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

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EXAMINER	
BOGGS, RUSSELL T	

ART UNIT	PAPER NUMBER
1663	

NOTIFICATION DATE	DELIVERY MODE
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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

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

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 5 May 2020.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☑ This action is **FINAL.** 2b) ☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 35,37-38,40-41,43-45 and 47-48 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 35,37-38,40-41,43-45 and 47-48 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☑ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

Notice of Pre-AIA or AIA Status

The present application is being examined under the pre-AIA first-to-invent provisions.

Status

1. Claims 35-48 as filed on 14 August 2019 were examined and rejected in an Office action mailed on 5 December 2019. Applicant responded on 5 May 2020, cancelling claims 36, 39, 42, and 46.

Claims 35, 37-38, 40-41, 43-45 and 47-48 are examined herein.

Withdrawal of Objections and Rejections

2. The objections to the specification are withdrawn in view of Applicant's amendments.

3. The objection to under the sequence rules is withdrawn in view of Applicant's amendments and submission of a sequence listing.

4. The objection to claim 37 is withdrawn in view of Applicant's amendments to the claim.

5. The rejection of claim 35 under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite is withdrawn in view of Applicant's amendments to the claim. The examiner apologizes for the mistake in quoting the limitation on page 4 of the Office action. Applicant correctly interpreted the rejection.

6. The rejection of claim 41 under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite is withdrawn in view of Applicant's amendments to the claim

7. The rejection of claims 35-39, 41 and 44-45 under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement regarding the recitation of "*Solanum* species plant line" is withdrawn in view of Applicant's amendments to the claims.

8. The rejection of claims 45 and 47-48 under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement is withdrawn in view of the rejection under 35 USC 103 *infra*.

9. The rejection of claims 45 and 47-48 under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, enablement requirement, is withdrawn in view of the rejection under 35 USC 103 *infra*.

10. The rejection of claims 41, 43-45 and 47-48 under 35 U.S.C. 103 in view of the Phumichai et al., Maris et al., and Watanabe et al. references is withdrawn in view of Applicant's amendments to the claims.

Any rejection or objection applying to a cancelled claim is rendered moot in view of the claim's cancellation.

Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 CFR 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim

remaining in the application. Any amendment of inventorship must be accompanied by a request under 37 CFR 1.48(b) and by the fee required under 37 CFR 1.17(i).

Information Disclosure Statement

11. The IDS submitted on 5 May 2020 and considered on 27 October 2020 has a lined-through reference: #2. The title for reference #2 is inconsistent with the journal citation in the IDS and no copy was provided with that journal citation. In fact, two copies of reference #5 were provided where reference #5 has the title associated with reference #2. The paper published with that journal citation, but not that title, is attached herewith and has been considered. de Vries et al. (2016) Open Agric 1:151-56.

Examiner's Notes

12. Citations to Applicant's specification are abbreviated herein "Spec."

Applicant submitted a declaration by the inventor Dr. Lindhout both in this application and in application serial no. 16/541,071. Documents are provided to the examiners via PE2E in black and white. Dr. Lindhout's declaration mentions color-coding on, for example, page 3. Further, the figure on page 4 is illegible in the copy provided to the examiner. Examiner Boggs contacted Rob Hanson and Sheila Walker by voicemail in application serial no. 16/541,071 pursuant to obtaining a better copy of the declaration but no reply was received.

Applicant responded to the request for information under 37 CFR 1.105. Response, pp. 22-25.

Claim Objections

13. Claims 14, 35 and 45 are objected to because of the following informalities.

Claims 35 and 45 are objected to because they recite limitation "S-locus inhibitor (*Sli*) gene from *Solanum chacoense*" once in claim 35 and twice in claim 45 The use of the parenthetical is objected to because of the potential of the parenthetical term having a different meaning than the rest of the limitation. It is acceptable practice to use a parenthetical to define an abbreviation for the remainder of the claim set. However the entire limitation is repeated in claim 45.

Claim 41 is objected to because it uses the word "in" in line 1 of part (d). Did Applicant mean "as a population" rather than "in a population"?

Claim 14 is objected to because in part (c) it produces (plural) seed and collects all the seeds but provides "a hybrid offspring seed." Then part (d) takes a multiplicity of the seeds. Applicant is requested to clarify the transitions between plural seeds and one seed in parts (c) and (d).

Claim 45 is objected to because in part (d) it collects seeds but provides "a hybrid potato seed." Applicant is requested to clarify the transitions between plural seeds and one seed in part (d).

Claim 45 is objected to because in part (c) the word "said" should be added between "and" and "second."

Appropriate correction is requested.

Claim Rejections - 35 USC § 112(a) (written description)

The following is a quotation of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), first paragraph:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

14. Claims 35, 37-38, 40-41, 43-44 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor or a joint inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention.

Due to Applicant's amendments to the claims, the rejection is modified from the rejection set forth in the Office action mailed 5 December 2019 as applied to the above-referenced claims. Applicant traverses the rejection in the paper filed 5 May 2020. Applicant's arguments have been fully considered but are not persuasive.

The Claims

Claim 35 is drawn to a plant of a potato line, where the potato 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 line are homozygous, where the said self-compatibility in the line comes from the *Sli* gene from *S. chacoense* wherein a copy of this gene is present in the deposited lines.

Dependent claim 37 requires that the plant comprises an additional agronomically desirable trait. Dependent claim 38 requires a certain amount of fresh weight of foliage. Dependent claim 40 reads on a seed or tuber of the plant.

Claim 41 is drawn to a method of producing 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 in six steps:

(1st) providing a first potato plant according to claim 35;

(2nd) providing a second potato plan that is a diploid *Solanum* plant with any level of homozygosity;

(3rd) cross-pollinating the two plants to provide a seed and then collecting the seed;

(4th) growing a multiplicity of the seeds and selecting a self-compatible plant;

(5th) backcrossing this self-compatible potato plant to the first potato plant 1 to 8 times; and

(6th) selecting for a fertile offspring plant to thus produce a diploid, fertile, self-compatible potato plant line that is at least 50% homozygous. The claim does not require that the plant produced has a yield of 200 grams of tuber per plant.

Dependent claim 43 requires that the plant used in the second part *supra* has an agronomically desirable trait. Dependent claim 44 reads on a plant line produced by the method.

Applicant's Description

Applicant describes the invention via deposited lines which are referred to by their breeders reference: AGVD1 (NCIMB 41663), AGVD2 (NCIMB 41664), AGVD3 (NCIMB 41665) and AGVD17 (NCIMB 41765). Spec., pp. 33-35.

In the specification, Applicant describes a breeding process according to the instant invention starting with a self-compatible line, e.g. AGVD1-3 or AGVD17. *Id.*, p. 37. Applicant states that "[n]o or at least little, attention is paid to the agronomical appearance of the plants at this stage." *Id.* One aspect of the recited breeding steps includes the possibility of adding a desired trait, in the instant case, vigor. *Id.*, p. 38. Continuing this aspect, after adding the trait, the hybrid is backcrossed to the self-compatible line while selecting for vigor. *Id.* After adding numerous traits, and performing test crosses, inbred elite diploid breeding lines can be obtained which can be used to produce commercially valuable hybrids. *Id.*, pp. 41-43.

Applicant reviews the steps: develop a "a vigorous fertile homozygous self-compatible diploid potato line;" "a diploid breeding line;" and then an "elite diploid potato breeding line." *Id.*, p. 40. On page 43, Applicant concludes that "[t]he above method now provides a breeding process for the production of homozygous breeding lines or elite breeding lines based on the background of population AGVD1, AGVD2, AGVD3 or AGVDI7, to which background successively additional alleles are added, thereby providing elite breeding lines wherein desirable traits are stacked." *Id.*, p. 43.

In particular, in Example 1, Applicant describes obtaining the IVP07-1001/4 line from publically available germplasm where the IVP07-1001/4 plants contained the *Sli* gene after repeated selfing. Spec., p. 44. Applicant cites to the 2006 Euphytica publication by Phumichai et al. as well as the 2005 Genome publication by Phumichai et al. regarding the source of the *Sli* gene. *Id.*, p. 44 & p. 57 (*citing* to Phumichai et al. (2005) Genome 48:977-94 and Phumichai et al. (2006) Euphytica 148:227-34 (2006)). Applicant describes that these plants are of no commercial value. *Id.* Applicant crossed

these plants to three different diploid donor lines, D1-3, in order to acquire commercially valuable traits carried by the donor lines. *Id.*, pp. 44-45. The F1 cross with D1 was selfed and the progeny deposited as AGVD1, which was estimated as 50% homozygous. *Id.*, p. 45. The F1(D1) was crossed with F1(D2) and the progeny deposited under AGVD2, and this was estimated to be 25% homozygous with respect to alleles originating from IVP07-1001/4. *Id.* The F1(D1) was backcrossed to obtain progeny 25% homozygous with respect to alleles originating from IVP07-1001/4; these were deposited as AGVD3. *Id.*

In Example 2, Applicant describes crossing the *Sli* gene donor with various publically available potato clones designated: IVP97-079-9, IVPAA-096-I8 and SH83-92-488 to generate clones D1, D2 and D3. *Id.*, pp. 47-50. Applicant describes this created a diploid trait donor lines containing the *Sli* gene with enhanced agricultural quality. *Id.* Applicant provides a more detailed description of the potato lines used in Table 6. *Id.*, pp. 57-58.

