. A., Kim, J., Hutten, R. C. B., and Visser, R. G. F. 2005. Dissection of foliage and tuber late blight resistance in mapping populations of potato. Euphytica . 2005; 143:75-83

Parniske, M., Hammond-Kosack, K. E., Golstein, C., Thomas, C. M., Jones, D. A., Harrison, K., Wulff, B. B., and Jones, J. D. 1997. Novel disease resistance specificities result from sequence exchange between tandemly repeated genes at the *Cf-4/9* locus of tomato. Cell 91:821-32.

Aardevo Exhibit 1010
Page 0863

Pel, M. 2010. Mapping, Isolation and Characterization of Genes Responsible for Late Blight Resistance .PhD thesis, Wageningen UR, Wageningen.

Pel, M. A., Foster, S. J., Park, T.-H., Rietman, H., van Arkel, G., Jones, J. D. G., Van Eck, H. J., Jacobsen, E., Visser, R. G. F., and Van der Vossen, E. A. G. 2009. Mapping and Cloning of Late Blight Resistance Genes from Solanum venturii Using an Interspecific Candidate Gene Approach. Molecular Plant-Microbe Interactions 22:601-615.

Pendinen, G., Gavrilenko, T., Jiang, J. M., and Spooner, D. M. 2008. Allopolyploid speciation of the Mexican tetraploid potato species *Solanum stoloniferum* and *S. hjertingii* revealed by genomic in situ hybridization. Genome 51:714-720.

Pink, D. 2002. Strategies using genes for non-durable disease resistance. Euphytica 124:227-236.

Rairdan, G. J., and Moffett, P. 2006. Distinct domains in the ARC region of the potato resistance protein Rx mediate LRR binding and inhibition of activation. Plant cell 18:2082-2093.

Rauscher, G. M., Smart, C. D., Simko, I., Bonierbale, M., Mayton, H., Greenland, A., and Fry, W. E. 2006. Characterization and mapping of *RPi-ber*, a novel potato late blight resistance gene from *Solanum berthaultii*. Theoretical and applied genetics 112:674-687.

Rehmany, A. P., Gordon, A., Rose, L. E., Allen, R. L., Armstrong, M. R., Whisson, S. C., Kamoun, S., Tyler, B. M., Birch, P. R. J., and Beynon, J. L. 2005. Differential recognition of highly divergent downy mildew avirulence gene alleles by *RPP1* resistance genes from two Arabidopsis lines. Plant Cell 17:1839-1850.

Rodriguez, A., and Spooner, D. M. 1997. Chloroplast DNA Analysis of Solanum bulbocastanum and S. cardiophyllum, and Evidence for the Distinctiveness of S. cardiophyllum Subsp. ehrenbergii (Sect. Petota). Syst. Botany 22:31-43.

Ronald, P. C., Salmeron, J. M., Carland, F. M., and Staskawicz, B. J. 1992. The cloned avirulence gene *Avrpto* induces disease resistance in tomato cultivars containing the *Pto* resistance gene. J. Bacteriol.174:1604-1611.

Rose, L. E., Bittner-Eddy, P. D., Langley, C. H., Holub, E. B., Michelmore, R. W., and Beynon, J. L. 2004. The maintenance of extreme amino acid diversity at the disease resistance gene, *RPP13*, in *Arabidopsis thaliana*. Genetics 166:1517-1527.

Rossi, M., Goggin, F. L., Milligan, S. B., Kaloshian, I., Ullman, D. E., and Williamson, V. M. 1998. The nematode resistance gene *Mi* of tomato confers resistance against the potato aphid. Proceedings of the National Academy of Sciences of the United States of America 95:9750-9754.

Rouppe van der Voort, J., Kanyuka, K., van der Vossen, E., Bendahmane, A., Mooijman, P., Klein-Lankhorst, R., Stiekema, W., Baulcombe, D., and Bakker, J. 1999. Tight physical linkage of the nematode resistance gene *Gpa2* and the virus resistance gene *Rx* on a single segment introgressed from the wild species *Solanum tuberosum subsp. andigena* CPC 1673 into cultivated potato. Mol. Plant-Microbe Interact. 12:197-206.

Sambrook, J., Fritsch, E. F., Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual (Second Edition).

Aardevo Exhibit 1010
Page 0864

Sandbrink, J. M., Colon, L. T., Wolters, P. J. C. C., and Stiekema, W. J. 2000. Two related genotypes of *Solanum microdontum* carry different segregating alleles for field resistance to *Phytophthora infestans*. Molecular Breeding 6:215-225.

Savenkov, E. I., Germundsson, A., Zamyatnin, A. A., Sandgren, M., and Valkonen, J. P. T. 2003. Potato mop-top virus: the coat protein-encoding RNA and the gene for cysteine-rich protein are dispensable for systemic virus movement in Nicotiana benthamiana. Journal of general virology 84 Part 4:1001-1005.

Schaart, J. G., Krens, F. A., Pelgrom, K. T. B., Mendes O., and Rouwendal, G. J. A. 2004. Effective production of marker-free transgenic strawberry plants using inducible site-specific recombination and a bifunctional selectable marker gene. Plant Biotechnology Journal 2:233-240.

Schornack, S., Ballvora, A., Gürlebeck, D., Peart, J., Ganal, M., Baker, B., Bonas, U., and Lahaye, T. 2004. The tomato resistance protein Bs4 is a predicted non-nuclear TIR-NB-LRR protein that mediates defense responses to severely truncated derivatives of AvrBs4 and overexpressed AvrBs3. Plant J. 37:46-60.

Schouten, H. J., and Jacobsen, E. 2008. Cisgenesis and intragenesis, sisters in innovative plant breeding. Trends in Plant Science 13:260-261.

Shan, W. X., Cao, M., Leung, D., and Tyler, B. M. 2004. The *Avr1b* locus of *Phytophthora sojae* encodes an elicitor and a regulator required for avirulence on soybean plants carrying resistance gene *Rps1b*. Molecular Plant Microbe Interactions 17:394-403.

Simko, I. 2002. Comparative analysis of quantitative trait loci for foliage resistance to *Phytophthora infestans* in tuber-bearing Solanum species. Am. J. Potato Res. 79:125-132.

Simons, G., Groenendijk, J., Wijbrandi, J., Reijans, M., Groenen, J., Diergaarde, P., Van der Lee, T., Bleeker, M., Onstenk, J., de Both, M., Haring, M., Mes, J., Cornelissen, B., Zabeau, M., and Vos, P. 1998. Dissection of the *Fusarium I2* gene cluster in tomato reveals six homologs and one active gene copy. Plant Cell 10:1055-68.

Skelsey, P., Kessel, G. J. T., Holtslag, A. A. M., Moene, A. F., and van der Werf, W. 2009. Regional spore dispersal as a factor in disease risk warnings for potato late blight: A proof of concept. Agricultural and Forest Meteorology 149:419-430.

Sliwka, J., Jakuczun, H., Lebecka, R., Marczewski, W., Gebhardt, C., and Zimnoch Guzowska, E. 2006. The novel, major locus Rpi-phu1 for late blight resistance maps to potato chromosome IX and is not correlated with long vegetation period. Theoretical and applied genetics 113:685-695.

Smilde, W. D., and Brigneti 2005. *Solanum mochiquense* chromosome IX carries a novel late blight resistance gene *Rpi-moc1*. Theoretical and applied genetics 110:252-258.

Song, J., Bradeen, J. M., Naess, S. K., Raasch, J. A., Wielgus, S. M., Haberlach, G. T., Liu, J., Kuang, H., Austin-Phillips, S., Buell, C. R., Helgeson, J. P., and Jiang, J. 2003. Gene RB cloned from Solanum bulbocastanum confers broad spectrum resistance to potato late blight. Proc Natl Acad Sci U S A 100:9128-33.

Spooner, D. M., Jansky, S. H., and Simon, R. 2009. Tests of Taxonomic and Biogeographic Predictivity: Resistance to Disease and Insect Pests in Wild Relatives of Cultivated Potato. Crop Sci. 49:1367-1376.

Aardevo Exhibit 1010
Page 0865

Spooner, D. M., van den Berg, R. G., Rodrigues, A., Bamberg, J. B., Hijmans, R. J., and Lara-Cabrera, S. I. 2004. Wild potatoes (*Solanum* section *Petota*; *Solanaceae*) of North and Central America. Amer. Soc. Plant Tax. Ann Arbor, Michigan.

Spooner, D. M., Sytsma, K. J., and Conti, E. 1991. Chloroplast DNA Evidence for Genome Differentiation in Wild Potatoes (Solanum Sect. Petota: Solanaceae). American Journal of Botany 78:1354-1366.

Stahl, E. A., Dwyer, G., Mauricio, R., Kreitman, M., and Bergelson, J. 1999. Dynamics of disease resistance polymorphism at the *Rpm1* locus of *Arabidopsis*. Nature 400:667-71.

Tan, M. Y. A., Hutten, R. C. B., Celis, C., Park, T. H., Niks, R. E., Visser, R. G. F., and van Eck, H. J. 2008. The *Rpi-mcd1* locus from Solanum microdontum involved in resistance to *Phytophthora infestans*, causing a delay in infection, maps on potato chromosome 4 in a cluster of NBS-LRR genes. Molecular Plant-Microbe Interactions 21:909-918.

Thomas, C. M., Jones, D. A., Parniske, M., Harrison, K., Balint-Kurti, P. J., Hatzixanthis, K., and Jones, J. D. 1997. Characterization of the tomato Cf-4 gene for resistance to Cladosporium fulvum identifies sequences that determine recognitional specificity in Cf-4 and Cf-9. Plant Cell 9:2209-24.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25:4876-4882.

Tian, M., Huitema, E., da Cunha, L., Torto Alalibo, T., and Kamoun, S. 2004. A Kazal-like extracellular serine protease inhibitor from Phytophthora infestans targets the tomato pathogenesis-related protease P69B. Journal of Biological Chemistry. 2004; 279:26370-26377.

Toxopeus, H. J. 1964. Treasure-digging for blight resistance in potatoes. Euphytica 13:206-222.

Tress, M. L., Martelli, P. L., Frankish, A., Reeves, G. A., Wesselink, J. J., Yeats, C., Ã"lason, P. l. Ä. s. l., Albrecht, M., Hegyi, H., Giorgetti, A., Raimondo, D., Lagarde, J., Laskowski, R. A., LÃ³pez, G., Sadowski, M. I., Watson, J. D., Fariselli, P., Rossi, I., Nagy, A., Kai, W., StÃ¸rling, Z., Orsini, M., Assenov, Y., Blankenburg, H., Huthmacher, C., RamÃ-rez, F., Schlicker, A., Denoeud, F., Jones, P., Kerrien, S., Orchard, S., Antonarakis, S. E., Reymond, A., Birney, E., Brunak, S. r., Casadio, R., Guigo, R., Harrow, J., Hermjakob, H., Jones, D. T., Lengauer, T., A. Orengo, C., Patthy, L. s., Thornton, J. M., Tramontano, A., and Valencia, A. 2007. The implications of alternative splicing in the ENCODE protein complement. Proceedings of the National Academy of Sciences 104:5495-5500.

Untergasser, A. 2006a. Cloning - Multiple Gateway Reaction II without a Mastermix. Untergasser's Lab. (access to the page: 11/2006) <http://www.untergasser.de/lab/protocols/lr_multiple_gateway_reaction_ii_v1_0.htm>.

Untergasser, A. 2006b. Cloning - Gateway BP-Reaction II. Untergasser's Lab. (access to the page: 11/2006) <http://www.untergasser.de/lab/protocols/bp_gateway_reaction_ii_v1_0.htm>.

van der Biezen, E. A., and Jones, J. D. 1998. The NB-ARC domain: a novel signalling motif shared by plant resistance gene products and regulators of cell death in animals. Current Biology 8:R226-7.

van der Fits, L., Deakin, E. A., Hoge, J. H. C., and Memelink, J. 2000. The ternary transformation system: constitutive virG on a compatible plasmid dramatically increases Agrobacterium-mediated plant transformation. Plant Mol. Biol. 43:495-502.

Aardevo Exhibit 1010
Page 0866

van der Hoeven, R., Ronning, C., Giovannoni, J., Martin, G., and Tanksley, S. 2002. Deductions about the Number, Organization, and Evolution of Genes in the Tomato Genome Based on Analysis of a Large Expressed Sequence Tag Collection and Selective Genomic Sequencing. Plant Cell 14:1441-1456.

van der Lee, T., De Witte, I., Drenth, A., Alfonso, C., and Govers, F. 1997. AFLP linkage map of the oomycete *Phytophthora infestans*. Fungal Genet. Biol. 21: 278-291.

van der Vossen, E. A. G., Gros, J., Sikkema, A., Muskens, M., Wouters, D., Wolters, P., Pereira, A., and Allefs, S. 2005. The *Rpi-blb2* gene from *Solanum bulbocastanum* is an *Mi-1* gene homolog conferring broad-spectrum late blight resistance in potato. Plant Journal 44:208-222.

van der Vossen, E., Sikkema, A., Hekkert, B. L., Gros, J., Stevens, P., Muskens, M., Wouters, D., Pereira, A., Stiekema, W., and Allefs, S. 2003. An ancient R gene from the wild potato species *Solanum bulbocastanum* confers broad-spectrum resistance to *Phytophthora infestans* in cultivated potato and tomato. Plant Journal 36:867-82.

van der Vossen, E. A. G., Rouppe van der Voort, J. N. A. M., Kanyuka, K., Bendahmane, A., Sandbrink, H., Baulcombe, D. C., Bakker, J., Stiekema, W. J., and Klein Lankhorst, R. M. 2000. Homologues of a single resistance-gene cluster in potato confer resistance to distinct pathogens: a virus and a nematode. Plant Journal 23:567-576.

van Engelen, F. A., Molthoff, J. W., Conner, A. J., Nap, J.-P., Pereira, A., and Stiekema, W. J. 1995. pBINPLUS: An improved plant transformation vector based on pBIN19. Transgenic Res. 4:288-290.

van Poppel, P.M.J.A, Jiang, R.H.J., Sliwka J., and Govers, F. 2009. Recognition of Phytophthora infestans Avr4 by potato R4 is triggered by C-terminal domains comprising W motifs. Molecular Plant Pathology 10:611-620.

van Poppel, P. M. J. A., Guo, J., van de Vondervoort, P. J. I., Jung, M. W. M., Birch, P. R. J., Whisson, S. C., and Govers, F. 2008. The Phytophthora infestans Avirulence Gene Avr4 Encodes an RXLR-dEER Effector. Molecular Plant-Microbe Interactions 21:1460-1470.

van West, P., Kamoun, S., van ' t Klooster, J. W., and Govers, F. 1999. Internuclear gene silencing in *Phytophthora infestans*. Mol. Cell 3:339-348.