Applicant describes creating a molecular marker platform, i.e. SNPs, in Example 3. *Id.*, pp. 50-58.

In Example 4, Applicant describes the development of homozygous diploid potato plants and then F1 hybrid progeny, as well as subsequent generations. *Id.*, pp. 58-62. Applicant describes that it is "often stated" that potatoes are very prone to inbreeding depression. *Id.*, p. 60. A cause of this might be deleterious alleles; Applicant potentially identified a few such deleterious alleles, with one allele almost being lethal. *Id.* However, no absolute lethal allele was present. *Id.* (heading at line 10).

Applicant concludes, on page 62, that

> It was found that after two generations of selfings homozygous diploid potato plants were obtained comprising alleles from both parents. Not a single marker out of 36 markers tested provided proof for a lethal allele. Furthermore, plants with more than 84% homozygous loci can be successfully crossed to obtain hybrid plants with at least five heterozygous chromosome regions. This means that hybrid seeds that are essentially uniform and that represent true potato seed have now been produced.

Id., p. 62.

The Scope of the Claims

In contrast to the limited description – four deposited lines – the claimed genus is quite large. Claim 35, for example, reads on any diploid fertile and self-compatible line created from any *Solanum* species that, for example, produces plants with a tuber yield of 200 grams per plant but Applicant only describes by deposit four lines.

To claim a genus under the written description requirement, Applicant is required to describe a representative number of species to reflect the variation within the genus or structures sufficient to define the genus. The factors to be considered include disclosure of complete or partial structure, physical and/or chemical properties, functional characteristics, structure/function correlation, methods of making the claimed product, or any combination thereof.

Applicant Fails to Describe a Representative Number of Species or Structural Features

Applicant provides written description – by deposit – for the deposited lines themselves. But does Applicant describe a representative number of species in comparison to the size of the genus claimed? In the instant application, Applicant only describes by deposit only four potato lines as compositions and for use in the claimed methods. All were created from the same *Sli* donor, the IVP07-1001/4 line, crossed to

three diploid donor lines. Spec., p. 44 & pp. 57-58. Applicant cites to Phumichai et al. (2006) as to the source of the *Sli* gene line. *Id.*, p. 44. The IVP07-1001/4 line does not appear to be a *S. tuberosum* line based on a review of the specification and both Phumichai et al. (2005) and Phumichai et al. (2006), but its identity is not directly stated. Applicant does not further characterize or describe the *Sli* gene.

Similarly, Applicant fails to describe the breeding history of the deposited lines in the specification. The most detailed descriptions of D1, D2 and D3 are found in Table 6, but Table 6 fails to provide the genetic background of the lines. Spec., p. 57.

Applicant fails to associate any genetic structure or structures required for the practice of the invention.

The Art

The art describes basic the approach of Applicant's invention. For example, in a 2009 publication, Jansky describes first obtaining homozygous diploids using a self-incompatibility inhibitor. Jansky (2009) "Breeding, Genetics, and Cultivar Development," Advances in Potato Chemistry and Technology, ed. J. Singh & L. Kaur, pp. 27-62, 46. Jansky teaches that "[h]ighly homozygous inbreds could be also be used for heterosis breeding. Jansky, p. 46. Jansky teaches that such an approach would be difficult. *Id.*

Phumichai et al. confirms the approach of Jansky, and documents the use of the *Sli* gene in the approach. Phumichai et al. (2005), abstract.

> Potato . . . is an auto- or segmental allo-tetraploid showing tetrasomic inheritance, which results in complicated genetic segregation. Since potatoes are propagated vegetatively, highly heterotic genotypes can be maintained, which facilitates the use of heterosis, an essential factor in potato breeding, particularly for tuber yield. Alternatively, open-pollinated

> true potato seed (TPS) can be used as a low-cost planting material for potato production.
>
> Highly homozygous inbreds, once amply produced, would be highly useful for the development of TPS varieties with genetic uniformity, as well as for the investigation of heterosis.
>
> A scheme for hybrid breeding using homozygous diploids and subsequent somatic hybridization has been proposed, for which the availability of highly homozygous diploid (or pure) lines is a key for success. In considering the use of pure lines for the development of genetically uniform [true potato seed] varieties, self-fertile, seed-propagated lines are highly desirable, and simple techniques to develop such lines are necessary.

Id., pp. 977-78 (citations omitted).

Finally, in 2016 Jansky et al. published an "Opinion & Policy" article calling for "Reinventing Potato as a Diploid Inbred Line-Based Crop." Jansky et al. (2016) Crop Sci. 56:1412-22. The authors call for the creation of "diploid inbred lines" (*id.*, p. 1413) with improved, e.g. tuber yield (*id.*, p. 1414) for use in creating inbred pools for use in heterosis. *Id.* The authors devote almost a page to the challenges faced. *Id.*, p. 1415. Then the authors addressed questions that must still be answered. *Id.* pp. 1415-18. One of the approaches cited by Jansky et al. is Dr. Lindhout's work – i.e. the instant claimed invention – which is described as being in the process of ongoing testing. *Id.*, pp. 1417-18. Therefore, approximately seven years after the filing date of the instant application, the problem of creating vigorous inbred diploid *S. tuberosum* plants apparently still exits.

Admissions in Applications and Art Relevant to the Scope of the Pending Claims

The claims read on broad genera of potato lines, but would all potato lines be active in the instant invention? What distinguishes a potato line that has 50%

homozygosity and a 200 gram tuber yield from another potato line falling outside the scope of the claims?. In the specification, Applicant states that

> It is often stated that repeated selfings in diploid potato will only result in weak plants due to inbreeding depression. This should be due to numerous deleterious alleles that reduce plant fitness, when these alleles are homozygously present. Therefore, we tested the segregation of 36 markers in the six F3 populations mentioned (see Figure 5).

Spec., p. 60. As discussed above, Applicant summarizes the result of the study as identifying some potentially, deleterious alleles, one of which is "very" deleterious, almost lethal. *Id.*

The declaration by Dr. Lindhout dated 15 August 2016 in the file history of US application serial no. 13/504,156 and supporting letters document how unique the potato lines used for the instant invention are. For example, one of the letters offered in support of Dr. Lindhout's declaration by Dr. J.D.G. Jones states that

> "[I]n the mid 2000s we attempted to produce hybrid potato ourselves. . . . 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.

Letter from Dr. Jonathan D.G. Jones (2012), p. 1 (appended to Decl. of Dr. Lindhout 15 August 2016, ("Decl. (2016)")) (underlining added).

In this declaration, Dr. Lindhout summarizes the background art prior to the claimed invention. Decl. (2016), pp. 3-4. In particular, Dr. Lindhout states that

> That as soon as a genotype becomes homozygous for negative alleles of which the negative is not compensated by functional paralogues, such genotypes might show very poor performance and low viability;
> That inbreeding depression is manifested as a reduced fitness with a loss in both yield and fertility;
> That inbreeding depression is mainly due to the presence of many (semi) lethal alleles, and alleles with a deleterious effect, that become visible

> more frequently at the diploid level when the maximum number of possible alleles is reduced from four to two, resulting in homozygosity of such alleles.
> That the chance of obtaining a progeny plant, by crossing different genotypes, which is not suffering from inbreeding depression in potato is infinitesimally small, and that starting from a heterozygous diploid potato genotypes, the chance of identifying a plant without homozygous deleterious alleles is, in practice, impossible . . .

Decl. (2016), p. 4.

In a similar vein, in a paper filed with the EPO, Applicant stated that "every expert in the field can confirm that due to inbreeding depression, the chances of successfully attaining the plant of the invention are close to zero. Hence, the production of such plants is generally considered impossible." EPO Publication No. EP 2 493 286 A2, Remarks to EPO (21 Dec. 2012), p. 8.

Furthermore, on 15 December 2011, approximately two years after the filing date of the instant application, Lindhout et al. published a paper in Potato Research which appears to correspond to the experiments set forth in the instant examples (all five inventors are listed as authors on the paper). Lindhout et al. (2011) Potato Res 54:301-12. The discussion in the paper is instructive. For example, Lindhout et al. describes that

> As a consequence of the high level of unfavourable alleles, techniques aimed at developing homozygotes in one step, although successful as a technology in various crops, certainly do not generate vigorous homozygous potatoes. For instance, if 20 of the 39,000 protein-encoding genes (PGSC 2011) on the potato genome harbor alleles causing severe fitness reduction, the chance to generate vigorous diploid selfed progenies without homozygous unfavourable loci from a parent genotype, that is heterozygous for these 20 loci, is only 0.3%.

Id., p. 303. Lindhout et al. concludes that "inbred lines were developed that combined self-compatibility with good agronomic performance. Although the number of such

plants is still low, the progress made in this breeding programme is significant." *Id.*, p. 304. As a possible reason for success, the authors offer that "lethal alleles do not occur at high frequency in our diploid potato germplasm. *Id.*, pp. 308-09. The authors continue that

> there are many loci with minor effects involved in these traits (Quantitative Trait Locus, QTL). Our current research focuses on identifying the crucial QTLs for these traits. The results will be applied in breeding and allow to increase efficiencies by using diagnostic markers for these traits.

Id., p. 309.

Claims 41-48 are included in this rejection largely because the only embodiment taught by Applicant uses the *Sli* gene and the *Sli* gene is not publically available. Although the *Sli* gene is present in Applicant's deposits, the claims do not reference the deposits. Furthermore, even though the *Sli* gene is present in the deposits, it is not further described.

Conclusion

Applicant's deposited lines are described and novel. However, in view of the above, especially including Applicant's assertion that "the production of such plants is generally considered impossible" *supra*, there is nothing in the claims to distinguish Applicant's deposited lines from other *Solanum* lines which fall outside the scope of the claimed tuber yield except for the tuber yield. Yet the claims read on a broad genus lines, provided they have a yield of 200 g per plant and are diploid and self-compatible. One of ordinary skill in the art is given no criteria in the claims to distinguish an "impossible" plant from and "possible" plant such as found in Applicant's deposited lines. Applicant, for example, fails to describe any distinguishing structure that is necessary and/or sufficient for the instant invention.

Therefore Applicant's invention appears to require both the *Sli* gene as represented by the deposits and other specific *S. tuberosum* diploid plants that are not described except potentially by reference to the deposit. Applicant 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. Applicant only describes one *Sli* gene donor line together with only a few diploid donor lines by deposits. Thus, Applicant fails to describe a representative number of species in comparison to the size of the genus claimed. The claims also fail to require any structure / function relationship that may be necessary and/or sufficient for a potato line to successfully be included in the claimed genus.

The specification therefore fails to provide an adequate written description to support the breadth of the claims. Applicant has not demonstrated possession to one of skill in the art of the instant invention as broadly as claimed at the time of filing. Thus one of skill in the art would not believe Applicant to be in possession of the genus as broadly as claimed especially in view of the discussion of the art by, e.g., Applicant before the EPO and the letter of Dr. Jones.

Dependent claims are included in this rejection because none provide further limitations obviating this rejection.

Applicant's Arguments

Applicant argues against the rejection. Response, pp. 17-19. Applicant provides a declaration from Dr. Lindhout ("Decl. Lindhout 2020"). *Id.* Applicant states that the declaration provides the breeding history. *Id.*, p. 17. Applicant points to the discussion in the declaration (paragraphs 13-15) regarding the genetic diversity of the deposited

lines. *Id.* Applicant continues to the discussion of Dr. Lindhout regarding correspondence with Dr. Jones. *Id.*, pp. 17-18. Applicant quotes from Dr. Jones letter to Dr. Lindhout. *Id.*, p. 18. Applicant turns to paragraph 34 of the declaration. *Id.* Applicant concludes that the rejection should be withdrawn. *Id.*, p. 19.

Response to Applicant's Arguments

Applicant provided the breeding history in the declaration of Dr. Lindhout but this breeding history is provided in Figure 1 of the declaration and Figure 1 is illegible in the copy provided to the examiner. Furthermore, Dr. Lindhout refers to color coding in his declaration – however the copy in the file history is in black and white. The examiner contacted Rob Hanson and Sheila Walker by voice-mail to attempt to obtain a better copy but no reply was received.

Applicant's argument relies on the declaration provided by Dr. Lindhout, the first listed inventor of the instant application. In particular, Applicant relies on a statement from Dr. Jones in a letter to Dr. Lindhout and discussed in the declaration in paragraphs 22-23. In particular, Dr. Jones wrote in the 2020 letter that "I never made a statement to that effect, and that my words are misinterpreted."

In response, Dr. Jones' statement in his 2020 letter is perplexing. First, the letter from Dr. Jones is not submitted as a declaration itself. Second, another letter from Dr. Jones was submitted by Applicant during prosecution of application serial no. 13/504,156, from which this application is a continuation. This previously submitted letter from Dr. Jones is quoted in the Office action of 5 December 2019. Is Dr. Jones in 2020 repudiating that letter? Where, in 2020, Dr. Jones wrote that "I never made a statement to that effect, and that my words are misinterpreted," Dr. Jones' did not

directly address the statements in his previous letter of 2012 and there is no evidence that Dr. Jones reviewed the letter prior the comment above. The previous letter written in 2012 speaks for itself. How is the 2012 letter by Dr. Jones that was submitted during prosecution inaccurate? How is it misinterpreted?

In the letter attached to the 2020 Declaration, Dr. Jones also cites to personal communications (presumably with Dr. Lindhout) and a publication by Dr. Lindhout et al. The "personal communications" are not dated and the publication is post-filing art.

The Federal Circuit finds that "the test for sufficiency is whether the disclosure of the application relied upon reasonably conveys to those skilled in the art that the inventor had possession of the claimed subject matter as of the filing date. *Ariad Pharm., Inc. v. Eli Lilly & Co.*, 598 F.3d 1336, 1351, 94 U.S.P.Q.2d 1161 (Fed. Cir. 2010) (citations omitted).

Thus the quoted passages from the recent letter of Dr. Jones are not persuasive with regard to the earlier letter of Dr. Jones. Even if Dr. Jones was persuasive in contradicting his earlier letter, his new position is irrelevant to the letter from Dr. Koornneef as seen below

In support of an argument against a rejection under 35 USC 103 in application serial no. 13/504,156, Applicant filed a declaration under 37 CFR 1.132 by Dr. Lindhout with supporting letters.

The supporting letters were initially filed in European Application EP2699684 (04 June 2014) and were copies therefrom. The letters teach that Applicant's invention, as broadly as claimed, was not fully described. For example, on page 2 of his letter, Dr. Koornneef states that

> Ever since Lindhout and his co-workers published their results in Potato Research volume 54 of 2011, showing high yielding hybrids from 87% homozygous F3 inbreds, I have been positively surprised that Solynta has managed to challenge this dogma. Solynta could successfully obtain essentially homozygous inbred lines by making use of genotypes that are selfcompatible and by choosing the right starting material that apparently is heterozygous for a lower number of deleterious alleles. This provides the basis of generating useful inbred lines that can become parents of F1 hybrids.

Letter from Koornneef (2012) p. 2 (underlining added). Earlier in the letter, Dr. Koornneef also states that "to start a project aiming at developing a good performing homozygous potato plant for the purpose of hybrid production is a daring undertaking with very little chance of success. *Id.*

Applicant's position in the European application, the quoted portions of the publication of Lindhout et al. (2011) (Office action 5 Dec. 2019, p. 15 and *supra*); and the review article by Jansky et al. (2016) (e.g. *Id.*, pp. 12-13 and *supra*) also support the instant rejection.

Furthermore, in application serial no. 13/504,156, Dr. Lindhout filed a declaration on 8 September 2016 to overcome a rejection under 35 USC 103 which stated in part, "[t]hat the chance of obtaining a progeny plant, by crossing different genotypes, which is not suffering from inbreeding depression in potato is infinitesimally small, and that starting from a heterozygous diploid potato genotypes, the chance of identifying a plant without homozygous deleterious alleles is, in practice, impossible. Decl. of Dr. Lindhout, app. ser. no. 13/504,156, signed 15 Aug. 2016.

Although opinion testimony must be considered, it is not controlling. MPEP § 716.01(c)(III). Thus although Dr. Lindhout and Dr. Jones assert the examiner is misinterpreting various teachings, Applicant's position is unconvincing.

The standard for a written description rejection (MPEP § 2163.04) and for considering a declaration (MPEP § 716.01(d)) is the preponderance of the evidence.. Under that standard, the rejection is maintained.

In summary, Dr. Lindhout in 2016 argued that the line was critical for the invention that it was more than just using the *Sli* gene. But now Dr. Lindhout argues that is the line isn't critical. Dr. Jones recanted his statement but without specific reference to his previous letter itself. For the reasons above, Dr. Jones' new position is not persuasive. Further, Dr. Jones' 2020 letter is not submitted as a declaration itself. The letter of Dr. Koornneef and the article of Jansky et al. remain un-contradicted on the record. Further, Applicant's previous positions argue against an adequate written description of the claimed invention. The undefined characteristics of the potato line are a critical part of the invention. Therefore one of ordinary skill in the art could not envision the claimed invention at the time of filing.

Claim Rejections - 35 USC § 112 (enablement)

15. Claims 35, 37-38, 40-41, and 43-44 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

The invention appears to employ novel plants described and enabled by the deposits where the deposited lines comprise the *Sli* gene which gene does not appear to be otherwise publically available. Since the plant is essential to the claimed invention

it must be obtainable by a repeatable method set forth in the specification or otherwise be readily available to the public. If the plant is not so obtainable or available, the requirements of 35 USC § 112 may be satisfied by a deposit of the seeds. A deposit of 625 seeds in the form of 25 packets of 25 seeds of each of the claimed embodiments is considered sufficient to ensure public availability. 1460 OG 263 (2019).

The specification does not disclose a repeatable process to obtain the plant and thus it is not apparent if the plant is readily available to the public.

(a) If a deposit is made under the terms of the Budapest Treaty, then a statement, affidavit or declaration by Applicant, or a statement by an attorney of record over his or her signature and registration number, or someone empowered to make such a statement, stating that the instant invention will be irrevocably and without restriction released to the public upon the issuance of a patent, would satisfy the deposit requirement made herein.