Villamon, F. G., Spooner, D. M., Orrillo, M., Mihovilovich, E., Pérez, W., and Bonierbale, M. 2005. Late blight resistance linkages in a novel cross of the wild potato species Solanum paucissectum (series Piurana). TAG Theoretical and Applied Genetics 111:1201-1214.

Visker, M., Keizer, L. C. P., Budding, D. J., Van Loon, L. C., Colon, L. T., and Struik, P. C. 2003. Leaf position prevails over plant age and leaf age in reflecting resistance to late blight in potato. Phytopathology 93:666-674.

Vleeshouwers, V. G. A. A., and Rietman, H. 2009. *In planta* expression systems. In: Oomycete Genetics and Genomics. K. H. Lamour and S. Kamoun, eds. John Wiley & Sons, Hoboken, New Jersey. p455-475.

Vleeshouwers, V. G. A. A., Rietman, H., Krenek, P., Champouret, N., Young, C., Oh, S.-K., Wang, M., Bouwmeester, K., Vosman, B., Visser, R. G. F., Jacobsen, E., Govers, F., Kamoun, S., and Vossen, E. A. G. V. d. 2008. Effector genomics accelerates discovery and functional profiling of potato disease resistance and *Phytophthora Infestans* avirulence genes. PLoS ONE 3 (8):e2875.

Aardevo Exhibit 1010
Page 0867

Vleeshouwers, V. G. A. A., van Dooijeweert, W., Govers, F., Kamoun, S., and Colon, L. T. 2000. The hypersensitive response is associated with host and nonhost resistance to *Phytophthora infestans*. Planta 210:853-864.

Vleeshouwers, V. G. A. A., van Dooijeweert, W., Keizer, L. C. P., Sijpkes, L., Govers, F., and Colon, L. T. 1999. A laboratory assay for *Phytophthora infestans* resistance in various Solanum species reflects the field situation. Eur. J. Plant Pathol. 105:241-250.

Vos, P., Simons, G., Jesse, T., Wijbrandi, J., Heinen, L., Hogers, R., Frijters, A., Groenendijk, J., Diergaarde, P., Reijans, M., Fierens, O. J., De, B. M., Peleman, J., Liharska, T., Hontelez, J., and Zabeau, M. 1998. The tomato Mi-1 gene confers resistance to both root-knot nematodes and potato aphids. Nature Biotechnology 16:1365.

Wang, M., Allefs, S., van den Berg, R. G., Vleeshouwers, V. G. A. A., van der Vossen, E. A. G., and Vosman, B. 2008. Allele mining in *Solanum*: conserved homologues of *Rpi-blb1* are identified in *Solanum stoloniferum*. Theor. Appl. Genet. 116:933-943Whisson, S. C., Boevink, P. C., Moleleki, L., Avrova, A. O., Morales, J. G., Gilroy, E. M., Armstrong, M. R., Grouffaud, S., van West, P., Chapman, S., Hein, I., Toth, I. K., Pritchard, L., and Birch, P. R. J. 2007. A translocation signal for delivery of oomycete effector proteins into host plant cells. Nature 450:115-118.

Whisson, S. C., van derLee, T., Bryan, G. J., Waugh, R., Govers, F., and Birch, P. R. J. 2001. Physical mapping across an avirulence locus of *Phytophthora infestans* using a highly representative, large-insert bacterial artificial chromosome library. Molecular Genetics And Genomics. Oct 266:289-295.

Win, J., Morgan, W., Bos, J., Krasileva, K. V., Cano, L. M., Chaparro-Garcia, A., Ammar, R., Staskawicz, B. J., and Kamoun, S. 2007. Adaptive Evolution Has Targeted the C-Terminal Domain of the RXLR Effectors of Plant Pathogenic Oomycetes. Plant Cell 19:2349-2369.

Wulff, B. B. H., Heese, A., Tomlinson-Buhot, L., Jones, D. A., de la Pena, M., and Jones, J. D. G. 2009. The Major Specificity-Determining Amino Acids of the Tomato Cf-9 Disease Resistance Protein Are at Hypervariable Solvent-Exposed Positions in the Central Leucine-Rich Repeats. Molecular Plant-Microbe Interactions 22:1203-1213.

Yu, W., Lamb, J. C., Han, F., and Birchler, J. A. 2006. Telomere-mediated chromosomal truncation in maize. Proceedings of the National Academy of Sciences 103:17331-17336.

Zhou, B., Qu, S., Liu, G., Dolan, M., Sakai, H., Lu, G., Bellizzi, M., and Wang, G.-L. 2006. The Eight Amino-Acid Differences Within Three Leucine-Rich Repeats Between Pi2 and Piz-t Resistance Proteins Determine the Resistance Specificity to Magnaporthe grisea. Molecular Plant-Microbe Interactions 19:1216-1228.

Zhu, S., Zhu, J., LI, Y., Nijenhuis, M., Bergervoet, M., Rietman, H., and Jacobsen, E.. 2010. Broad Spectrum Resistance from *Rpi-blb1* Homologous *R*-genes Has Been Broken by *Phytophthora infestans* Isolates Collected from *Solanum stoloniferum*. Acta Horticulturae Sinica, 37(2): 241–246

Aardevo Exhibit 1010
Page 0868

SUMMARY

Potato is consumed worldwide and represents the fourth most important staple food crop after rice and wheat. Potato cultivars display a large variety of color, shape, taste, cooking properties and starch content but are all derived from the same species; *Solanum tuberosum*. Potato breeding is an economic important activity for international breeding companies, but also plays an important role in breaking the circle of poverty for small farmers. In the Andean region, most farmers use many different potato genotypes combined with farming practices transmitted orally over thousands of years.

The most prominent menace to potato production is Late Blight caused by the oomycete *Phytophthora infestans* which destroys leaves, stems and tubers. Differences of breeding methods between the potato grown in South America and in the rest of the world are related to differences in the consequences of Late Blight infection. In the 19^{th,} century, entire potato fields in Ireland were devastated while in South America *P. infestans* proliferation was readily inhibited. This difference is found in the biodiversity reserve such as that of the Chiloé archipelago in Chile where local small farmers cultivate about 200 varieties of native potato. Obviously, the genetic diversity of cultivated native potato acts as a shield against this versatile pathogen. Inspired by this model to solve the problems raised by the extensive use of potato monoculture, large scale growers and breeders need to maintain genetic diversity in the European staple food crops.

In exploring the South American native potato collection, *Solanum demissum* and later on *Solanum bulbocastanum* appeared to be a source of resistance genes (*Rpi*) to *P. infestans*. The *S. demissum Rpi* genes were transmitted to potato breeding clones by traditional introgression breeding. However the fading of their ability in providing effective resistance against Late Blight infection was witnessed within a decade. In the pursuit to provide a hopefully more durable protection in existing potato cultivars, breeding scientists proposed to directly introduce South American native potato *Rpi* genes in modern potato varieties by using a so-called cisgenic approach. This is in contrast to transgenic plants which can contain genes which have originated from non related genera or even different kingdoms. Breeding of cisgenic plants is on its way to public acceptance because of its inherent resemblance to natural crossing and because efforts are made by the scientific community to explain the principles of cisgenesis.

Lessons were learned from the flexibility of *P. infestans* to overcome the effect of newly introduced *Rpi* genes and, therefore, efforts are still ongoing to discover and clone new *Rpi* genes from native

Aardevo Exhibit 1010
Page 0869

potatoes. With this in mind, a new family of *Rpi* genes represented by *Rpi-blb3*, *Rpi-abpt*, *R2, R2-like* and *Rpi-mcd1.1* were characterized in clones derived from *S. bulbocastanum*, *S. demissum, S. edinense* and *S. microdontum.* We accomplished in this research the physical isolation of these genes, the molecular characterization of their functionality and the allelic distribution in the Petota collection.

Rpi-blb3, Rpi-abpt, *R2, R2-like* and *Rpi-mcd1.1* belong to the potato linkage group IV and all contain signature sequences characteristic of LZ-NBS-LRR proteins. The closest known *R* gene so far is RPP13 from *Arabidopsis thaliana w*hich shares an amino-acid sequence similarity of 35%. The LRR domains of Rpi-blb3, Rpi-abpt, R2 and R2-like proteins are highly homologous, whilst LZ and NBS domains are more polymorphic with those of R2 being the most divergent. All four Rpi genes recognize the recently identified RXLR effector protein PiAVR2 which is secreted by *P.infestans* in the cytoplasm of plant cells during the infection process. Unlike *Rpi-blb3, Rpi-abpt, R2* and *R2-like*, the *S. microdontum* resistance gene *Rpi-mcd1.1* does not interact with PiAVR2 and provides a different resistance spectrum. *Rpi-mcd1.1* shares 90% nucleotide identity with *Rpi-blb3* and polymorphic nucleotides are mainly located in the LRR region.

The *S. bulbocastanum* haplotypes of *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* were discovered in several Mexican diploid as well as polyploid species closely related to *S. bulbocastanum*. These three resistance genes occurred in different combinations and frequencies in *S. bulbocastanum* accessions and their distribution is confined to Central America. A selected set of genotypes was tested for their response to the avirulence effectors IPIO-2, Avr-blb2 and Pi-Avr2, which interact with *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3,* respectively, as well as by disease assays with a diverse set of isolates. Using this approach some accessions could be identified that contain novel, yet unknown, Late Blight resistance factors in addition to the *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* genes

Analysis of the sequences obtained in different allele mining strategies suggests an evolution of the major late blight locus on linkage group IV through recombination and point mutations. The identification of the repeats and amino acids in the LRR domain which are specific for PiAVR2 recognition was possible by making use of the sequence information provided by the alleles.

Finally the results, described in this thesis, have been discussed in a potato/ *P. infestans* co-evolution context.

Aardevo Exhibit 1010
Page 0870

SAMENVATTINGI

Aardappel wordt wereldwijd gegeten en is het op twee na belangrijkst voedingsgewas na rijst en tarwe. Aardappelrassen laten een grootte verscheidenheid aan kleur, vorm, smaak, kookeigenschappen en zetmeelgehalten zien, maar zijn allemaal afkomstig van dezelfde soort: *Solanum tuberosum*. Aardappelveredeling is voor internationale georiënteerde veredelingsbedrijven een economisch belangrijke activiteit, maar het speelt ook een belangrijke rol bij het doorbreken van de armoedecirkel waar kleine boeren zich in bevinden. In de Andes gebruiken boeren verschillende aardappelgenotypen gecombineerd met landbouwtechnieken die al duizenden jaren mondeling worden doorgegeven.

De belangrijkste bedreiging voor de aardappelproductie is de Aardappelziekte die wordt veroorzaakt door de oömyceet *Phytophthora infestans* die bladeren, stengels en knollen aantast en verwoest. Verschillen tussen de manier waarop aardappel wordt gecultiveerd in Zuid-Amerika en de rest van de wereld houdt verband met de verschillen in de consequenties van *Phytophthora* infecties. In de 19de eeuw werden hele aardappelvelden in Ierland verwoest terwijl in Zuid-Amerika de verspreiding van *P. infestans* gemakkelijk werd tegengehouden. Dit verschil wordt verklaard door de grote hoeveelheid biodiversiteit zoals die te vinden is in de Chiloé archipelago in Chili waar de plaatselijke bevolking ongeveer 200 verschillende variëteiten van de inheemse aardappel verbouwen. De genetische diversiteit van de gecultiveerde inheemse aardappel gedraagt zich als een schild tegen dit onberekenbare pathogeen. Geïnspireerd door dit model zouden landbouwers en veredelaars de genetische diversiteit in de Europese aardappel meer moeten gebruiken, om problemen te voorkomen die veroorzaakt worden door het te intensief gebruikt van aardappel als monocultuur.

Tijdens het verkennen van de Zuid-Amerikaanse inheemse aardappelcollectie, *bleken Solanum demissum* en later ook *Solanum bulbocastanum* een bron te zijn van resistentiegenen tegen *P. infestans (Rpi)*. De *S. demissum Rpi* genen werden overgebracht in aardappelveredelingsklonen door traditionele introgressie veredeling. Toch werd doorbreking van deze mogelijkheid, om effectieve resistentie tegen de Aardappelziekte te verschaffen, binnen een decennium waargenomen. Met als doel om een hoopvolle en meer duurzame bescherming in bestaande cultivars te verkrijgen, stelden wetenschappers in de plantenveredeling voor, om inheemse Zuid-Amerikaanse aardappel *Rpi* genen direct in moderne aardappelrassen te introduceren via de zogenaamde cisgenese aanpak. Hierbij worden resistentiegenen van aardappel zelf of van kruisbare wilde soorten gebruikt. Dit in tegenstelling tot de transgene aanpak, waarbij planten genen geïntroduceerd kunnen krijgen die afkomstig zijn van niet gerelateerde geslachten of zelfs andere rijken. Veredelen van cisgenese planten is goed op weg naar publieke

Aardevo Exhibit 1010
Page 0871

acceptatie door de impliciete gelijkenis met natuurlijke kruisingen en omdat de principes van cisgenese vanuit de wetenschappelijke gemeenschap goed uitgelegd kunnen worden.

Door de flexibele eigenschap van *P. infestans*, om de bescherming van de nieuw geïntroduceerde *Rpi* genen te overwinnen, wordt nog steeds getracht nieuwe meer duurzame *Rpi* genen te ontdekken en te kloneren uit inheemse aardappelen. Hierdoor is er een nieuwe familie van *Rpi* genen gekarakteriseerd die vertegenwoordigd wordt door *Rpi-blb3*, *Rpi-abpt*, *R2, R2-like* en *Rpi-mcd1.1* die afkomstig zijn van *S. bulbocastanum*, *S. demissum, S. edinense* en *S. microdontum.* Tijdens dit onderzoek zijn we erin geslaagd deze genen fysiek te isoleren, de moleculaire karakterisatie van hun functionaliteit te bepalen en hun allelische verdeling in de Petota collectie in kaart te brengen.