(b) If a deposit has not been made under the Budapest Treaty, then in order to certify that the deposit meets the criteria set forth in 37 CFR 1.801-1.809 and MPEP 2402-2411.05, Applicant may provide assurance of compliance by statement, affidavit or declaration, or by someone empowered to make the same, or by a statement by an attorney of record over his or her signature and registration number showing that:

(i) during the pendency of this application, access to the invention will be afforded to the Commissioner upon request;

(ii) all restrictions upon availability to the public will be irrevocably removed upon granting of the patent in accordance with 37 CFR § 1.808(a)(2);

(iii) the deposit will be maintained in a public depository for a period of 30 years or 5 years after the last request or for the effective life of the patent, whichever is longer;

(iv) a test of the viability of the biological material at the time of deposit (see 37 CFR § 1.807); and,

(v) the deposit will be replaced if it should ever become inviable.

In addition, the identifying information set forth in 37 CPR 1.809(d) should be added to the specification, if not already present. See 37 CPR 1.801 - 1.809 [MPEP 2401-2411.05] for additional explanation of these requirements.

Applicant has deposited the seeds (see e.g. pp. 33-34 of the instant specification). Examiner notes that, *inter alia*, a statement, affidavit or declaration by Applicant, or a statement by an attorney of record over his or her signature and registration number, or someone empowered to make such a statement, stating that the instant invention will be irrevocably and without restriction released to the public upon the issuance of a patent is required.

Applicant did not address this rejection. It is maintained.

Claim Rejections - 35 USC § 112 (scope of enablement)

16. Claims 35, 37-38, 40-41, 43-44 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, because the specification, while being enabling for the deposited cell lines, does not reasonably provide enablement for forms of self-compatibility and all potato lines as broadly as claimed. The specification does not

enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

Due to Applicant's amendments to the claims, the rejection is modified from the rejection set forth in the Office action mailed 5 December 2019 as applied to the above-referenced claims. Applicant traverses the rejection in the paper filed 5 May 2020. Applicant's arguments have been fully considered but are not persuasive.

The invention features plants with novel characteristics. Since these plant characteristics are essential to the claimed invention they must be obtainable by a repeatable method set forth in the specification or otherwise be readily available to the public. If such a plant is not so obtainable or available, the requirements of 35 USC § 112 may be satisfied by a deposit of the seeds. Deposits under NCIMB accession numbers 41663, 41664, 41665 and 41765 were made. However the claims are not drawn solely to the deposits.

The Claims

Claim 35 is drawn to a plant of a potato line, where the potato 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 line are homozygous,
where the said self-compatibility in the line comes from the *Sli* gene from *S. chacoense* wherein a copy of this gene is present in the deposited lines.

Dependent claim 37 requires that the plant comprises an additional agronomically desirable trait. Dependent claim 38 requires a certain amount of fresh weight of foliage. Dependent claim 40 reads on a seed or tuber of the plant.

Claim 41 is drawn to a method of producing 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 in six steps:

(1st) providing a first potato plant according to claim 35;

(2nd) providing a second potato plan that is a diploid *Solanum* plant with any level of homozygosity;

(3rd) cross-pollinating the two plants to provide a seed and then collecting the seed;

(4th) growing a multiplicity of the seeds and selecting a self-compatible plant;

(5th) backcrossing this self-compatible potato plant to the first potato plant 1 to 8 times; and

(6th) selecting for a fertile offspring plant to thus produce a diploid, fertile, self-compatible potato plant line that is at least 50% homozygous. The claim does not require that the plant produced has a yield of 200 grams of tuber per plant.

Dependent claim 43 requires that the plant used in the second part *supra* has an agronomically desirable trait. Dependent claim 44 reads on a plant line produced by the method.

The Scope of the Claims

In contrast to the limited description – four deposited lines – the claimed genus is quite large. Claim 35, for example, reads on any diploid fertile and self-compatible line created from any *Solanum* species that, for example, produces plants with a tuber yield of 200 grams per plant but Applicant only describes by deposit four lines.

In contrast to the four deposited lines – the claimed genus is quite large. Claim 35, for example, reads on any diploid fertile and self-compatible line created from any

Solanum species that, for example, produces plants with a tuber yield of 200 grams per plant but Applicant only has deposited four lines. ~~The method claims also read on any method of creating such a line, but Applicant only describes methods using the *Sli* gene.~~

The Federal Circuit in *In re Wands* lists eight considerations for determining whether or not undue experimentation would be necessary to practice an invention. *In re Wands*, 858 F2d 731, 738, 8 USPQ2d 1400, 1406 (Fed. Cir. 1988). These factors are: the quantity of experimentation necessary, the amount of direction or guidance presented, the presence or absence of working examples of the invention, the nature of the invention, the state of the prior art, the relative skill of those in the art, the predictability or unpredictability of the art, and the breadth of the claims. *Id.*

Applicant's Teachings

Applicant deposited lines designated by breeders reference and deposit number: AGVD1 (NCIMB 41663), AGVD2 (NCIMB 41664), AGVD3 (NCIMB 41665) and AGVD17 (NCIMB 41765). Spec., pp. 33-35.

In the specification, Applicant teaches a breeding process according to the instant invention starting with a self-compatible line, e.g. AGVD 1-3 or AGVD17. *Id.*, p. 37. Applicant teaches that "[n]o or at least little, attention is paid to the agronomical appearance of the plants at this stage." *Id.* One aspect of the breeding steps taught by the specification includes the possibility of adding a desired trait, such as vigor. *Id.*, p. 38. After adding such a trait, the hybrid is backcrossed to the self-compatible line while selecting for, e.g. vigor. *Id.* After adding numerous traits, and performing test crosses, inbred elite diploid breeding lines can be obtained which can be used to produce commercially valuable hybrids. *Id.*

Applicant reviews the steps: develop a "a vigorous fertile homozygous self-compatible diploid potato line;" "a diploid breeding line;" and then an "elite diploid potato breeding line." *Id.*, p. 40. On page 43, Applicant concludes that "[t]he above method now provides a breeding process for the production of homozygous breeding lines or elite breeding lines based on the background of population AGVD1, AGVD2, AGVD3 or AGVDI7, to which background successively additional alleles are added, thereby providing elite breeding lines wherein desirable traits are stacked." *Id.*, p. 43.

In the experiments that underlie the claimed invention, Applicant teaches obtaining the IVP07-1001/4 line from publically available germplasm where the IVP07-1001/4 plants contained the *Sli* gene after repeated selfing in Example 1. Spec., p. 44. Applicant cites to the 2006 Euphytica publication by Phumichai et al. (Euphytica 148:227-34 (2006)) as well as the 2005 Genome publication by Phumichai et al. Genome 48:977-94 (2005)) regarding the source of the *Sli* gene. *Id.*, p. 44 & p. 57 Applicant teaches that these plants are of no commercial value. *Id.* Applicant crossed these plants to three different diploid donor lines, D1-3, in order to acquire commercially valuable traits carried by the donor lines. *Id.*, pp. 44-45. The F1 cross with D1 was selfed and the progeny deposited as AGVD1, which was estimated as 50% homozygous. *Id.*, p. 45. The F1(D1) was crossed with F1(D2) and the progeny deposited under AGVD2, and this was estimated to be 25% homozygous with respect to alleles originating from IVP07-1001/4. *Id.* The F1(D1) was backcrossed to obtain progeny 25% homozygous with respect to alleles originating from IVP07-1001/4; these were deposited as AGVD3. *Id.*

In Example 2, Applicant teaches crossing the *Sli* gene donor with various publically available potato clones designated: IVP97-079-9, IVPAA-096-I8 and SH83-92-488 to generate clones D1, D2 and D3. *Id.*, p. 47. Applicant teaches this created a diploid trait donor lines containing the *Sli* gene with enhanced agricultural quality. *Id.*, pp. 46-50. Applicant provides a more detailed description of the potato lines used in Table 6. *Id.*, pp. 57-58.

Applicant teaches creating a molecular marker platform, i.e. SNPs, in Example 3. *Id.*, pp. 50-58.

In Example 4, Applicant teaches the development of homozygous diploid potato plants and then F1 hybrid progeny, as well as subsequent generations. *Id.*, pp. 58-62. Applicant teaches that it is "often stated" that potatoes are very prone to inbreeding depression. *Id.*, p. 60. A cause of this might be deleterious alleles; Applicant potentially identified a few such deleterious alleles, with one allele almost being lethal. *Id.*

In Example 4, Applicant teaches the development of homozygous diploid potato plants and then F1 hybrid progeny, as well as subsequent generations. *Id.*, pp. 58-62. Applicant describes that it is "often stated" that potatoes are very prone to inbreeding depression. *Id.*, p. 60. A cause of this might be deleterious alleles; Applicant potentially identified a few such deleterious alleles, with one allele almost being lethal. *Id.* However, no absolute lethal allele was present. *Id.* (heading at line 10).

Applicant concludes, on page 62, that

> It was found that after two generations of selfings homozygous diploid potato plants were obtained comprising alleles from both parents. Not a single marker out of 36 markers tested provided proof for a lethal allele. Furthermore, plants with more than 84% homozygous loci can be successfully crossed to obtain hybrid plants with at least five heterozygous

> chromosome regions. This means that hybrid seeds that are essentially uniform and that represent true potato seed have now been produced.

Id., p. 62.

The Art

The art generally teaches this breeding strategy, or similar strategies. For example, Jansky teaches first obtaining homozygous diploids using a self-incompatibility inhibitor. J Jansky, "Breeding, Genetics, and Cultivar Development," Advances in Potato Chemistry and Technology, ed. J. Singh & L. Kaur, pp. 27-62 (June, 2009), p. 46. Jansky teaches that "[h]ighly homozygous inbreds could be also be used for heterosis breeding. Jansky, p. 46. Jansky teaches that such an approach would be difficult. *Id.*

Phumichai et al. confirms the approach of Jansky, and documents the use of the *Sli* gene in the approach. Phumichai et al., Genome 48:977-84 (2005), abstract.

> Potato . . . is an auto- or segmental allo-tetraploid showing tetrasomic inheritance, which results in complicated genetic segregation. Since potatoes are propagated vegetatively, highly heterotic genotypes can be maintained, which facilitates the use of heterosis, an essential factor in potato breeding, particularly for tuber yield. Alternatively, open-pollinated true potato seed (TPS) can be used as a low-cost planting material for potato production.
>
> Highly homozygous inbreds, once amply produced, would be highly useful for the development of TPS varieties with genetic uniformity, as well as for the investigation of heterosis.
>
> A scheme for hybrid breeding using homozygous diploids and subsequent somatic hybridization has been proposed, for which the availability of highly homozygous diploid (or pure) lines is a key for success. In considering the use of pure lines for the development of genetically uniform [true potato seed] varieties, self-fertile, seed-propagated lines are highly desirable, and simple techniques to develop such lines are necessary.

Id., pp. 977-78 (citations omitted).

Finally, in 2016 Jansky et al. published an "Opinion & Policy" article calling for "Reinventing Potato as a Diploid Inbred Line-Based Crop." Jansky et al. (2016) Crop Sci. 56:1412-22. The authors call for the creation of "diploid inbred lines" (*id.*, p. 1413) with improved, e.g. tuber yield (*id.*, p. 1414) for use in creating inbred pools for use in heterosis. *Id.* The authors devote almost a page to the challenges faced. *Id.*, p. 1415. Then the authors addressed questions that must still be answered. *Id.* pp. 1415-18. One of the approaches cited by Jansky et al. is Dr. Lindhout's work – i.e. the instant claimed invention – as undergoing ongoing testing. *Id.*, pp. 1417-18. Therefore, approximately seven years after the filing date of the instant application, the problem of creating vigorous inbred diploid *S. tuberosum* plants apparently still exits.

Claims 41-48 are included in this rejection largely because the only embodiment taught by Applicant uses the *Sli* gene and the *Sli* gene is not publically available. Although the *Sli* gene is present in Applicant's deposits, the deposits have not been perfected with respect to this application.

Revisiting the Scope of the Claims

Applicant claims a large genus of potato lines. Both Jansky and Spooner et al. describes the breadth of the of the claimed genus as extremely large. *See*, e.g., Jansky (2009) "Breeding, Genetics, and Cultivar Development," pp. 27-29; Spooner et al. (2014) Bot Rev 80:283-383, 286-94, Table 1. Applicant's invention in claim 35, for example, reads on any potential potato line that is diploid, fertile, self-compatible and where the potato line has a yield of 200 g per plant.

In view of the arguments presented above, success in practicing Applicant's claimed invention is unpredictable and depends on the genotype of the potato lines.

Therefore, extensive guidance would be required to enable the invention as broadly as claimed. Such guidance is not provided and the claims encompass a broad, unpredictable genus.

The specification therefore does not enable one skilled in the art to make and use members of the species of the claimed genus other than the deposited lines. *See*, for example, MPEP 2164.06(a)(II), citing to *Ex parte Jackson*, 217 USPQ 804, 806 (Bd. App. 1982). The issue before the Board in *Ex parte Jackson* was:

> The issue squarely raised by this rejection is whether or not a description of several newly discovered strains of bacteria having one particularly desirable metabolic property in terms of the conventionally measured culture characteristics and a number of metabolic and physiological properties would enable one of ordinary skill in the relevant art to independently discover additional strains having the same specific, desirable metabolic property, i.e., the production of a particular antibiotic.
>
> To state the issue somewhat differently, it is whether a verbal description of a new species would enable one of ordinary skill in the relevant art to obtain strains of that species over and above the specific strains made available through deposit in one of the recognized culture depositories.

Ex parte Jackson, 217 USPQ 804, 806 (Bd. App. 1982). In the instant application, the issue is similar to the one before the Board in that Applicant made deposits with unique characteristics but not taught how to make and use other lines similar to the deposited lines. In *Ex parte Jackson*, the Board held that the deposit, without a detailed explanation of how to obtain more species, did not enable the generic claims. *Id.*, p. 808. The board found that "the degree of experimentation involved in locating new [organisms] apart from deposited cultures is undue in light of the enablement requirement of 35 U.S.C. 112." *Id.* The *Jackson* decision was considered and distinguished by the Federal Circuit in the *In re Wands* decision in view of the routine experimentation required to obtain hybridomas that were before the bar in the Wands

application. *In re Wands*, 858 F2d 735-37, 8 USPQ2d at 1403-04. Therefore, in particular in view of the *Ex parte Jackson* decision, the generic scope of the claims reading on the genus as set forth above is not enabled. Obtaining further species falling within the scope of the generic claims would require undue experimentation.

Furthermore, in 1966, the Supreme Court held that "[t]he basic quid pro quo contemplated by the Constitution and the Congress for granting a patent monopoly is the benefit derived by the public from an invention with substantial utility. Unless and until a process is refined and developed to this point-where specific benefit exists in currently available form, there is insufficient justification for permitting an application to engross what may prove to be a broad field. *Brenner v. Manson*, 383 U.S. 519, 534-35, 148 USPQ 689, 695 (1966).

Dependent claims are included in this rejection because none provide further limitations obviating this rejection.

Additionally, Applicant provides minimal teachings and/or guidance regarding the *Sli* gene, which appears to be an essential part of the claimed invention.

Applicant's Arguments

Applicant argues against the rejection. Response, pp. 19-21. Applicant provides a declaration from Dr. Lindhout ("Decl. Lindhout 2020"). *Id.*

Applicant points to the discussion in the declaration (paragraphs 13-17) regarding the genetic diversity of the deposited lines. *Id.*

Applicant continues to the discussion of Dr. Lindhout regarding correspondence with Dr. Jones. *Id.*, p. 20. Applicant quotes from Dr. Jones letter to Dr. Lindhout. *Id.*, p. 18. Applicant concludes that the rejection should be withdrawn. *Id.*, pp. 20-21.

Response to Applicant's Arguments

Applicant sole argument is the declaration of Dr. Lindhout. E.g., Response, p. 20. The declaration under 37 CFR 1.132 filed 16 July 2020 is insufficient to overcome the rejection of the above claims based upon 35 USC 112(a) as set forth in the last Office action because of the following reasons.

Dr. Lindhout, in paragraph 13, asserts that the lines D1-D3 represent a very large genetic variation. In fact, in paragraph 14, Dr. Lindhout assets that the "genetic background of the plants of D1-D3 is thus extremely diverse." Further, "D1-D3 are not unique and do not represent any specific genotype of potato lines." Thus, "any *S. tuberosum* is equably suitable for use as a starting material in a method of the invention." Decl. 2020, para. 15.

In contrast to this position, in application serial no. 13/504,156, Dr. Lindhout filed a declaration on 8 September 2016 to overcome a rejection under 35 USC 103 which stated in part, "[t]hat the chance of obtaining a progeny plant, by crossing different genotypes, which is not suffering from inbreeding depression in potato is infinitesimally small, and that starting from a heterozygous diploid potato genotypes, the chance of identifying a plant without homozygous deleterious alleles is, in practice, impossible. Decl. of Dr. Lindhout, app. ser. no. 13/504,156, signed 15 Aug. 2016.

Returning to the instant declaration, in paragraphs 16-17, Dr. Lindhout presents the results of an undated study which is asserted to indicate that only 4% haplotypes were unique to lines D1, D2 and D3. *Id.*, para. 17. Dr. Lindhout continues the argument in paragraphs 18-20. Dr. Lindhout quotes from an earlier declaration submitted in US application serial no. 13/504,156.

Dr. Lindhout then turns to a letter that he received from Dr. Jones. Dr. Jones wrote his 2020 letter "I never made a statement to that effect, and that my words are misinterpreted."

In response, Dr. Jones' statement in his 2020 letter is perplexing. First, the letter from Dr. Jones is not submitted as a declaration itself. Second, a letter from Dr. Jones was submitted by Applicant during prosecution. This letter from Dr. Jones is quoted in the Office action. Is Dr. Jones to repudiating that letter? Where Dr. Jones wrote that "I never made a statement to that effect," Dr. Jones' did not directly address the statements in his previous letter and there is no evidence that Dr. Jones reviewed the letter prior the comment above. The previous letter speaks for itself. How is the letter by Dr. Jones that was submitted during prosecution inaccurate? How is it misinterpreted?

In the letter attached to the 2020 Declaration, Dr. Jones also cites to personal communications (presumably with Dr. Lindhout) and a publication by Dr. Lindhout et al. The "personal communications" are not dated and the publication is post-filing art, both with respect to the instant application and the 13/504,156 application. The MPEP requires that the specification must be enabling as of the filing date. MPEP § 2164.05(a).