Rpi-blb3, Rpi-abpt, *R2, R2-like* en *Rpi-mcd1.1* behoren tot de aardappel koppelingsgroep IV en hebben allemaal een specifieke sequentie die karakteristiek is voor LZ-NBS-LRR eiwitten. Het meest gelijkende R gen dat tot nu toe bekend is, is RPP13 van *Arabidopsis thaliana* die wat betreft aminozuur sequentie 35% ermee overeenkomt. De LRR domeinen van de Rpi-blb3, Rpi-abpt, R2 en R2-like eiwitten zijn zeer homoloog, terwijl de LZ en NBS domeinen het meest polymorf zijn waarbij die van R2 het meest divergent is. Alle vier de *Rpi* genen herkennen het recent geïdentificeerde RXLR effectoreiwit PiAVR2 welke door *P. infestans* in het cytoplasma van de plantencel wordt uitgescheiden tijdens het infectieproces. In tegenstelling tot *Rpi-blb3, Rpi-abpt, R2* en *R2-like*, gaat het *S. microdontum* resistentiegen *Rpi-mcd1.1* geen interactie aan met PiAVR2 en heeft een ander resistentiespectrum. *Rpi-mcd1.1* komt op DNA nucleotide niveau voor 90% overeen met *Rpi-blb3* en de polymorfe nucleotiden zijn vooral gelokaliseerd in de LRR regio.

De *S. bulbocastanum* haplotypen van *Rpi-blb1, Rpi-blb2* en *Rpi-blb3* werden ontdekt in een aantal Mexicaanse diploïde en polyploïde soorten die verwant aan *S. bulbocastanum* zijn. Deze drie resistentiegenen komen in verschillende combinaties en frequenties voor in *S. bulbocastanum* accessies en hun verspreiding is beperkt tot Centraal Amerika. Een geselecteerde set genotypen was getest op hun reactie op de avirulentie effectoren IPIO-2, Avr-blb2 en Pi-Avr2, welke een interactie aangaan met *Rpi-blb1*, *Rpi-blb2* en *Rpi-blb3*, respectievelijk. Deze set was ook getest na inoculatie met verschillende isolaten. Met deze aanpak konden enkele accessies geïdentificeerd worden die nieuwe, nog onbekende, *Phytopthora* resistentiefactoren bevatten naast de al bekende *Rpi-blb1, Rpi-blb2* en *Rpi-blb3* genen.

Analyse van de sequenties verkregen uit de verschillende "allele mining" strategieën suggereren het optreden van een evolutieproces van dit belangrijke *Phytopthora* resistentielocus, dat op recombinaties en puntmutaties gebaseerd is. Door gebruik te maken van de sequentie informatie van deze allelen

Aardevo Exhibit 1010
Page 0872

konden repeats en aminozuren in het LRR domein geïdentificeerd worden die specifiek voor de PiAVR2 herkenning zijn.

In het laatste hoofdstuk worden de resultaten die in dit proefschrift beschreven zijn in de context van een co-evolutie van aardappel/ *P. infestans* besproken.

Aardevo Exhibit 1010
Page 0873

RESUME

La pomme de terre joue un rôle clé dans le système alimentaire mondial. C'est la principale denrée alimentaire non céréalière du monde. Dans les pays développés, la consommation de pommes de terre augmente considérablement et représente plus de la moitié de la récolte mondiale. Comme elle est facile à cultiver et que sa teneur énergétique est élevée, c'est une culture commerciale précieuse pour des millions d'agriculteurs. Elle est vivement recommandée pour atteindre la sécurité alimentaire et elle peut aider les agriculteurs à faibles revenus et les consommateurs vulnérables à surmonter la crise actuelle des disponibilités alimentaires et de la demande mondiales.

La pomme de terre est consommée dans les Andes depuis 8 000 ans environ, les agriculteurs sont parvenus à sélectionner et à améliorer les premiers spécimens de ce qui allait donner, au fil des millénaires, une diversité inouïe de tubercules. Bien qu'offrant à ses consommateur un choix de couleurs, forme, saveur, propriétés de cuisson, de contenu en amidon etc..La pomme de terre que nous connaissons, l'espèce *Solanum tuberosum*, ne contient en réalité qu'une infime partie de la diversité génétique contenue dans les sept espèces reconnues et dans les 5 000 variétés de pommes de terre qui sont encore de nos jours cultivées dans les Andes.

Cette absence de diversité génétique chez les clones de tubercules cultivés en Amérique du Nord et en Europe a pour conséquence de les rendre extrêmement vulnérables: si un ravageur ou une maladie s'attaquait à une plante, il pouvait se propager rapidement aux autres. Un exemple fut le mildiou de la pomme de terre causé par l'oomycète *Phytophthora infestans*, qui dévasta les champs de pommes de terre d'Europe continentale, de la Belgique à la Russie. Mais le pays le plus affecté fut l'Irlande, où la pomme de terre représentait plus de 80 pour cent de la ration énergétique. Entre 1845 et 1848, le mildiou de la pomme de terre ravagea trois récoltes, provoquant une famine qui causa la mort d'un million de personnes.

De toute évidence, la diversité génétique de la pomme de terre ainsi que les méthodes agricoles pratiquée dans la cordières des Andes agissent comme un bouclier contre les différents bio agresseurs. Inspiré par ce modèle pour résoudre les problèmes soulevés par l'utilisation intensive de la monoculture de pommes de terre et des épidémies de mildiou, agriculteurs et ingénieurs agronomes pensent qu'il est nécessaire de maintenir la diversité génétique dans les cultures de pomme de terre, en mettant au point des variétés plus productives et résistantes aux maladies..

Aardevo Exhibit 1010
Page 0874

En explorant le contenu génétique des pommes de terres indigènes collectionnées a l occasion de différentes expéditions en en Amérique du Sud dans les années 60, il fut découvert de nouvelles espèces contenant une variétés de gènes de résistances au mildiou (Rpi : resistance à *Phytophthora infestans*), qui sont *Solanum demissum* et, plus tard, *Solanum bulbocastanum* . Les gènes de résistance au mildiou présent dans le matériel génétique de *S. demissum*. ont été transmis aux nouvelle variétés de pomme de terre par sélection traditionnelle. L effet souhaité en champs fut de courte durée car la résistance aux mildiou apportée par les nouveaux gènes fut contournée en une décennie par une adaptation rapide de *P. infestans*.

Une autre solution pour rapidement fournir une protection totale, espérons-le, plus durable aux varietes existantes de pommes de terre est d'y introduire directement les gènes de résistances indigènes d'origine sud américaine en utilisant une approche dite cisgenic. Les plantes cisgeniques sont modifiees exclusivement par des gènes provenant du meme genre de plante, à l'inverse des plantes transgéniques qui elles, peuvent contenir des gènes qui provenant de genre non apparentés ou même du monde animal. L'acceptation des plantes cisgenic par le public est en bonne voie grâce aux efforts déployés par la communauté scientifique pour expliquer les principes de cisgenesis ainsi que du fait que ces plantes soient fortement comparable à celle obtenues par sélection traditionnelle.

Des leçons ont été tirées sur la flexibilité de P. infestans pour contourner l'effet de resistance procurer par les gènes nouvellement introduits et, par conséquent, les efforts sont maintenus pour en découvrir et en cloner de nouveaux. Dans cet esprit, une nouvelle famille de gènes de résistance aux mildiou représentée par *Rpi-blb3*, *Rpi-abpt*, *R2*, *R2-like* et *Rpi-mcd1.1* ont été caractérisés dans des clones issus des especes de pommes de terre *S.bulbocastanum* , *S.demissum*, *S.edinense* et *S.microdontum*. Nous avons accompli dans cette recherche l'isolement physique de ces gènes, la caractérisation moléculaire de leurs fonctionnalités ainsi que leur distribution allelic dans la collection de pommes de terre sud américaine dénommée « Petota ».

Les gènes *Rpi-blb3*, *Rpi-abpt*, *R2*, *R2-like* et *Rpi-mcd1.1* sont localisés sur le chromosome numéro IV de la pomme de terre et tous contiennent des séquences caractéristiques des protéines appartenant a la catégorie LZ-NBS-LRR (LZ : leucine zipper, NBS : nucleotide binding site, LRR : leucine rich repeat). Le gène de résistance le plus similaire est *RPP13* provenant de la plante modèle *Arabidopsis thaliana*, avec un taux de similitude des séquence protéiques de 35 %. Les domaines LRR des genes *Rpi-blb3*, *Rpi-abpt*, *R2* et *R2-like* sont très homologues, tandis que les domaines LZ et BNS sont plus polymorphes avec ceux de R2, ce dernier étant le plus divergent. Les protéines issue des quatre gènes reconnaissent la protéine de type RXLR récemment identifiée, PiAVR2, qui est sécrétée par *P.infestans* dans le cytoplasme des cellules végétales au cours du processus d'infection. Contrairement à *Rpi-blb3*,

Aardevo Exhibit 1010
Page 0875

Rpi-abpt, *R2* et *R2-like*, la proteine issue du gène de résistance provenant de *S.microdontum*, *Rpi-mcd1.1,* n'interagit pas avec PiAVR2 et fournit un spectre de résistance différent. *Rpi-mcd1.1* est similaire à 90 % au niveau nucléotidique à *Rpi-blb3* et les nucléotides polymorphes sont principalement situées dans la région LRR.

Les haplotypes *Rpi-blb1*, *Rpi-blb2* et *Rpi-blb3* à l'origine identifiés dans l'èspèce *S. bulbocastanum*, ont été découverts dans plusieurs espèces mexicaines diploïdes et polyploïdes étroitement apparentées à *S. bulbocastanum*. Différentes combinaisons des trois gènes de résistance ont été trouvées et leur distribution est limitée à l'Amérique centrale. Un jeu de génotypes a été sélectionné et testé pour leur réponse aux protéines secretees par *P. infestans*, IPIO-2, Avr-blb2 et PI-Avr2, qui interagissent avec Rpi-blb1, Rpi-blb2 et Rpi-blb3, respectivement. Leurs niveaux de résistance à différent isolats de *P. infestans* ontaussi été évalués. Cette approche as permis d' identifier de nouvelles especes pouvant contenir des facteurs de résistance au mildiou encore inconnus, en plus de *Rpi-blb1*, *Rpi-blb2* et *Rpi-blb3*.

L'analyse des séquences obtenues à partir des différentes stratégies d'« allele mining » suggère une évolution du locus de résistance au mildiou localisé sur le chromosome IV, par recombinaison et mutations ponctuelles. En explorant les différences existant entre les différent allèles, nous avons identifié quelles parties du domaine LRR, sont importantes dans la reconnaissance de PiAVR2.

Enfin, les résultats décrits dans cette thèse ont été discuté dans un contexte de co-évolution entre la pomme de terre et *P. infestans*.

Aardevo Exhibit 1010
Page 0876

ACKNOWLEDGEMENTS

Here comes the last piece of this thesis. The most popular one, where I would like to sincerely acknowledge my colleagues, friends and families who supported my choices and helped me to realize my dreams.

I arrived in Wageningen the 4th of February 2005, thanks to **Richard** who replied positively to my request to do an internship of six months at the Laboratory of Plant Breeding. Richard, thank you to have given me that first opportunity and later on for the trust you had in me to conduct and finish my PhD successfully.

Evert!! How is life?!!?? Thank you for this wonderful interaction we have, you have the wisdom and the patience of what we call in Africa a "sage". More than my promotor, you are the wise man with who I can talk about life, science, the past, the present, and the future. I keep on learning from you. Thank you to accept me the way I am and for your precious advises.

Edwin, Thank you to have been my mentor. You taught me the most important word in science "repeat" and through that word, the ethic of science, and more. You pushed me beyond what I though were my limits, and always put me in front new challenges. These years working with you were a real pleasure.

Ben, without you, the third Chapter wouldn't have been finished today. Thank you for your efficient and fruitful supervision. **Vivianne**, Thank you for your constructive remarks and the time you spent in reading and correcting my chapters despite your overbooked agenda.

Annie, I will never forget your warm welcome in the entrance of Dijkgraaf when I arrived for the first time in Wageningen. Together with **Letty**, you helped me with the Dutch administration and always had answers to all of my questions. The first day, you helped me to find a bike (essential in the Netherlands). You introduced me to **Herma** and **Krit** who brought me to the supermarket to buy my first Dutch groceries. Thank you! **Isolde** and **Marjon**, Thank you to have shared your office and daily working life with me. I keep a nice memory of this period at Binnenhaven 5. You all contributed to make me feel good in this new environment which encouraged me to stay longer in the Netherlands.

Annelies, **Marjon**, **Marian**, **Maarten**, **Gert**, **Koen**, **Marcel** thank you for your friendliness in the lab and your always appreciated advises in any protocol. You are the Kings and the Queens of the lab!!! A special thanks to the `real` potato breeders **Ronald** and **Dirkjan** and to the green fingers of plant breeding and the guarantor of nice healthy plants, **Bertus**, **Gerda**, **Henk**, and **Michel**.

Aardevo Exhibit 1010
Page 0877

Thierry, **Benoit** and **Peter**, your advices were precious at the time I was trying to figure out what to do after my Master. Thank you! **Mariame**, **Jeroen**, **Shital**, **Ningwen**, **Bjorn**, **Björn**, **Antoine**, **Pierre-Yves**, **Estelle**, **Krissana**, **Paula**, **Cesar**, **Nicolas**, **Mathieu** thank you for your friendship!! We shared lunches and coffee breaks but also celebrated any occasion together, house warming parties, new jobs, new cars, birthdays, publications, wedding, births….That was fun!!

Nicolas, **Estelle**, **Mathieu** and **Hendrik**, my "late blight resistance group" fellows. Here we are now, all finishing our PhD. We went through many different phases and learned a lot from each other. Thank you to be who you are and to have shared this wonderful experience.

Krissana and **Nicolas**, thank you for your sincere friendship. From colleagues we became close friends. Our differences brought us closer. Nowadays, we could have a nice scientific discussion punctuated with what our respective daughters have done recently. We could never have imagined that four years ago!!! Amazing life!!!

Mandy and **Rod!!!** What a nice adventure we are having now with our charity projects. Thank you to make this dream possible.

Hey **Remko!!** Thank you for your artistic touch, in my thesis… in my house… in our friendship. You are now officially the artistic "griot" of the family Lokossou-van Herpen!!!