Thus the quoted passages from the recent letter of Dr. Jones are not persuasive with regard to the earlier letter of Dr. Jones. Even if Dr. Jones directly addressed his previous letter, his new position has no weight regarding the letter from Dr. Koornneef which was also submitted in Applicant's argument against the 103 rejection. Both supporting letters, Dr. Jones and Dr. Koornneef were initially filed in European

Application EP2699684 (04 June 2014). The letters teach that Applicant's invention, as broadly as claimed, was not fully enabled. For example, on page 2 of his letter, Dr. Koornneef states that

> Ever since Lindhout and his co-workers published their results in Potato Research volume 54 of 2011, showing high yielding hybrids from 87% homozygous F3 inbreds, I have been positively surprised that Solynta has managed to challenge this dogma. Solynta could successfully obtain essentially homozygous inbred lines by making use of genotypes that are selfcompatible and by choosing the right starting material that apparently is heterozygous for a lower number of deleterious alleles. This provides the basis of generating useful inbred lines that can become parents of F1 hybrids.

Letter from Koornneef (2012) p. 2 (underlining added). Earlier in the letter, Dr. Koornneef also states that "to start a project aiming at developing a good performing homozygous potato plant for the purpose of hybrid production is a daring undertaking with very little chance of success. *Id.*

In a similar vein, in a paper filed with the EPO, Applicant stated that "every expert in the field can confirm that due to inbreeding depression, the chances of successfully attaining the plant of the invention are close to zero. Hence, the production of such plants is generally considered impossible." EPO Publication No. EP 2 493 286 A2, Remarks to EPO (21 Dec. 2012), p. 8.

Furthermore, on 15 December 2011, approximately two years after the filing date of the instant application, Lindhout et al. published a paper in Potato Research which appears to correspond to the experiments set forth in the instant examples (all five inventors are listed as authors on the paper). Lindhout et al. (2011) Potato Res 54:301-12. The discussion in the paper is instructive. For example, Lindhout et al. describes that

> As a consequence of the high level of unfavourable alleles, techniques aimed at developing homozygotes in one step, although successful as a technology in various crops, certainly do not generate vigorous homozygous potatoes. For instance, if 20 of the 39,000 protein-encoding genes (PGSC 2011) on the potato genome harbor alleles causing severe fitness reduction, the chance to generate vigorous diploid selfed progenies without homozygous unfavourable loci from a parent genotype, that is heterozygous for these 20 loci, is only 0.3%.

Id., p. 303. Lindhout et al. concludes that "inbred lines were developed that combined self-compatibility with good agronomic performance. Although the number of such plants is still low, the progress made in this breeding programme is significant." *Id.*, p. 304. As a possible reason for success, the authors offer that "lethal alleles do not occur at high frequency in our diploid potato germplasm. *Id.*, pp. 308-09. The authors continue that

> there are many loci with minor effects involved in these traits (Quantitative Trait Locus, QTL). Our current research focuses on identifying the crucial QTLs for these traits. The results will be applied in breeding and allow to increase efficiencies by using diagnostic markers for these traits.

Id., p. 309.

In summary, Dr. Lindhout in 2016 argued that the potato line selected was critical for the invention - that it was more than just using the *Sli* gene. But now Dr. Lindhout argues that is the line isn't critical. Dr. Jones recanted his statement but without specific reference to his previous letter itself. For the reasons above, Dr. Jones' new position is not persuasive. Further, Dr. Jones' 2020 letter is not submitted as a declaration itself. The letter of Dr. Koornneef and the article of Jansky et al. remain un-contradicted on the record. Additionally, Applicant's previous positions argue against the claimed invention fully enabled. The undefined characteristics of the potato line are a critical part of the invention.

Therefore Applicant fails to provide sufficient guidance and thus the claimed invention requires undue experimentation without a reasonable expectancy of success. The general standard for a prima facie rejection and for considering a declaration (MPEP § 716.01(d)) is the preponderance of the evidence. E.g., 37 CFR 1.56(b). Under that standard, the rejection is maintained.

Claim Rejections - 35 USC § 112(d)

The following is a quotation of 35 U.S.C. 112(d):

> (d) REFERENCE IN DEPENDENT FORMS.—Subject to subsection (e), a claim in dependent form shall contain a reference to a claim previously set forth and then specify a further limitation of the subject matter claimed. A claim in dependent form shall be construed to incorporate by reference all the limitations of the claim to which it refers.

The following is a quotation of pre-AIA 35 U.S.C. 112, fourth paragraph:

> Subject to the following paragraph [i.e., the fifth paragraph of pre-AIA 35 U.S.C. 112], a claim in dependent form shall contain a reference to a claim previously set forth and then specify a further limitation of the subject matter claimed. A claim in dependent form shall be construed to incorporate by reference all the limitations of the claim to which it refers.

17. Claim 37 is rejected under 35 U.S.C. 112(d) or pre-AIA 35 U.S.C. 112, 4th paragraph, as being of improper dependent form for failing to further limit the subject matter of the claim upon which it depends, or for failing to include all the limitations of the claim upon which it depends. Claim 37 depends from claim 35 but requires an additional agronomically desirable trait. Art that could apply to the plant of claim 37 but might not apply to the plant of claim 35. Thus claim 37 is broader in scope than claim 35 and fails to further limit the claim.

Applicant may cancel the claim(s), amend the claim(s) to place the claim(s) in proper dependent form, rewrite the claim(s) in independent form, or present a sufficient showing that the dependent claim(s) complies with the statutory requirements.

Applicant addressed the rejection on page 21 of the response. Applicant pointed to the amendment that removed the word "additional" but Applicant added the word "further" suggesting that a trait is being added to the plant of claim 35.

It appears that deleting the word "further" should obviate this rejection. Claim 37 would not be rejected under 35 USC 112(d) if it is merely articulating and claiming a property of the plant of claim 35.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

18. Claims 45 and 48 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Phumichai & Hosaka (2006) Euphytica 149:251-58 in view of Birhman & Hosaka (2000) Genome 43:495-502.

This is a new rejection necessitated by Applicant's amendments to the claims.

The factual inquiries set forth in *Graham v. John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

a. Determining the scope and contents of the prior art.

b. Ascertaining the differences between the prior art and the claims at issue.

c. Resolving the level of ordinary skill in the pertinent art.

d. Considering objective evidence present in the application indicating obviousness or nonobviousness.

Claim 45 is drawn, according to the preamble, to a method of producing a uniform hybrid potato that is diploid, fertile, self-compatible by crossing two potato plants, both of which have the *Sli* gene as documented by Phumichai et al. (see e.g. Dec. Lindhout 2020, para. 24) where in each of the parents at least 50% of the genomic loci are homozygous.

No reference explicitly teaches the required limitations of claim 45. However, several publications by the Hosaka lab teaches various combinations of the limitations. One of ordinary skill in the art would be motivated to follow the teachings of the above references and, given the published results, would have a reasonable expectation of success.

Thus Phumichai & Hosaka (2006) teaches selfing diploid potato clones using the *Sli* gene for obtaining self-compatible potato lines. Phumichai & Hosaka (2006), pp. 252-53. Beginning with the 2H22 family, obtained from the diploid clone 97H32—6, 33 of 62 loci were homozygous. *Id.*, p. 253. This is "at least 50%." Phumichai also teaches continued crosses using this starting material. *Id.*, p. 252. Birhman & Hosaka teaches using different potato lines to add traits to a reference line with the *Sli* gene. Birhman & Hosaka, p. 496. Therefore claims 45 and 48 are obvious.

Applicant's Argument & Response

Applicant expected that the claim amendments would overcome art rejections by using limitations from claim 35 in claim 45.

Claims 45 and 47-48 are still rejected over the prior art. The examiner also notes that there is no mention of tuber yield in claim 45.

19. Claim 47 is rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Phumichai & Hosaka (2006) Euphytica 149:251-58 in view of Birhman & Hosaka (2000) Genome 43:495-502 in further view of Bradshaw et al (2006) Potato Res 49:49-65.

As seen above, claim 45 is obvious in view of Phumichai & Hosaka (2006) and Birhman & Hosaka. Neither reference teaches adding by crosses any of the traits listed in claim 47. Bradshaw et al., however, teaches crossing traits such as disease resistance (p. 52) into potato plants. Such crosses would have a reasonable expectation of success. Therefore claim 47 is obvious.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.
Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

The USPTO internet Web site contains terminal disclaimer forms which may be used. Please visit http://www.uspto.gov/forms/. The filing date of the application will

determine what form should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

20. Claims 35, 37-38, 40-41, 43-45 and 47-48 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-19 of US Patent No. 10,524,436 B2. Although the conflicting claims are not identical, they are not patentably distinct from each other.

The instant application is a continuation where Applicant is seeking broader coverage than the issued claims. As such the instant pending claims represent genera encompassing the species claims in the above Patent. Thus the pending claims are obvious in view of the allowed claims.

Applicant stated that a terminal disclaimer will be filed if allowable subject matter is obtained. Response, pp. 21-22. The rejection is maintained.

Conclusion

21. No claim is allowed.

The following reference from the Hosaka lab are also made of reference.

(1) Phumichai et al. (2005) Genome 48:977-94 also teaches crossing potato plants to obtain highly homozygous lines.

(2) Phumichai et al. (2006) Euphytica 148:227-34 teaches crossing potato plants with the *Sli* gene to a various diploid potatoes.

(3) Phumichai et al. (2004) Mem Grad School Sci Technol Kobe Univ 22:115-21 teaches various breeding histories with potatoes containing the *Sli* gene.

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to RUSSELL T BOGGS whose telephone number is (571)272-2805. The examiner can normally be reached on Monday - Friday, 0800 to 1830 Mtn.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Shubo (Joe) Zhou, can be reached on 571-272-0724. The fax phone

number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/RUSSELL T BOGGS/
Examiner, Art Unit 1663

Notice of References Cited	Application/Control No. 16/541,071	Applicant(s)/Patent Under Reexamination Lindhout et al.	
	Examiner RUSSELL T BOGGS	Art Unit 1663	Page 1 of 2

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-10524436-B2	01-2020	Lindhout et al.	A01H1/02	1/1
	B					
	C					
	D					
	E					
	F					
	G					
	H					
	I					
	J					
	K					
	L					
	M					

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	de Vries et al. (2016) Open Agric 1:151-56
*	V	Birhman & Hosaka (2000) Genome 43:495-502
*	W	Phumichai & Hosaka (2006) Euphytica 149:251-58
	X	Bradshaw et al (2006) Potato Res 49:49-65

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Notice of References Cited	Application/Control No. 16/541,071	Applicant(s)/Patent Under Reexamination Lindhout et al.	
	Examiner RUSSELL T BOGGS	Art Unit 1663	Page 2 of 2

U.S. PATENT DOCUMENTS

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NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Phumichai et al. (2004) Mem Grad School Sci Technol Kobe Univ 22:115-21
*	V	Phumichai et al. (2006) Euphytica 148:227-34
	W	Phumichai et al. (2005) Genome 48:977-94
*	X	Lindhout et al. (2011) Potato Res 54:301-12

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/541,071	Lindhout et al.
	Examiner	Art Unit
	RUSSELL T BOGGS	1663

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
see searches in parent application 13/504,156	11/27/2019	RTB
NPL search as per attached beginning	11/23/2019	RTB
EAST search as per attached	11/27/2019	RTB
STN search as per attached	11/23/2019	RTB
EAST search as per attached	10/27/2020	RTB
review references from the Hosaka lab	11/02/2020	RTB

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

/RUSSELL T BOGGS/ Examiner, Art Unit 1663	

Printed on: 20201001

Toward the development of highly homozygous diploid potato lines using the self-compatibility controlling *Sli* gene

Chalermpol Phumichai, Motoyuki Mori, Akira Kobayashi, Osamu Kamijima, and Kazuyoshi Hosaka

Abstract: Cultivated diploid potatoes ($2n = 2x = 24$) are self-incompatible, but can be altered to become self-compatible using the *Sli* gene. Previously, a diploid clone 97H32–6 was selfed up to S_3 using the *Sli* gene. To explore the usefulness of the *Sli* gene for the production of highly homozygous diploid potatoes, 2 S_4 families from the above 97H32-6 derived S_3 lines (inbred series A) and 3 S_5 families by continuous selfings from a different F_1 (= S_0) plant (inbred series B) were developed. The level of heterozygosity and the location of heterozygous loci on the genetic map were investigated using RFLP and AFLP markers. The average heterozygosity levels of the originally heterozygous loci decreased from 100% in S_0 to 10.7% in S_4 and 8.6% in S_5 (inbred series A and B, respectively). The average rate of reduction in heterozygosity per generation (38.4% and 38.5% for inbred series A and B, respectively) was lower than the theoretically expected rate (50%). However, none of the loci or chromosome sections was exclusively heterozygous in the advanced self-progeny. Thus, highly homozygous and seed-propagated diploid potatoes could be obtained by repeated selfing using the *Sli* gene.

Key words: Selfing, heterozygosity, diploid potato, *Sli* gene, *Solanum*.

Résumé : Les pommes de terre diploïdes cultivées ($2n = 2x = 24$) sont autoincompatibles mais cela peut être modifié grâce au gène d'autocompatibilité *Sli*. Antérieurement, 1 clone diploïde, 97H32-6, a été autofécondé jusqu'en S_3 à l'aide du gène *Sli*. Afin d'explorer l'utilité de ce gène pour la production de pommes de terre diploïdes fortement homozygotes, 2 familles S_4 dérivées des lignées S_3 mentionnées plus haut (série A de lignées autofécondées) et 3 familles S5 obtenues par autofécondation continue d'une plante F_1 (=S_0) différente (série B) ont été produites. Le niveau d'hétérozygotie et la position des locus hétérozygotes sur la carte génétique ont été déterminés à l'aide de marqueurs RFLP et AFLP. Les niveaux moyens d'hétérozygotie pour les locus initialement hétérozygotes sont passés de 100 % en S_0 à 10,7 % en S_4 et 8,6 % en S_5 chez les séries A et B, respectivement. Les taux moyens de réduction de l'hétérozygotie à chaque génération (38,4 % et 38,5 % respectivement) étaient plus faibles que l'attente théorique (50 %). Cependant, aucun des locus ou segments chromosomiques n'était exclusivement hétérozygote chez les lignées autofécondées. Ainsi, des pommes de terre largement homozygotes et reproduites via les graines ont été obtenues par autofécondation répétée à l'aide du gène *Sli*.

Mots clés : autofécondation, hétérozygotie, pomme de terre diploïde, gène *Sli*, *Solanum*.

[Traduit par la Rédaction]

Introduction

Potato (*Solanum tuberosum* L., $2n = 4x = 48$) is an auto- or segmental allo-tetraploid showing tetrasomic inheritance, which results in complicated genetic segregation (Howard 1970; Ross 1986; Matsubayashi 1991). Since potatoes are propagated vegetatively, highly heterotic genotypes can be maintained, which facilitates the use of heterosis, an essential factor in potato breeding, particularly for tuber yield (Ross 1986; Peloquin et al. 1989). Alternatively, open-pollinated true potato seed (TPS) can be used as a low-cost planting material for potato production (Shonnard and Peloquin 1991). However, the introduction of TPS in developed countries is difficult because of the lack of homogeneity in tuber shape and size (Ross 1986). Highly homozygous inbreds, once amply produced, would be highly useful for the development of TPS varieties with genetic uniformity, as well as for the investigation of heterosis (Birhman and Hosaka 2000).

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Aardevo Exhibit 1010
Page 0963

To obtain homozygous lines, tetraploid potatoes can be selfed, but the selfing causes marked tuber yield reduction and loss of fertility, since these traits are controlled largely by heterotic genetic effects (Krantz 1924, 1946; Mendiburu and Peloquin 1977; Golmirzaie et al. 1998*a*; 1998*b*). The use of closely related diploid species and dihaploid potatoes is hampered by gametophytic self-incompatibility controlled by multiallelic *S* genes (Pushkarnath 1942; Pandey 1962; Cipar et al. 1964*a*). Self-compatible variants have often been described in essentially self-incompatible diploid species (Pushkarnath 1942; Cipar et al. 1964*b*; De Jong and Rowe 1971; Olsder and Hermsen 1976; De Jong 1977; Cappadocia et al. 1986; Birhman 1986). However, as far as we could determine, these variants were not successively selfed over generations.

Monoploidization by parthenogenetic reduction using a *Solanum phureja* haploid inducer or anther culture has been extensively conducted (Irikura 1975; van Breukelen et al. 1975; Foroughi-Wehr et al. 1977; Uijtewaal et al. 1987*a*). Spontaneously doubled or colchicine-doubled monohaploids (complete homozygous diploids) were often isolated from monohaploids of dihaploid *S. tuberosum* (van Breukelen et al. 1977; Uijtewaal et al. 1987*b*), *S. phureja* Juz. et Buk. (Veilleux et al. 1985), or *Solanum chacoense* Bitter (Cappadocia et al. 1986). However, most of these homozygous diploids were either male or female sterile (van Breukelen et al. 1977; Uijtewaal et al. 1987*b*; Singh et al. 1988; Paz and Veilleux 1999), except for a few exceptional self-fertile plants (Wenzel et al. 1979; Cappadocia et al. 1986).

A scheme for hybrid breeding using homozygous diploids and subsequent somatic hybridization has been proposed, for which the availability of highly homozygous diploid (or pure) lines is a key for success (Meyer et al. 1992). In considering the use of pure lines for the development of genetically uniform TPS varieties, self-fertile, seed-propagated lines are highly desirable, and simple techniques to develop such lines are necessary.

Recently, we found a dominant allele (the *S* locus inhibitor gene *Sli*) from a self-compatible variant in a wild diploid species, *S. chacoense*, which can alter self-incompatible plants to self-compatible ones (Hosaka and Hanneman 1998*a*). The *Sli* gene was mapped at the most distal end of chromosome 12 (Hosaka and Hanneman 1998*b*), and its possible tight linkage with a recessive lethality gene was proposed (Hosaka and Hanneman 1998*a*). Birhman and Hosaka (2000) introduced the *Sli* gene by crossing various cultivated diploid potatoes and successfully developed S_2 progeny. The S_2 inbreds were later accidentally pollinated with bulked pollen grains from other S_2 plants, producing F_1 hybrids from which a new series of inbred progeny was developed (Phumichai et al. 2004). Restriction fragment length polymorphism (RFLP) analysis was carried out to monitor the inbreeding process of this new inbred series from F_1 (= S_0) to S_3 progeny, the results of which suggested a possible association between fertility and heterozygosity, resulting in a much higher observed percentage of heterozygosity in S_3 progeny than theoretically expected (Phumichai et al. 2004).