Mariame, **Virginia**, **Brigitte**, **Soifia** and **Edwige**, my ladies band and Maelya's aunties. Thank you for your friendship, your support and your prayers. We shared lots of emotions together and I hope to share more in the future. We are more than friends, we are family!

Tonton Theo et **Tata Marie Ange**, mes parents d'adoption. Merci de m'avoir accueillie au sein de votre famille, de m'avoir protégée et guidée dans mes premiers pas, un peu précoces, à la recherche de mon indépendance. **Penelope** et **Greg**, merci d'avoir cru en moi. Votre soutien fut essentiel, presque vital.

Papa et **Maman**, c'est grâce à vous que je suis arrivée au bout de mes études. Votre amour, vos conseils et encouragements ainsi que votre sagesse ont fait de moi ce que je suis. Merci, du plus profond de mon cœur. **Nounou**, mon petit frère adoré, merci d'être là, à l'écoute, dans mes moments de joie ou de peine. Toujours prêt à aider, quoiqu' il en coute. Prends soin de toi !! **Natasha**, merci pour ta patience, ta douceur et ton humour. J'espères te revoir bientôt. Merci a mes amis et ma famille au **Togo**, en **France** et au **Laos**. De prés ou de loin, vous m'avez tous encouragée et couverte de bénédictions.

When I started my PhD, scientific research was the core of my life, working late in the nights and weekends. And I met **Teun**, my angel on earth, who makes me realize that the sky was blue, birds were singing, flowers were colorful and beautiful. It was in spring 2006. We got married, two times, in Togo and in the Netherlands….nine months later **Maelya** was born. It was the 1st of July 2009. Teun, thank you for this wonderful gift you gave me, your LOVE;

Aardevo Exhibit 1010
Page 0878

Thank you for your patience, your tolerance, your energy, your support in this life. **Teun** and **Maelya**, you are the core of my life. I love you.

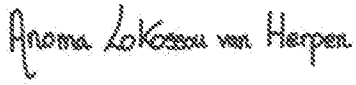
Ria, **Riny**, **Jan**, **Nelly**, **Maarten**, **Melina**, **Wendy**, **Nathalie**, **Iris** en **Mike,** dank jullie wel**.** Jullie hebben jullie armen en hart geopend en me warm en onvoorwaardelijk ontvangen in jullie familie.

This acknowledgment part wouldn't be complete without a special part to **those who left**.

I first would like to thank you **Bud** for your humor and positive attitude in all circumstances, until the end. Rest in peace.

Thank you to the anonymous **donator** who made a legacy gift to Wageningen University fund and permitted to finance my project. I hope that I fulfill his/her wish.

To finish, Thank you to my **ancestors**, **family** and **friends** who kept an eye on me and guide me on the good tracks through this life. M' do Akpe ne mawu. Akpe ne mia togbe o. Agnigban ne fa na mi.



Aardevo Exhibit 1010
Page 0879

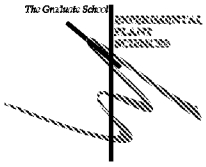


CURRICULUM VITAE

Anoma Akuvi Lokossou was born on September the 7th, 1978 in Nice located on the sunny Mediterranean coast of France. In 1981, she moved with her family to Lomé, the capital of Togo. After she completed her high academic school at the "Lycée français de Lomé" in 1996, she returned to Nice to study biology at the University of Sofia Antipolis. In 2002, she continued with plant biotechnology and bioingeniery study at the University of Paul Sabatier in Toulouse (France). In February 2005, Prof. Dr. Richard Visser gave her the opportunity to do a 6 months internship in Wageningen University at the Laboratory of Plant Breeding (The Netherlands). She worked in the team of Dr. Krit Raemakers on improving the in vitro genetic transformation of Cassava and analyzing the protein content of transgenic pea plants. After she obtained her master degree in September 2005, she started her PhD in Plant breeding under the supervision of Prof. Dr. Richard Visser, Prof. Dr. Ir. Evert Jacobsen and Dr. Edwin van der Vossen. The results of her research are described in this thesis.

Aardevo Exhibit 1010
Page 0880

Education Statement of the Graduate School
Experimental Plant Sciences



Issued to: Anoma A. Lokossou
Date: 7 June 2010
Group: Laboratory of Plant Breeding, Wageningen University

1) Start-up phase	*date*
► **First presentation of your project**	
Late blight resistance genes: Rpi-blb3, Rpi- abpt, R2-like	Mar 20, 2006
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
Genetic Analysis, Tools and Concepts	Oct 31-Nov 19, 2005
► **Laboratory use of isotopes**	
Radiation expert level 5B	Mar 19, 2007
Subtotal Start-up Phase	*9.0 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD Student day, Wageningen University	Sep 19, 2006
EPS PhD Student day, Wageningen University	Sep 13, 2007
EPS PhD Student day, Leiden University	Feb 26, 2009
► **EPS theme symposia**	
EPS theme 4 symposium 'Genome Plasticity', Wageningen University	Feb 09, 2005
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', University of Amsterdam	Feb 02, 2007
► **NWO Lunteren days and other National Platforms**	
NWO-ALW meeting, Lunteren	Apr 02-03,2007
NWO-ALW meeting, Lunteren	Apr 07-08, 2008
NWO-ALW meeting, Lunteren	Apr 08-09, 2009
► **Seminars (series), workshops and symposia**	
Seminar Sophien Kamoun	Oct 05, 2005
Seminar Tony Conner at Keygene	Jun 29, 2005
Seminar Dr. Kurt H. Lamour: "Phytophthora capsici: the key to unlocking Oomycete genetics and host specificity".	Nov 06, 2006
Seminar Dr. Roger Hellens: Identification and functional analysis of transcription factor genes from apple and their role in fruit quality-related traits	Feb 14, 2007
Research day (Plant breeding)	2007
Research day (Plant breeding)	Mar 2009
► **Seminar plus**	
► **International symposia and congresses**	
Oomycete Molecular Genetics Network Wageningen	May 04-07, 2006
MPMI (Italy: Sorrento)	Jul 21 -27, 2007
Sol meeting (Koln)	Oct 12-16, 2008
► Bio-exploit meeting	Feb 25-27, 2009
► **Presentations**	
Poster presentation at Oomycete Molecular Genetics Network Wageningen	May 04-07, 2006
Poster presentation at lunteren	Apr 02-03,2007
Poster presentation at Sorento	Jul 21 -27, 2007
Oral presentation at lunteren	Apr 07-08, 2008
Poster presentation at 5th Solenaceae genome workshop	Oct 12-16, 2008
► **IAB interview**	Sep 13, 2007
► **Excursions**	
Avebe excursion	Sep 2006
Subtotal Scientific Exposure	*13.6 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Signaling in plant development	Jun 19-21, 2006
Gateway to gateway technology	Nov 20-24, 2006
International PhD summerschool "Environmental signaling: Arabidopsis as a model"	Aug 27-29, 2007
► **Journal club**	
► Literature discussion "Plant Breeding"	2005-2009
► **Individual research training**	
Subtotal In-Depth Studies	*6.0 credits**

4) Personal development	*date*
► **Skill training courses**	
6th Frans-Nederlandse Ontmoetingsdagen: Promoveren zonder grenzen (Paris)	May 22-24, 2006
PhD competence assessment	Jun 02, 2006
Teaching and supervising Thesis students	Jun 13-14, 2006
Personal Efficacy	Sep 27-Oct 25, 2006
Toefle test (personal training)	Apr 26, 2008
Course Career perspectives	Oct 20-Nov 24, 2009
► **Organisation of PhD students day, course or conference**	
Lab trip of Plant breeding laboratory	Jun 2006
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*6.6 credits**

TOTAL NUMBER OF CREDIT POINTS*	**35.2**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study*

Aardevo Exhibit 1010
Page 0881

Cover Art / Photo CV / Invitation:

Remko Zijlstra 2010

www.RemkoZijlstra.vpweb.nl

Ezel1@live.nl

Remko Zijlstra created this cover inspired by the history of the potato combined with reading this thesis summary.

The cover depicts the journey of the potato across time, presented by a serie of maps, from the front to the back, as a "come back" to the origin.

This project was financed by a legacy to the Wageningen University Funds (WUF)

Printed by Wöhrmann Print Service, Zutphen, The Netherlands

Aardevo Exhibit 1010
Page 0882

EXHIBIT C

Plant Pathology (2007) **56**, 150–155 Doi: 10.1111/j.1365-3059.2006.01489.x

Occurrence of the *R1* allele conferring resistance to late blight in potato R-gene differentials and commercial cultivars

B. R. Trognitz* and F. Ch. Trognitz

Bioresources Dept., ARC Seibersdorf research GmbH (ARC-sr), 2444 Seibersdorf, Austria

A total of 23 Scottish and 14 Dutch potato R-gene differentials as well as five Austrian, two Dutch and two German commercial potato cultivars were screened for the *R1* allele conferring resistance to *Phytophthora infestans* carrying *Avr1*, via PCR amplification and sequencing. A single 1400 bp fragment with complete sequence identity to the corresponding part of the *R1* allele, was obtained from genomic DNA of all potato R-gene differential clones whose denomination indicates *R1* or a combination of *R1* and other major resistance factors. The *R1* allele was detected, as expected, in all these clones. This fragment also occurred in one Austrian and one German cultivar. Unexpectedly, the same *R1* allele also was detected within all R5, R6 and R9 differentials.

Keywords: international R-gene differential set, *Phytophthora infestans*, *R1* allele

Introduction

The potato-late blight pathogen, *Phytophthora infestans*, forms physiologic races that are able to circumvent resistance determined by specific *R* genes of *Solanum*. Accordingly, the solanaceous hosts of *P. infestans*, including potato, tomato, pepper, pepino dulce and many other species, have evolved multiple *R* genes for race-specific resistance to late blight. Major *R* genes of plants have often been detected in segregating progenies by their highly heritable resistance phenotype. The total number of *R* genes for resistance to late blight in *Solanum* spp. is unknown; they may exceed considerably those represented by the current international set of eleven R-gene differentials (Black *et al.*, 1953; Malcolmson & Black, 1966).

The *R1* gene, characterized by Ballvora *et al.* (2002), confers resistance to strains of *P. infestans* carrying *Avr1* (Van der Lee *et al.*, 2001). *R1* was introduced into the common potato (*S. tuberosum*) from the wild hexaploid, *S. demissum* via cross hybridization and selection for the R1 phenotype. This utilization of potato genetic resources commenced by potato researchers in the 19th century unfortunately has not been fully documented. It is not known whether *R1* was introduced into potato breeding stocks from only one or several different genetic resources. Early accounts (Schick, 1932) refer to a limited number of genetic resources from *S. demissum* and *S.* × *edinense*, its natural hybrid with the cultivated potato that had been used for hybridization experiments by Broili. The German plant pathologist and breeder K.O. Müller described the use of clonal hybrid selections that he labelled 'Ef-Stamm' ('Edinense fraglich'; compare Müller, 1951) referring to their possible taxonomic identity with *S.* × *edinense*. This and other clonal samples, perhaps of a common descent, had been distributed among several potato breeders across the world before 1930.

In 1953, a consortium of potato breeders and pathologists established a set of potato R-gene differentials that would allow for the detection of discriminant avirulence phenotypes (Black *et al.*, 1953). This set comprised clonal selections made individually by W. Black of Scotland, by C. Mastenbroek, the Netherlands, and by W.R. Mills and L.C. Peterson, USA. Other workers at other institutions even had developed individual sets of potato differential stocks maintained as botanical seed (Schick & Schick, 1959). While none of these sets represented all *R* genes and combinations of *R* genes known at that time (by their phenotype), there were several potato clones of possibly diverse origin representing one single factor. This arose as these pioneering late blight researchers maintained differential isolates of *P. infestans* to assay the resistance phenotype of potato segregants containing unknown factors for race-specific resistance. For example, the *P. infestans* isolates breaking R1 resistance were capable of causing disease on the clonal selections namely, 1085(6) (Black's differential), 43154·5 (Mastenbroek), and Essex (Mills and Peterson), all of which attained the status (by their phenotype) of international R1 differentials (Black *et al.*, 1953).

*E-mail: bodo.trognitz@arcs.ac.at

Accepted 14 July 2006

© 2006 The Authors
Journal compilation © 2006 BSPP

Aardevo Exhibit 1010
Page 0884

The international set of differentials (Black *et al.*, 1953) has been maintained clonally and was amended until the late 1960 s (Eide *et al.*, 1959; Black & Malcolmson, 1965; Malcolmson & Black, 1966; Malcolmson, 1969). It comprises clones for the detection of 11 single late blight resistance factors (classified as R1–R11) originating from *S. demissum*.

Although the *R1* gene is overcome by the majority of present-day *P. infestans* populations (Ross 1986; for examples compare Deahl *et al.*, 1991; Andrivon *et al.*, 1994; Drenth *et al.*, 1994; Forbes *et al.*, 1997; Dorrance *et al.*, 1999; Hermansen *et al.*, 2000; Elansky *et al.*, 2001), it has been of interest as a model to plant geneticists. Besides its most obvious role of producing race-specific resistance, it has been suspected to play a role in quantitative field resistance (Darsow *et al.*, 1987; Stewart *et al.*, 2003). Both race-specific and quantitative resistance in the field may, in part, employ the same mechanisms; the basic difference could be the rate and intensity of expression of resistance pathways (Ordoñez *et al.*, 1998; Gebhardt & Valkonen, 2001). The *R1* gene belongs to a large group of structurally related *R1*-like *R* genes and *R* gene analogues whose members appear to be frequent in *Solanum* spp. (Trognitz & Trognitz, 2005).

To assess the power of selectivity of the *R1*-specific PCR primers proposed by Ballvora *et al.* (2002) to tag unequivocally the *R1* allele, these primers have been applied to potato R-gene differential clones and several contemporary and older potato cultivars that are used in breeding. Whether the R1 phenotype of the respective potato R-gene differential clones is reliably associated with the occurrence of the *R1* allele was also determined.

Materials and methods

Plant material

Clones of two potato differential sets (Table 1) were included. The Scottish set of Black's differentials was kindly provided by Dr. H. Stewart, SCRI, Dundee, UK. An incomplete Dutch set has been maintained at the International Potato Centre as described in Trognitz (1998). A copy of this set was provided as stocks *in vitro* by Dr. K. Schüler, Institute of Plant Genetics and Crop Plant Research, External Branch North, Gross Lüsewitz, Germany. In addition, samples of genomic DNA of the Scottish differentials R5, R6, and R9 were kindly provided by Dr. Renata Lebecka, IHAR, Mlochow, Poland). Commercial seed tubers of Austrian, Dutch and German cultivars (Table 1) were obtained from Niederösterreichische Saatbau, Meires, Austria. Plants were maintained pathogen-free *in vitro* and grown in pots in the glasshouse at ARC-sr, Seibersdorf, Austria, according to standard procedures.

Molecular biological procedures

Total DNA was isolated from different plants grown *in vitro* or glasshouse following the instructions of the supplier (DNeasy DNA extraction kit). The *R1*-specific PCR primers 76-2sf2 and 76-2SR identified on *R1* cDNA by Ballvora *et al.* (2002) were applied as indicated by these authors, and ethidium bromide-stained PCR products were visualized on 1%-agarose gels.

For sequencing of DNA fragments, PCR products were cleaned on Sephadex columns; 1 μL of PCR product was mixed with 2 μL BigDye (V 3) reagent (Applied Biosystems), 2 μL buffer, and 0·5 μM *R1*-specific forward or reverse primer. Following the sequence reaction, the cleaned product was mixed with 10 μL HIDI (Applied Biosystems) for processing in an ABI 3100 Genetic Analyser (Applied Biosystems). Forward and reverse sequences were assembled and analysed in Sequencher software (GeneCode).

Detection of plant resistance phenotype

Detached leaflet assays were performed following the procedure of Trognitz (1998). Lateral leaflets were collected from fully expanded top leaves of adult plants. One Petri dish containing four leaflets that had been collected from at least two different clonal plants per genotype represented an experimental unit. A paper towel was folded to fit in the bottom of a Petri dish and wetted to serve as a moisture reservoir. Sporangial inoculum was prepared from infected potato tuber slices (cv. Naglerner Kipfler) and it was applied with a hand-held pump sprayer at a concentration of 20 000 sporangia/mL. The samples were incubated at 18°C for 4–8 days until symptoms were fully developed. Percentage total leaflet area diseased and degree of sporulation were assessed. For all genotypes, tests were repeated on a minimum of two dates. For the inoculations, a 'race 0' isolate of *P. infestans* (provided by W. Flier, Plant Research International, Wageningen, The Netherlands) avirulent on all 11 R-gene differentials but pathogenic on the universal suscept, r0 was used.

Results and discussion

The primers reliably amplified one fragment of the 1400 bp size expected for *R1* (Ballvora *et al.*, 2002; Gebhardt *et al.*, 2004) from 16 R-gene differential clones and two cultivars (Table 1, Fig. 1). The sequences of all fragments proved fully identical with the corresponding part of the *R1* allele at the *R1* locus (accession No. AF447489), regardless of the potato cultivar analysed.

Detached leaflet tests with race 0 gave a resistance reaction on differentials carrying R1–R11 and combinations thereof and susceptibility on the universal suscept. Two of the nine commercial cultivars genotyped were tested with race 0 and of these, Evita was susceptible and this is in accordance with the absence of the *R1* fragment. Linzer Delikatess, also was susceptible in the assays, although it carries the *R1* allele. This suggests that *R1* of Linzer Delikatess either may be suppressed by an unknown intervening genetic element (El-Kharbotli *et al.*, 1996; Ordoñez *et al.*, 1997; Trognitz, 1998), or that an additional unknown, synergistic element required for the function of

Aardevo Exhibit 1010
Page 0885

Table 1 Potato cultivars and late blight R-gene differentials, tested for presence of the *R1* allele and for resistance to a race 0 isolate of *Phytophthora infestans*

Country of origin	R-gene differential	Clone/cultivar	*R1* fragment	Resistant to race 0
Scotland	Universal susceptible	Craig's Royal	No	No
	R1	Craig's Snow White	Yes	Yes
	R2	1512 c(16)	No	Yes
	R3	Pentland Ace	No	Yes
	R4	1563 c(14)	No	(Yes)[a]
	R5	3053–18	**Yes**[b]	Yes
	R6	XD2–21	**Yes**	Yes
	R7	2182ei(7)	No	Yes
	R8	2424a(5)	No	Yes
	R9	2573(2)	**Yes**	Yes
	R10	3681ad(1)	No	Yes
	R11	5008ab(6)	No	Yes
	R1R2	1647b(1)	Yes	Yes
	R1R3	2070(32)	Yes	Yes
	R1R4	1506b(9)	Yes	Yes
	R2R3	1682 c(1)	No	Yes
	R2R4	2070(31)	No	Yes
	R3R4	1488b(1)	No	Yes
	R1R2R3	2070(59)	Yes	Yes
	R1R2R4	2070(69)	Yes	Yes
	R1R3R4	2070(4)	Yes	Yes
	R2R3R4	2070(50)	No	Yes
	R1R2R3R4	2070(54)	Yes	Yes
Netherlands	R1	CEBECO-43154-5, CIP800986	Yes	Yes
	R2	CEBECO-44158-4, CIP800987	No	Yes
	R3	CEBECO-4642-1, CIP800988	No	Yes
	R4	CEBECO-4431-5, CIP800989	No	(Yes)[a]
	R8	Black 2424a(5), CIP800993	No	Yes
	R9	Black 2573, CIP800994	**Yes**	Yes
	R10	Black 3618ad(1), CIP800995	No	Yes
	R11	Black 5008ab(6), CIP800996	No	Yes
	R1R2	CIP800997	Yes	Yes
	R1R3	CIP800998	Yes	Yes
	R2R3	CIP801000	No	Yes
	R3R4	CIP801002	No	Yes
	R1R2R3	CIP801003	Yes	Yes
	R2R3R4	CIP801005	No	Yes
		Arletta (1998)[c]	No	n.d.
		Kuras (1996)	No	n.d.
Austria		Evita (1995)	No	No
		Gabriella (2000)	No	n.d.
		Linzer Delikatess (1975)	Yes	No
		Romina (1989)	No	n.d.
		Rosita (1999)	No	n.d.
Germany		Laura (1998)	Yes	n.d.
		Quarta (1979)	No	n.d.

[a]The race 0 isolate produced small, slowly sporulating lesions on the R4 differentials from different sources indicating that it may possess incomplete avirulence for this resistance factor.
[b]Unexpected phenotype indicated in bold-face. n.d.; not done.
[c]Year of release indicated in parentheses.

R1 is absent in this cultivar. The German cultivar Laura (launched in 1998) also has the *R1* allele; however, its phenotype of resistance to race 0 was not tested. According to varietal assessment results (Anonymous, 2001), the level of susceptibility to foliage blight of Laura (*R1* carrier, second-early maturity) in the field is low–intermediate. For comparison, this level of susceptibility corresponds to a score of 4 on the German 1–9 scale of blight susceptibility (Anonymous, 2003). Laura proved more field-resistant than the other second-early, non-*R1*-carrying cultivars

Aardevo Exhibit 1010
Page 0886

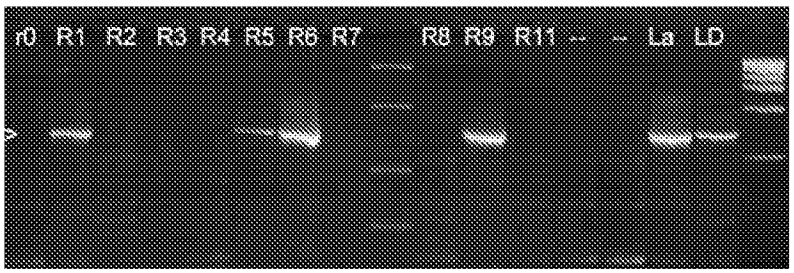


Figure 1 PCR-amplified *R1* fragment (1400 bp, marked at left) from genomic DNA of potato R-gene differentials and cvs Laura and Linzer Delikatess using *R1*-specific primers. r0, universal susceptible differential (Craig's Royal); R1–R11, Scottish R-gene differentials; La, cv. Laura; LD, cv. Linzer Delikatess. Size standards: centre, Fermentas FastRuler; at right, Invitrogen high DNA mass ladder.

Table 2 Summary of *Phytophthora infestans* avirulence phenotypes on R-gene differentials from published studies on isolates collected between 1983 and 1998

Source	Total isolates	Avirulent on R1	Avirulent on R1 and virulent on R5, R6, R9	Country	Year of collection	Host
Hermansen *et al.*, 2000	374	24	0	Finland, Norway	1990–96	potato
Andrivon *et al.*, 1994	23	1	0	France	1991	potato
Lebreton & Andrivon, 1998	62	1	0	France	1995/96	potato, tomato
Drenth *et al.*, 1994	253	not indicated		Netherlands	1981–91	
Schöber & Turkensteen, 1992	77	12	0	Netherlands	1988	potato
Flier & Turkensteen, 1999	36	7	0	Netherlands	1995	potato
Sujkowski *et al.*, 1996	95	2	0	Poland	1985–91	potato
Elansky *et al.*, 2001	191	5	not indicated	Russia	1997/98	potato, tomato
Abu-El Samen *et al.*, 2003[a]	21	9	1 (on R6)	USA	1994/95	potato
Deahl *et al.*, 1991	10	1	0	USA, Canada	1983–89	potato, tomato
Dorrance *et al.*, 1999	60	4	3 (1 on R5, 1 on R6, 1 on R5+R6)	USA	1996/97	potato, tomato, nightshades
Goodwin *et al.*, 1995	32	6	1 (R5), (R6 not done)	USA, Canada, Mexico	1989–94	potato, tomato
Rivera-Peña, 1990	78	23	9 (5 on R5, R6 not done, 4 on R9)	Mexico	1985/86	wild potatoes
Forbes *et al.*, 1997	108	18	1 (R5)	Ecuador	1993	potato
Oyarzun *et al.*, 1998	60	0	0	Ecuador	1993–95	potato
Oyarzun *et al.*, 1998	60	57	0	Ecuador	1994–96	tomato
Total[b]	1287	170	15			

[a]These authors investigated single-zoospore isolates from two single-lesion isolates; all other authors had single-lesion isolates.
[b]Excluding Drenth *et al.*, 1994.

investigated, Evita and Quarta, which possess an intermediate susceptibility level (score 5). Romina (early) also is intermediately susceptible, whereas the late-maturing cv. Kuras is resistant in the field. Both early-maturing cultivars, Rosita and the silent *R1* carrier Linzer Delikatess display an intermediate-pronounced level of susceptibility in the field (score 6). The first-early cultivar Gabriella has been scored intermediately susceptible and no results are available for Arletta from the same source (Anonymous, 2001).

The experiment reported here confirms that the *R1* gene is distributed across all differentials specific for the R1 factor and for all combinations of R1 with other factors. Therefore, there is a good chance that the R1 phenotype of resistance is expressed by the functional *R1* allele as isolated by Ballvora *et al.* (2002). Gebhardt *et al.* (2004) detected this allele also by its distinctive 1400 bp PCR fragment in several accessions of the Mesoamerican *S. demissum* and in one accession of *S. stoloniferum*.

The occurrence of the *R1* allele in the R5, R6 and R9 differentials was unexpected (Table 1, Fig. 1). Because the phenotype resulting from interaction with the universally avirulent race 0 does not allow discrimination among different *R* genes, it cannot be assessed whether and to what extent *R1* may contribute to the resistance phenotype displayed by these three R-gene differential clones.

In Table 2, results of 15 studies on avirulence of *P. infestans* isolates collected throughout more than one decade are summarized. Only 170 out of 1287 isolates tested (13%) were avirulent on the R1 differentials and only 15 of these 170 isolates (9%) were virulent on R5, R6, or R9 differential clones. The latter were exclusively reported in studies from the Americas where R-gene differential clones different from those analysed in this study may have been partly used (as an example see Goodwin *et al.*, 1995). However, as the status of *P. infestans* avirulence generally has been assessed by the resistance phenotypes of R-gene differential clones, it is possible that those

Aardevo Exhibit 1010
Page 0887

multiply-virulent pathotypes may, in part, be artefacts for which inadvertent selection has occurred. This could be verified once additional R-gene differentials of R5, R6, and R9 devoid of *R1* become available. Such additional differentials could easily be obtained by selecting self- and cross-progenies of the existing differentials against R1 using the molecular markers now available (Gebhardt *et al.*, 2004).

Two out of nine commercial potato cultivars tested positive for the presence of *R1* (Table 1) indicating that *R1* still occurs within contemporary European potato breeding stocks although races breaking it down had already appeared in Europe by 1932 (Lehmann, 1937). Selection for this gene was suspended half a century ago when the widespread breakdown of the resistance to several of these *R* genes was recognized (Ross, 1986). The presence of *R1*, however, is understandable considering the narrow pedigree-breeding schemes widely applied, the long period of generation turnover in potato breeding (Gebhardt *et al.*, 2004), and no directed selection against *S. demissum* *R* genes. Notwithstanding, there is evidence that defeated *R* genes still may contribute to an overall increased level of resistance in the field (Darsow *et al.*, 1987; Stewart *et al.*, 2003). Therefore, breeders could be encouraged to use *R* genes for resistance to late blight particularly when these are easily selected as in the case of the *R1* allele.

Acknowledgements

This work was supported by grants from the Austrian Ministry of Agriculture (BMLFUW 1235), the government of Lower Austria (ND 80–2001), and ARC-sr (1·56·00096). We are grateful to Zsuzsanna Portik for performing some of the PCR, José Avendaño-Corcoles for resistance phenotyping, Agnes Burg and Anton Grahsl for maintenance of plants, and to an anonymous reviewer for valuable comments on the manuscript.

References

Abu-El Samen FM, Secor GA, Gudmestad NC, 2003. Genetic variation among asexual progeny of *Phytophthora infestans* detected with RAPD and AFLP markers. *Plant Pathology* **52**, 314–25.

Andrivon D, Beasse C, Laurent C, 1994. Characterization of isolates of *Phytophthora infestans* collected in northwestern France from 1988 to 1992. *Plant Pathology* **43**, 471–8.

Anonymous, 2001. *Saatkartoffeln aus Niederösterreich.* Sortenkatalog 2001. Niederösterreichische Saatbaugenossenschaft, Meires, Austria: NÖS.

Anonymous, 2003. *Beschreibende Sortenliste Kartoffeln 2003.* Bundessortenamt, Hannover, Germany: Deutscher Landwirtschaftsverlag.

Ballvora A, Ercolano MR, Weiss J, Meksem K, Bormann CA, Oberhagemann P, Salamini F, Gebhardt C, 2002. The *R1* gene for potato resistance to late blight (*Phytophthora infestans*) belongs to the leucine zipper/NBS/LRR class of plant resistance genes. *The Plant Journal* **30**, 361–71.

Black W, Malcolmson JF, 1965. New races of *Phytophthora infestans* (Mont.) de Bary and their complementary R-genes from *Solanum demissum* Lindl. *American Potato Journal* **42**, 256.

Black W, Mastenbroek C, Mills WR, Peterson LC, 1953. A proposal for an international nomenclature of races of *Phytophthora infestans* and of genes controlling immunity in *Solanum demissum* derivatives. *Euphytica* **2**, 173–8.

Darsow U, Göbel S, Götz E, Oertel H, Schüler K, 1987. R-Gene und relative Resistenz der Kartoffelknolle gegen *Phytophthora infestans* (Mont.) de Bary. *Archiv für Züchtungsforschung* **17**, 387–97.

Deahl KL, Goth RW, Young R, Sinden SL, Gallegly MG, 1991. Occurrence of the A2 mating type of *Phytophthora infestans* in potato fields in the United States and Canada. *American Potato Journal* **68**, 717–25.

Dorrance AE, Inglis DA, Derie ML, Brown CR, Goodwin SB, Fry WE, Deahl KL, 1999. Characterization of *Phytophthora infestans* populations in western Washington. *Plant Disease* **83**, 423–8.

Drenth A, Tas ICQ, Govers F, 1994. DNA fingerprinting uncovers a new sexually reproducing population of *Phytophthora infestans* in the Netherlands. *European Journal of Plant Pathology* **100**, 97–107.

Eide CJ, Bonde R, Gallegly ME, Graham K, Mills WR, Niederhauser J, Wallin JR, 1959. Report of the late blight investigations committee. *American Potato Journal* **36**, 421–3.

El-Kharbotli A, Pereira A, Stiekema WJ, Jacobsen E, 1996. Race specific resistance against *Phytophthora infestans* in potato is controlled by more genetic factors than only R-genes. *Euphytica* **90**, 331–6.

Elansky S, Smirnov A, Dyakov Y, Dolgova A, Filippov A, Kozlovsky B, Kozlovskaya I, Russo P, Smart C, Fry W, 2001. Genotypic analysis of Russian isolates of *Phytophthora infestans* from the Moscow region, Siberia and far east. *Journal of Phytopathology* **149**, 605–11.

Flier WG, Turkensteen LJ, 1999. Foliar aggressiveness of *Phytophthora infestans* in three potato growing regions in the Netherlands. *European Journal of Plant Pathology* **105**, 381–8.

Forbes GA, Escobar XC, Ayala CC, Revelo J, Ordoñez ME, Fry BA, Doucett K, Fry WE, 1997. Population genetic structure of *Phytophthora infestans* in Ecuador. *Phytopathology* **87**, 375–80.

Gebhardt C, Ballvora A, Walkemeier B, Oberhagemann P, Schüler K, 2004. Assessing genetic potential in germplasm collections of crop plants by marker-trait association: a case study for potatoes with quantitative variation of resistance to late blight and maturity type. *Molecular Breeding* **13**, 93–102.

Gebhardt C, Valkonen JPT, 2001. Organization of genes controlling disease resistance in the potato genome. *Annual Review of Phytopathology* **39**, 79–102.

Goodwin SB, Sujkowski LS, Fry WE, 1995. Rapid evolution of pathogenicity within clonal lineages of the potato late blight disease fungus. *Phytopathology* **85**, 669–76.

Hermansen A, Hannukkala A, Hafskjold Naerstad R, Brurberg MB, 2000. Variation in populations of *Phytophthora infestans* in Finland and Norway: mating type, metalaxyl resistance and virulence phenotype. *Plant Pathology* **49**, 11–22.

Lebreton L, Andrivon D, 1998. French isolates of *Phytophthora infestans* from potato and tomato differ in phenotype and genotype. *European Journal of Plant Pathology* **104**, 583–94.

Aardevo Exhibit 1010
Page 0888

Lehmann H, 1937. Das heutige Ausgangsmaterial für die Züchtung *Phytophthora*-widerstandsfähiger Kartoffeln. *Der Züchter* **9**, 29–35.

Malcolmson JF, 1969. Races of *Phytophthora infestans* occurring in Great Britain. *Transactions of the British Mycological Society* **53**, 417–23.

Malcolmson JF, Black W, 1966. New R-genes in *Solanum demissum* Lindl. and their complementary races of *Phytophthora infestans* (Mont.) de Bary. *Euphytica* **15**, 199–203.

Müller KO, 1951. Über die Herkunft der W-Sorten, ihre Entwicklungsgeschichte und ihre bisherige Nutzung in der praktischen Kartoffelzüchtung. *Zeitschrift für Pflanzenzüchtung* **29**, 366–87.

Ordoñez ME, Forbes GA, Trognitz BR, 1997. Resistance to late blight in potato. A putative gene that suppresses R genes and is elicited by specific isolates. *Euphytica* **95**, 167–72.

Ordoñez ME, Forbes GA, Trognitz BR, 1998. Relationship between ineffective R genes and expansion rate of lesions on potato caused by *Phytophthora infestans*. *Plant Pathology* **47**, 130–6.

Oyarzun PJ, Pozo A, Ordoñez ME, Forbes GA, 1998. Host specificity of *Phytophthora infestans* on tomato and potato in Ecuador. *Phytopathology* **88**, 265–71.

Rivera-Peña A, 1990. Wild tuber-bearing species of Solanum and incidence of *Phytophthora infestans* (Mont.) de Bary on the western slopes of the volcano Nevado de Toluca. 3. Physiological races of *Phytophthora infestans*. *Potato Research* **33**, 349–55.

Ross H, 1986. *Potato Breeding-Problems and Perspectives. Advances in Plant Breeding* 13. Berlin and Hamburg, Germany: Paul Parey.

Schick R, 1932. Über das Verhalten von *Solanum demissum*, *Solanum tuberosum* und ihren Bastarden gegenüber verschiedenen Herkünften von *Phytophthora infestans*. (Vorläufige Mitteilung zur Frage der biologischen Spezialisierung von Phytophthora infestans.) *Der Züchter* **4**, 233–37.

Schick R, Schick E, 1959. Die Differenzierung der verschiedenen Rassen der *Phytophthora infestans* auf Sämlingen von *S. demissum* (Lindl.) und *S. stoloniferum* (Schlecht. et Bouche). *Der Züchter* **29**, 220–5.

Schöber B, Turkensteen LJ, 1992. Recent and future developments in potato fungal pathology. *Netherlands Journal of Plant Pathology* **98**, 73–83.

Stewart HE, Bradshaw JE, Pande B, 2003. The effect of the presence of R-genes for resistance to late blight (*Phytophthora infestans*) of potato (*Solanum tuberosum*) on the underlying level of field resistance. *Plant Pathology* **52**, 193–8.

Sujkowski LS, Goodwin SB, Fry WE, 1996. Changes in specific virulence in Polish populations of *Phytophthora infestans*: 1985–1991. *European Journal of Plant Pathology* **102**, 555–61.

Trognitz BR, 1998. Inheritance of resistance in potato to lesion expansion and sporulation by *Phytophthora infestans*. *Plant Pathology* **47**, 712–22.

Trognitz FCh, Trognitz BR, 2005. Survey of resistance gene analogs in *Solanum caripense*, a wild relative of potato and tomato, and update on *R* gene genealogy. *Molecular Genetics and Genomics* **274**, 595–605.

Van der Lee Th, Robold A, Testa A, van't Klooster JW, Govers F, 2001. Mapping of avirulence genes in *Phytophthora infestans* with markers selected by bulked segregant analysis. *Genetics* **157**, 949–56.

Aardevo Exhibit 1010
Page 0889

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Willem Hendrik Lindhout *et al.*	Group Art Unit: 1663
Serial No.: 16/541,071	Examiner: Russell T. Boggs
Filed: August 14, 2019	Atty. Dkt. No.: VONL:004USC1
For: Hybrid Seed Potato Breeding	Confirmation No.: 4348

DECLARATION OF WILLEM HENDRIK LINDHOUT, PH.D. UNDER 37 C.F.R. § 1.132

I, Willem Hendrik Lindhout, hereby declare as follows:

1. I am a Founder and Director of R&D at Florere B.V. and Agventure B.V., operating under the tradename Solynta, and am an inventor on the patent application identified above ("the Application").

2. I hold an MSc degree in Biology, an MSc degree in Chemistry, and a Ph.D. in Molecular Biology from the University of Leiden, in the Netherlands. Until 2006, I was an Associate Professor of Plant Breeding at Wageningen University in the Netherlands. During this time period, I published more than 100 scientific publications related to plant breeding, served as the Coordinator of Bachelors and Masters Studies in Plant Sciences at Wageningen University, and served as the National Coordinator of Tomato Genomics Research as part of the Centre for Biosystems Genomics (CBSG). Previously, I served as Director of R&D at De Ruiter Seeds. After the acquisition of De Ruiter Seeds by Monsanto, I was appointed Monsanto's Global Breeding Lead of the Vegetable Seed Division.

3. I understand that the United States Patent and Trademark Office is in the process of evaluating the patentability of U.S. patent application serial No. 16/541,071 and am familiar with the contents of the Office Action issued by the United States Patent and Trademark Office on December 5, 2019.

4. I am providing the present Declaration to provide evidence demonstrating that the dihaploid lines designated D1, D2, and D3 are genetically diverse and are representative of the entire range of genomic diversity in the *Solanum tuberosum* germplasm that is currently available and that the application therefore provides a sufficient number of working examples to support the plants as presently claimed and the wide applicability of the claimed methods across diverse *S. tuberosum* germplasm.

5. I am further providing the present Declaration to show that the Examiner is misinterpreting the letter of Dr. Jonathan D G Jones of 11 December 2012 in support of the assertion that the claimed method is not shown to work in *S. tuberosum* lines other than lines D1, D2, and D3, or the deposits made in connection with the application. In particular, I will provide further evidence to support that the success in practicing the claimed invention is not unpredictable and does not depend on the genotype of the potato lines used.

6. Finally, I am providing the present Declaration to show that the Examiner is misinterpreting the publication of Jansky et al. (2016) "Reinventing Potato as a Diploid Inbred Line-Based Crop." Crop Sci. 56:1412-22, when concluding therefrom that, approximately seven years after the filing date of the instant application, the problem of creating vigorous inbred diploid *S. tuberosum* plants apparently still existed. In particular, I will provide evidence that the Applicant was in possession of the invention as broadly as claimed at the time of filing.

Aardevo Exhibit 1010
Page 0891

Genetic diversity of lines D1, D2, and D3

7. Diploid potato lines D1, D2, and D3 (IVP97-079-9, IVPAA-096-18, and SH83-92-488, respectively, as indicated in Table 6 of the application) are heterozygous lines of a diploid potato breeding program at the Department of Plant Breeding, Wageningen University, the Netherlands. This program spans a period of over 50 years, and was initiated using a wide range of dihaploids generated from tetraploid potato cultivars in the 1960's.

8. In support of the diverse genetic background and wide applicability of the plants and methods as claimed, the complete pedigree showing the generation of diploid clones D1, D2, and D3 is provided as Figure 1 below. A majority of the information of Figure 1 is available from the public Potato Pedigree Database (https://www.plantbreeding.wur.nl/PotatoPedigree/index.html).

9. In the pedigree, the original tetraploid (2n = 4x = 48) *Solanum tuberosum* cultivars are shown in green, the dihaploids (2n = 2x = 24) generated from these tetraploids are shown in red, the diploid breeding lines of the program that are ancestors of D1, D2 and D3 are shown in orange, and diploid lines D1-D3 are shown in yellow. Red colored lines show parental mother, blue colored lines show parental father. The tetraploid cultivars (green) represent commercial cultivars, such as Chippewa, Femmema, Grata, Merrimack and Katahdin. Names of the diploid breeding lines (orange) often include a reference to their production year (e.g. DH63-72, the dihaploid from Maritta was developed in 1963).

Aardevo Exhibit 1010
Page 0892

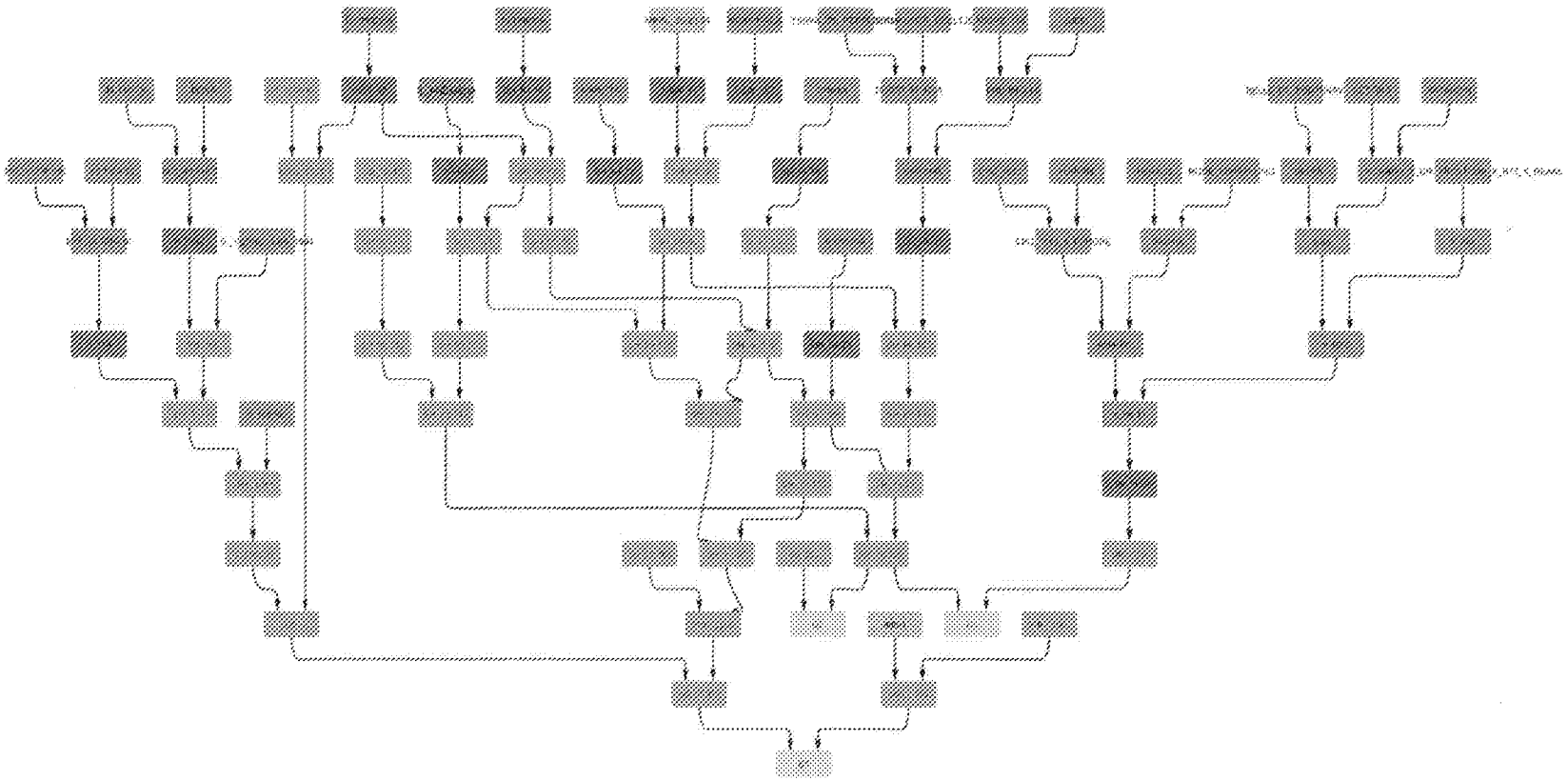


Figure 1. Pedigree of the diploid breeding lines D1, D2 and D3 (yellow) as described in the Application.

Aardevo Exhibit 1010
Page 0893

10. D1 is a diploid resulting from a cross between IVP92-053 (mother) and IVP92-027 (father). These parental lines were produced in 1992. The mother line is a descendant of dihaploids generated from the original tetraploid cultivars "Primura", "Grata", "Fennema", "Chippewa", "Maritta", "Minn-20-20-35" "Merrimack", "Mittelfrühe", "Bona", and wild relative "*S. andigena*".

11. D2 is a diploid resulting from a cross between DB-207 (mother) and SH_76-128 (father). The father line is a descendant of dihaploids generated from the original tetraploid cultivars "Chippewa", "Fennema", "Maritta", "Minn-20-20-35" "Merrimack", "Grata", "Primura", "Sirtema", and wild relative "*S. andigena*". "Sirtema" finds ancestry in cultivars "Fruhmolle", "Jubel", "Berlikumer Geeltje", and "Tinwald's Perfection".

12. D3 is a diploid resulting from a cross between DH_80-8 (mother) and SH_76-128 (father). The mother line is a descendant of a dihaploid generated from the original tetraploid cultivars "Furore", "Rode Eersteling", "Herald", "Saskia", and "Amaryl" as paternal ancestors, and "McIntosh", "Record", "Katadhin", "Belle de Fontenay", "Belka" and "Bea" as maternal ancestors.

13. D1, D2, and D3 represent such large genetic variation, that the use of each of these plants in crosses yield offspring plants with a wide variation in tuber characteristics as skin color and pattern (white to deep purple, solid color, multi-colored), flesh color and color pattern (solid, ringed, white to deep purple), tuber shape (oval spherical, flat, long), tuber "eye" configuration (depth, number, location, color), tuber starch content, fry quality, cook quality, as well as non-tuber characteristics such as flower color, etc. This is typical for dihaploids derived from tetraploid potatoes. The same was observed in an F2 population created by self-pollinating a

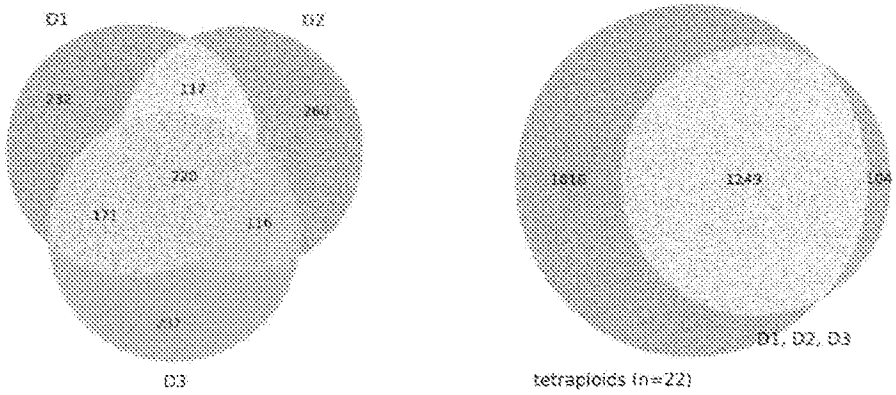
single F1 plant from a cross between two diploid potato clones (Jansky et al. 2015. Am. J. Botany 102: 1213): "*The tuber shape and color variability derived from self-pollinating a single diploid F 1 plant approaches that of the landrace collections maintained in potato genebanks*".

14. The genetic background of the plants of D1-D3 is thus extremely diverse. In fact, all *S. tuberosum* diploids, whether produced 60 years ago, today, or in the future, contain a random sample of the genes of the *S. tuberosum* tetraploid(s) from which they were derived. In that sense, D1-D3 are not unique and do not represent any specific genotype of potato lines.

15. Therefore, any *S. tuberosum* diploid is equally suitable for use as starting material in a method of the invention, that is, to serve as a recipient of the *Sli*-gene to produce a self-compatible diploid line that can be selfed in order to increase its level of homozygosity.

16. As a further substantiation of the diverse genetic background and wide applicability of the plants and methods as claimed, I provide results of a study on the genetic variation between lines D1, D2, and D3, as well as the genetic variation between these lines and a random set of 22 commercial tetraploid *Solanum tuberosum* varieties (*i.e.* Vr808, Vitabella, Spunta, Sarpo Mira, Russet Burbank, Nicola, Miss Mignonne, Lady Claire, Innovator, Colomba, Brooke, Bionica, Ivpx-041-01, Avito, Altus, Lady Anna, Barcelona, Toronto, Taisiya, Mondial, Melody, and Madrid). The genetic variation was determined by using 426 multi-allelic markers that were proportionally and randomly distributed over the twelve chromosomes. These results are summarized in Figure 2, below.

Aardevo Exhibit 1010
Page 0895



A B

Figure 2. Haplotype overlap between individual diploid lines D1-D3 (A); Haplotype overlap between the combination of the diploid lines D1-D3 and 22 random tetraploids (B).

17. It was found that lines D1, D2, and D3 represent a total of 1353 haplotypes, of which only 220 haplotypes (16%) were shared by all three (Figure 2A). By contrast, the 22 tetraploids (comprising a total of 22x4=88 genomes) represented a total of 2267 haplotypes (Figure 2B), of which no less that 1249 (55%) were also present in lines D1, D2, and D3 (together comprising only 3x2=6 genomes). Only 104 haplotypes (4%) were unique to lines D1, D2, and D3, which is a small fraction of the wide genetic variety between the 25 clones compared (Figure 2B). That D1, D2, and D3 share only a fraction of their alleles shows that they are genetically diverse. That no less than 96% of the alleles of D1, D2, and D3 is also present in the 22 commercial tetraploids shows that D1, D2, and D3 represent a random sample of *Solanum tuberosum*.

18. For these reasons, lines D1-D3 should be considered to be representative of the genetic diversity that is present in a large group of commercial tetraploid *Solanum tuberosum* varieties presently available to breeders.

Aardevo Exhibit 1010
Page 0896

19. It is characteristic of current potato breeding that the four genomes of tetraploid parents are mixed in each generation. Because all homologous chromosomes can pair with each other (quadrivalence), these four genomes are repeatedly mixed up. The three diploid lines D1, D2 and D3 are a good example thereof, and show how this mixing results in a wide genetic variation. Again, D1-D3 are not unique and do not represent any specific genotype of potato lines. Other than the Examiner appears to suggest, there is no such thing as a "genetically unique potato" or a "genetically diverse potato". Each genotype is a sample from a great raffle of possibilities and is therefore just as good as any other sample. That is also why D1, D2 and D3 have such a large overlap with tetraploid potatoes as shown in Figure 2B.

20. Thus, (i) the D1-D3 pedigree is rooted in a wide variety of tetraploid cultivars, (ii) no specific "sample" from tetraploid potato germplasm was taken and no selection occurred when choosing D1-D3, (iii) that the four genomes of tetraploid germplasm was randomly sampled is evidenced by the showing that the genetic variation within D1-D3 almost entirely overlaps with that of 22 tetraploid cultivars, and (iv) crosses with D1-D3 result in a segregation of a wide variation of tuber and non-tuber characteristics. This demonstrates that lines D1-D3 as starting material for introgression of the *Sli*-gene and the development of self-compatible vigorous diploids of the invention, represent three fully independent working examples to demonstrate the applicability within diverse *Solanum tuberosum* germplasm to one of skill in the art of plant breeding.

Practicing the claimed invention is predictable in view of the teaching in the patent application and does not depend on the genotype of the potato lines used

21. I reiterate the comments I made in paragraph 16 of my Declaration dated January 29, 2019, submitted in U.S. patent application serial No. 13/504,156, now U.S. Patent No. 10,524,436:

> *"More recent, independent research, using completely different diploid S. tuberosum clones as recipient of the Sli gene, has confirmed that it is possible to generate fertile, essential homozygous S. tuberosum potato lines when selecting for fertile offspring in self progenies (Jansky et al., 2014. J Plant Registrations, doi:10.3198/ jpr2013.05.0024crg; Jansky et al., 2016 Crop Sci 56: 1412-1422). This independent research supports my statement that the specific starting material is not essential to the success of the claimed method of producing the Solanum tuberosum plants, and further supports the effectiveness of the method."*

Thus, independent research published in 2014 shows that D1, D2 or D3 may be substituted with entirely different diploid clones, such as DM1-3 and US-W4.

22. I further understand from the Office Action that the Examiner cites the letter of Dr. Jonathan D G Jones of 11 December 2012 for the argument that the claimed method would not credibly work in *Solanum tuberosum* lines other than lines D1, D2, and D3. As I consider this to not be consistent with the conclusions of Dr. Jones, I asked Dr. Jones to further explain the paragraph referred to by the Examiner. The following is a quote from the response letter I received from Dr. Jones:

"I herewith inform you [...] that I never made a statement to that effect, and that my words are misinterpreted. What I stated is that we attempted to test the Sli gene in S. phureja, but were not successful, and that we found that, after only a few rounds of selfing with the original material from Hosaka, inbreeding depression was already so extensive that we terminated the program, because we were unable to propagate the material. We drew no specific conclusions at the time, other than that we considered that it might have been due to the poor germplasm we worked with. However, I was well aware at the time of my letter that this was not the case, as I was convinced from personal communications, and your scientific publication in Potato Research (2011) Vol. 54:301–312, that we should have persisted inbreeding and selecting plants with seed carrying berries.

I considered that your method was widely applicable to potato, and you had certainly shown that it was widely applicable to S. tuberosum."

The letter of Dr. J.D. Jones is attached as **Exhibit A**.

23. It is therefore incorrect to conclude that Dr. Jones's letter supports the Examiners assertion that the success of the method was due to a specific selection of diploid germplasm, *i.e.* the diploid *Solanum tuberosum* lines D1-D3. On the contrary, nothing suggests that this is the case. In fact, I believe that the claimed methods can be successfully used in species other than *Solanum tuberosum*. As *Solanum tuberosum* is a natural tetraploid, deleterious genes can be hidden in the tetraploid genome, and are maintained in the dihaploid genome of *Solanum tuberosum* diploid plants, making diploid *S. tuberosum* a more "difficult" potato.

Aardevo Exhibit 1010
Page 0899

24. As a showing of what Dr. Jones and others will have observed, I point to Example 2 of the Application, where the self-compatible diploid *Sli*-gene donor (H, for Hosaka – the same *Sli*-gene donor to which Dr. Jones refers) was crossed with the three self-incompatible diploid potato clones D1, D2, and D3. Some of the characteristics of the early stage breeding plants are provided in Table 2 of the Application, reproduced below.

Table 2. Some characteristics of winter grown plants of a diploid potato breeding program to introduce the *Sli* gene.

	# plants	compatibility	yield (gram per SC plant)	
		frequency (%)	average	variation
Sli donor	2	100	22*	0 - 43
F1 (HxD3)	9	89	443**	358 - 654
BC1 (Hx(HxD1)	49	81	206**	0 - 524
BC1 (Hx(HxD2)	64	72	237**	28 - 543
F2 selfed (HxD1)	23	22	252**	121 - 376
F2 selfed (HxD2)	20	25	69**	0 - 201

* from tuber

** from seedling

25. As can be seen from this Table, the two individual *Sli* donor plants (referred to in Table 6 of the application as "H" or "07-1004-1") were fully self-compatible, and crosses HxD1, HxD2 and HxD3 all resulted in the generation of self-compatible offspring plants, albeit in various degrees. Yet, self-compatible plants were found in all instances, indicating successful *Sli*-gene introgression from parent "H". Even in the F1 of cross HxD3, which yielded very few seeds, 8 out of 9 plants (89%) were found to be self-compatible.

26. As also shown in Table 2 above, selfings of initial hybrids (F2 selfed (HxD1); F2 selfed (HxD2)) resulted in an overall decrease in self-compatibility towards 22-25%. This is attributed to inbreeding depression.

27. As shown in Table 3 of the Application, which is reproduced below, of the eight self-compatible plants of the F1(HxD3) noted above, eight selfed populations were prepared from seed to generate a total of 1602 field plants. Of these, only ten plants (about 0.6%) were self-compatible, and of these ten self-compatible plants, only seven showed sufficient seed set. Fertility is thus greatly if not especially affected by inbreeding depression in the early crosses.

Table 3. Breeding scheme to introduce self-compatibility (SC) into diploid potato. In bold the most promising materials are indicated.

P	F1 (HxD)	F2: selfed (HxD)				BC1S1: selfed (Rx(HxD))			
genome	plants (DxH)	Populations tested	plants in the field	self compatible plants	plants selected*	populations tested	plants in the field	self compatible plants	plants selected
	#	#	#	#	#	#	#	#	#
H = SSt donor									
D1	33	16	2480	± 50	7	28	2521	< 10	1
D2	45	28	**5588**	**> 500**	**52**	24	1380	< 10	1
D3	8	8	1602	± 10	7				

* sufficient seedset

28. This means that only four in 1,000 plants were selected and 99.6% of the offspring were discarded. The relevance of this step is indicated in the application on page 37, line 27 – page 38, line 7:

"No, or at least little, attention is paid to the agronomical appearance of the plants at this stage. They may look withered and frail, with only a few flowers. Nonetheless, the selfing is repeated at least three times, but preferably four or five or even six to eight times. In this manner, a line of essentially homozygous, fertile, self-compatible, and diploid potato plants is obtained, essentially by selfing and selecting for plants that produce fertile offspring.

Next, an agronomically desirable trait is added to the essentially homozygous, fertile, self-compatible, diploid potato genotype by crossing a plant of that line with a plant of an unrelated essentially heterozygous diploid potato line, preferably a line containing an agronomically desirable trait. This essentially heterozygous line (which term merely reflects that the line is not an essentially homozygous line as used herein) functions as a donor for an agronomically desirable trait, in this case vigour."

Thus, the plants that were not discarded but used for further breeding were the frail, fertile plants, while the vigorous less fertile plants were discarded.

29. The Examiner appears to suggest that prior to the invention selection of progeny plants for subsequent crosses based on a plant's fertility was a well-understood and routine activity. However, this was not at all the case in conventional potato breeding. Selection for fertile offspring or persistent self-compatibility in the selfed progeny of inbred diploid potato has shown to require the generation of large numbers of plants and selecting those that are increasingly homozygous and fertile. From the large population, the plants that held potential of yielding fertile progeny were shown to be the seemingly useless plants which often carried only a few berries, each having only a few seeds. This meant that the vigorous and abundantly

Aardevo Exhibit 1010
Page 0902

flowering plants were not the plants to be selected as these were the plants that were the least homozygous. Given the observed reduction in self-compatibility after every subsequent inbreeding step, and the large number of plants required to identify the homozygous fertile plants after three or four steps of inbreeding (not to mention that such plants may look withered, frail, and utterly invaluable, and that a markers system needed to be developed in order to test homozygosity), there was nothing obvious to one of ordinary skill in the art to proceed with such an inbreeding program prior to the invention, let alone have any expectation of success in obtaining the plants as presently claimed.

Possession of the Invention

30. The publication of Jansky et al. (2016) "Reinventing Potato as a Diploid Inbred Line-Based Crop." Crop Sci. 56:1412-22, fully supports the findings of the present inventors. In fact, it advocates the radical change to the potato breeding industry using an inbred-line-based breeding system: "*We call on leaders of the public and private organisations to come together to explore the feasibility of this radical and exciting new strategy in potato breeding*". The publication describes at great length why classic potato breeding is not effective, and further describes that attempts at the tetraploid level were never successful: "*Efforts to self-pollinate potato at the tetraploid level were initiated in the mid-twentieth century in response to the success of the inbred-hybrid methods developed in maize. Low yield and poor fertility were observed in S5 and S6 generations, so this effort was terminated. [...]. Consequently, these efforts to self-pollinate tetraploid potato clones never actually tested the value of creating inbred lines to eliminate deleterious alleles and fix desirable ones*" (emphasis added).

31. This is entirely in line with what I have stated before: Potato experts were so discouraged by all failures that no further attempts to produce homozygous lines by inbreeding were made prior to the current invention.

32. It is important to note that more than mere scientific proof is needed to change an established crop production system that has existed for hundreds of years. The authors of Jansky et al. 2016 did not question the possibility of producing fertile and vigorous homozygous diploids and methods for their production as claimed by the present inventors. They indicated that much work still needed to be done before a diploid inbred line breeding system could replace the conventional potato breeding system. As explained on page 1414, right column, first §, such a system would require the assignment of inbreds to heterotic pools within each market class (red, white, russet, specialty) that can be crossed to combine important traits, such as time to senescence, tuber yield, flesh texture, tuber composition, disease resistance, and maintenance of tuber quality in storage.

33. This leaves untouched the fact that the present inventors have provided the pivotal ingredients for the new potato crop production system: fertile and vigorous homozygous diploids. In showing that the inventors were in possession of such plants, I simply refer to the deposits, notably line AGVD17.

34. Using the technology of the instant application, the present inventors were able to develop the experimental test-hybrids into a potato hybrid breeding system. This is exemplified by publications of de Vries et al. in 2016 (Open Agriculture. 2016; 1: 151–156), wherein hybrids tested in East-Africa showed a yield of up to 29 ton/ha, and of Lindhout et al. in 2017 (Hybrid potato breeding for improved varieties. In: Achieving sustainable cultivation of potatoes Vol. 1

Aardevo Exhibit 1010
Page 0904

Breeding, nutritional and sensory quality. Edt Burleigh & Dodds, Science publishing, Cambridge, UK), wherein the scientific principles and applied aspects of hybrid potato breeding are provided. (copies enclosed). In Lindhout et al. in 2017, it is indicated that (Page 18):

> *"Since the first crosses in 2008, the Solynta research efforts have been focused on the development of a hybrid potato breeding system. This research has now reached the stage where hybrid potato breeding will become reality. This has recently been supported by two leading potato breeding companies in EU, KWS and HZPC, who have also expressed their conviction that hybrid potato breeding will be future main breeding system (KWS, 2016). In addition, 21 leaders in the industry and potato science in the United States have expressed their opinion on ' Reinventing potato as a diploid inbred line-based crop ' with a scientific base for diploid hybrid potato breeding (Jansky et al., 2016)."*

35. Furthermore, Stockem et al. in 2020 Potato Res. DOI 10.1007/s11540-019-09444-x, describing experiments of 2016, confirmed that diploid hybrids can exhibit the same average yields as an established commercial cultivar (Abstract), despite the fact that such diploid hybrids were never selected for high yielding capacity under field conditions (Results section "Seedling Tuber Production").

36. Therefore nothing in the sentence of Jansky et al. 2016 "*Diploid inbred line breeding in potato is being tested by the private sector in Europe (Lindhout et al., 2011)*" bears any relation to a suggestion that, approximately seven years after the filing date of the instant application, the problem of creating vigorous inbred diploid *S. tuberosum* plants apparently still existed. On the

contrary, in the paper of Jansky et al. 2016, the existence of a breeding program based on diploid inbred lines is only confirmed.

37. All the above is further supported by independent development of diploid *Sli*-gene-containing *S. phureja* and *S. tuberosum* inbreds wherein the *Sli-gene* was introgressed from *S. chacoense* M6 into diploid DM1-3 and US-W4 (Jansky et al. 2014. Journal of Plant Registrations 8:195–199), showing that D1, D2 or D3 can successfully be substituted with diploid clones DM1-3 and US-W4 (page 198, right column, second §), and by the publication of Jansky and Spooner 2018 "The Evolution of Potato Breeding", Plant Breeding Reviews, Volume 41, First Edition. Edited by Irwin Goldman. John Wiley & Sons, Inc., which states on page 201:

> *"Next, Pim Lindhout, in the Netherlands, reported on the development of diploid inbred lines with acceptable tuber shape and size (Lindhout et al., 2011). F3 plants were uniform for tuber quality and yield, so they were intercrossed to make F1 hybrid prototypes. Some F1 hybrids were uniform and exhibited good tuber quality. This proof of concept paper has set the stage for the development of diploid F1 hybrids in potato."*

Aardevo Exhibit 1010
Page 0906

38. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: May 1st, 2020

Willem Hendrik Lindhout

EXHIBIT A

John Innes Centre
Norwich Research Park
Colney • Norwich NR4 7UH • UK
T +44 (0) 1603 450400 F +44 (0) 1603 450011
admin@TSL.ac.uk www.TSL.ac.uk

16th April 2020

Dear Pim,

I write to you in response to your request for explanation of a paragraph in my letter of 11 December 2012, which letter was attached to an Inventor's declaration that you submitted on 15 August 2016, and which letter is now part of the patent prosecution proceedings in the case of your US application 16/541,071.

I understand that the US Examiner is putting weight on the paragraph wherein I state:

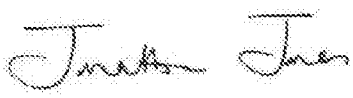
"*(I)n the mid 2000s we attempted to produce hybrid potato ourselves. No successes had ever been reported with S. tuberosum. In order to work at the diploid level, we back-crossed the S. chacoense Sli- gene into Solanum phureja. In addition, we selfed the lines that carry the Sli- gene. However, we never succeeded in developing any promising material in the form of fertile homozygotes that could be useful for hybrid breeding and that could tolerate recurrent selfing. We considered that this was due to our inability to cross the Sli- self-compatibility locus into good enough or diverse enough diploid Solanum germplasm.*" (underlining added by Examiner).

In particular, I understand that the Examiner interprets my statement as a showing that your method of recurrent inbreeding to recover the vigor of the diploid potatoes only works in selected *Solanum* germplasm, i.e. that your success depended on the genotype of your diploid potato lines.

I herewith inform you, and you may share this with the Examiner, that I never made a statement to that effect, and that my words are misinterpreted. What I stated is that we attempted to test the *Sli* gene in *S. phureja*, but were not successful, and that we found that, after only a few rounds of selfing with the original material from Hosaka, inbreeding depression was already so extensive that we terminated the program, because we were unable to propagate the material. We drew no specific conclusions at the time, other than that we considered that it might have been due to the poor germplasm we worked with. However, I was well aware at the time of my letter that this was not the case, as I was convinced from personal communications, and your scientific publication in Potato Research (2011) Vol. 54:301–312, that we should have persisted inbreeding and selecting plants with seed carrying berries.

I considered that your method was widely applicable to potato, and you had certainly shown that it was widely applicable to *S. tuberosum*.

Best regards,



Prof Jonathan D G Jones FRS

Electronic Acknowledgement Receipt

EFS ID:	40339763
Application Number:	16541071
International Application Number:	
Confirmation Number:	4348
Title of Invention:	Hybrid Seed Potato Breeding
First Named Inventor/Applicant Name:	Willem Hendrik Lindhout
Customer Number:	125771
Filer:	Amy Dunstan
Filer Authorized By:	
Attorney Docket Number:	VONL:004USC1
Receipt Date:	20-AUG-2020
Filing Date:	14-AUG-2019
Time Stamp:	16:51:31
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	VONL_004USC1_SIDS.pdf	131490 14a1ab22d4a083ff0718bcd6876f1230bb3477e5	no	2

Warnings:

Information:					
2	Information Disclosure Statement (IDS) Form (SB08)	VONL_004USC1_SB08.pdf	819838 bf3f78661e14c20750b8b5cfa2c7ab3d81e088e4	no	3
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
3	Non Patent Literature	VONL_006US_RNFOA.pdf	12268589 5b8d48a545f60574024cadad9fb9e36d086a3716	no	206
Warnings:					
Information:					
		Total Files Size (in bytes):	13219917		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Willem Hendrik Lindhout *et al.*	Group Art Unit: 1663
Serial No.: 16/541,071	Examiner: Russell T. Boggs
Filed: August 14, 2019	Atty. Dkt. No.: VONL:004USC1
For: HYBRID SEED POTATO BREEDING	Confirmation No.: 4348

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Commissioner:

In compliance with the duty of disclosure under 37 C.F.R. § 1.56, it is respectfully requested that this Supplemental Information Disclosure Statement be entered and the documents listed on attached Form PTO/SB/08 be considered by the Examiner and made of record. Copies of the listed documents required by 37 C.F.R. § 1.98(a)(2) are enclosed for the convenience of the Examiner.

In accordance with 37 C.F.R. §§ 1.97(g), (h), this Supplemental Information Disclosure Statement is not to be construed as a representation that a search has been made, and is not to be construed to be an admission that the information cited is, or is considered to be, material to patentability as defined in 37 C.F.R. § 1.56(b).

Applicants certify that no item of information contained in this Supplemental Information Disclosure Statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the Supplemental Information Disclosure Statement was known to any individual designated in 37 C.F.R. § 1.56(c) more than three months prior to the filing of this Supplemental Information Disclosure Statement in view of the July 16, 2020, filing date of the Response to Non-Final Office Action regarding U.S. Application No. 14/112,747.

No fees are believed to be due in connection with the filing of this Supplemental Information Disclosure Statement. Should any fees under 37 C.F.R. §§ 1.16 to 1.21 be deemed necessary for any reason relating to these materials, the Commissioner is authorized to deduct the appropriate fees from Dentons US LLP Deposit Account No.: 19-3140/VONL:004USC1.

Respectfully submitted,

/Amy Dunstan/

Amy Dunstan
Reg. No. 61,069
Agent for Applicants

Dentons US LLP
2000 McKinney Avenue, Suite 1900
Dallas, Texas 75201
(214) 259-0965

Date: August 20, 2020

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Willem Hendrik Lindhout *et al.*	Group Art Unit: 1663 Examiner: Russell T. Boggs
Serial No.: 16/541,071	Atty. Dkt. No.: VONL:004USC1
Filed: August 14, 2019	
For: HYBRID SEED POTATO BREEDING	Confirmation No.: 4348

STATUS INQUIRY

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

On June 30, 2020, Applicant filed a Petition to Make Special Under 37 C.F.R. §§ 707.02 and 708.01(i), in the above-referenced application. On July 17, 2020, the Office of Petitions issued a Granted Petition Decision placing the application status as ***"considered 'special' by the examiner,"*** and to be examined on an expedited basis (emphasis added). Applicant filed a Response to Office Action on May 5, 2020, but has not received a reply to this or any further correspondence. Applicant therefore respectfully requests an update on the status of the application and that the examination be expedited pursuant to the special status accorded to the application.

Respectfully submitted,

/Robert E. Hanson/

Robert E. Hanson
Reg. No. 42,628
Attorney for Applicants

Dentons US LLP
2000 McKinney Avenue, Suite 1900
Dallas, Texas 75201
(214) 259-0931

Date: September 24, 2020