In this study, 2 S_4 families from the above S_3 progeny and 3 S_5 families of a new inbred series were developed, and their inbreeding processes were monitored using RFLP and AFLP markers. The specific objectives were as follows: (*i*) to understand the process of inbreeding in terms of inbreeding depression as measured by fertility; (*ii*) to measure the degree of heterozygosity reduction and identify heterozygous loci that resist universal homozygosity; and (*iii*) to discuss the usefulness of *Sli* gene in developing of highly homozygous lines of diploid potato.

Materials and methods

Plant material

The first series of inbred lines (inbred series A) was obtained from a diploid clone 97H32–6 (composed of 75% cultivated and 25% wild germplasm with 100% cultivated cytoplasm), which was self-compatible due to the function of the *Sli* gene originally derived from a self-compatible variant of *S. chacoense* (Hosaka and Hanneman 1998*a*). Two randomly chosen S_4 families, 3H94 (15 plants) and 3H100 (15 plants), were analyzed for heterozygosity by DNA analysis and compared with that of their S_1 (15 plants from the 2H21 family) and S_3 (15 plants from the 2H22 family) ancestors (Fig. 1). The S_2 family was not available for DNA analysis.

The second series of inbred lines (inbred series B) were started from 2 advanced diploid clones, WB922236–2 and WB922236–3 (full sibs of W822227–1 × W882204–8 that possess at least 37.5% of the chromosomal and 100% of the cytoplasmic information from *S. phureja* PI 225683) by crossing with the pollen of *Sli* gene donors F_1-1, 97H32–6, 97H32–7, or 97H32–14 (the last 3 clones were full sibs descended from F_1-1; see Fig. 1). Three randomly chosen S_5 families, 3H86 (15 plants), 3H88 (15 plants), and 3H5 (12 plants), were used for DNA analysis; their pedigrees are shown in Fig. 1. Their parental populations, S_1 (12 plants from family 3H2), S_2 (14 plants from 3H3), and S_4 (7 plants from 2H32 and 16 plants from 2H34) were used for comparison. The S_3 family was not available for DNA analysis.

Plants were grown in a green house in the fall and winter or in an insect-proof screen house in the spring. To ensure optimal growing conditions for flowering, light was supplemented to maintain 16 h of daylight from October to March, while from April to July, plants were grown in natural daylight. To advance selfed generations quickly, only seedlings, except for the initial population of inbred series B, were used without harvesting tubers. Self-pollinations were made on as many flowers as possible until the plants set berries during the season.

RFLP analysis

Total DNA isolation and RFLP detection procedures were all as described in Hosaka and Hanneman (1998*b*). Samples of total DNA from S_1 plants (from 3H2 and 2H21 families) were digested with restriction endonucleases *Dra*I, *Eco*RI, *Eco*RV, or *Hin*dIII and surveyed to obtain heterozygous loci in S_0 plants. Only heterozygous loci detected with specific probe and enzyme combinations were used to analyze the subsequent inbred progeny. RFLP probes used were either single-copy tomato probes obtained from Dr. S.D. Tanksley, Cornell University (Tanksley et al. 1992) or potato probes (Hosaka and Spooner 1992).

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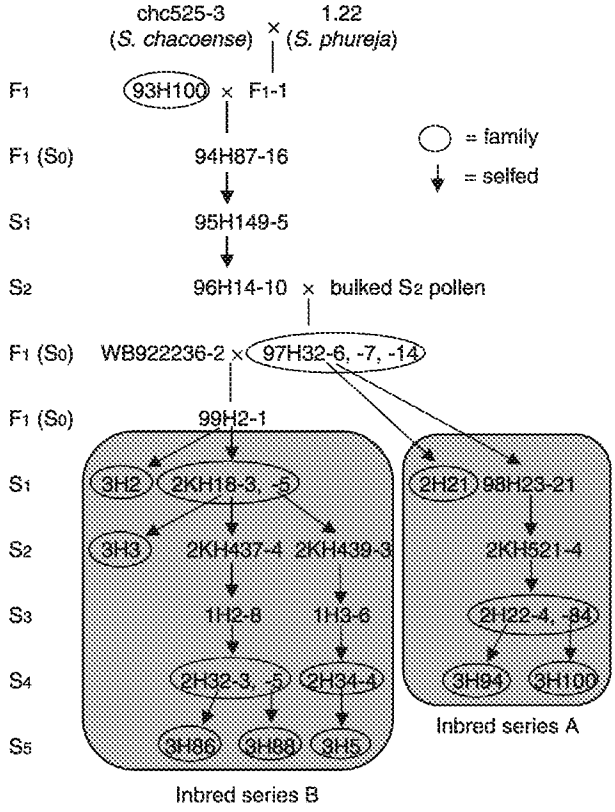
Page 0964

Fig. 1. Two inbred series, A and B, developed from 97H32–6.



AFLP analysis

The same sets of DNA samples used for RFLP analysis were also used for AFLP analysis using procedures that are essentially the same as the protocols used in the AFLP® Analysis System (Invitrogen, Carlsbad, Calif.), described previously (Phumichai et al. 2004).

Data analysis

The locus-based observed percentage of heterozygosity (LOPH) was given by the percentage of the heterozygous RFLP loci for each individual plant, and the mean of the family was calculated. For the parental clone, the LOPH was estimated from the percentage of segregating loci in its selfed progeny using RFLP or AFLP markers. Section-based observed percentage of heterozygosity (SOPH) was defined as the percentage of the sections (each of 12 chromosomes was trisected into sections *a*, *b*, and *c*, based on the map length) containing 1 or more heterozygous RFLP loci, and calculated in the same way as the LOPH was for parents and their progeny. For RFLP loci, segregation ratios of homozygotes and heterozygotes were compared with the expected segregation ratios of 1:1 and 1:2:1, respectively, using chi-square tests.

Results

Production of selfed progeny

The initial selfings up to 96H14–10 were reported by Birhman and Hosaka (2000). Reproductive traits and heterozygosity levels in subsequent selfing progeny from 97H32–6 (S_0) to the 2H22 family (S_3) in inbred series A were investigated by Phumichai et al. (2004). For this study, 424 S_4 plants of 7 families derived from the 2H22 family by selfing were raised, of which only 30 plants flowered and produced enough pollen grains for self-pollination during the season. As many flowers as possible were self-pollinated (a total of 132 flowers), but only 8 plants produced seeds (Table 1).

Production of inbred series B was started with 5040 F_1 plants from 7 families: WB922236–2 × F_1-1 (294 plants), WB922236–2 × 97H32–6 (808 plants), WB922236–2 × 97H32–7 (510 plants), WB922236–2 × 97H32–14 (687 plants), WB922236–3 × F_1-1 (1050 plants), WB922236–3 × 97H32–7 (1087 plants), and WB922236–3 × 97H32–6 (604 plants). Only a fraction of these flowered, among which 68 (1.3%) formed tubers. Since a drastic decrease in progeny number as a result of poor fertility and growth had been experienced, we saved an additional 141 plants that did not flower but formed good tubers. These 209 plants grown from tubers became an initial population for selfing in inbred series B. The result of self-pollinations for each generation is summarized in Table 1. As the selfed generation proceeded, many families failed to produce selfed progeny, resulting in an arrival at S_5 only from 99H2–1, 1 of the 14 F_1 hybrids from WB922236–2 × 97H32–6. Forty-three S_5 plants successfully produced selfed seeds.

Fertility and self-compatibility

In the selfed progeny of inbred series A, the percentage of available plants for self-pollination drastically decreased from 62.6% in S_1 to 7.1% in S_4 (Table 1). Many S_4 plants did not form buds, and even if buds were formed, they dropped at the very young developmental stage. The percentage of selfers (= plants that set seeds by self-pollination) among pollinated plants decreased steadily from 82.5% in S_1 to 26.7% in S_4.

Such steady decreases in either the percentage of self-pollinated plants or the percentage of selfers were not found in inbred series B. Both percentages fluctuated greatly among generations, with the average decrease being 32.5% for pollinated plants and 43.0% for selfers from S_0 to S_5. Many S_5 plants flowered, but most of them showed very poor or no pollen shedding.

Heterozygosity in inbred series A

Out of 123 single-copy RFLP probes surveyed, 62 on 34 chromosome sections (Fig. 2A) segregated in the S_1 population (2H21), which were used to monitor the subsequent inbreeding process. Since some loci were localized together within a short map distance or cosegregated, SOPH was thereafter more weighted than LOPH.

The S_2 clone 2KH521–4 showed a slightly lower SOPH (52.9%), or an even higher LOPH (50.0%) than those of the parental S_1 population (the mean SOPH of 57.4%, or LOPH of 49.6%), indicating no reduction in heterozygosity from S_1 to S_2. Thus, 2KH521–4 and its parental clone 98H23–21 were probably unintentional selections with higher heterozygosity in S_2 and S_1 populations, respectively (Phumichai et al. 2004). In contrast, 2H22–4 and 2H22–84, showing SOPH values of 20.6% and 17.7%, respectively, were likely unintentional selections, with lower heterozygosity in the S_3 fam-

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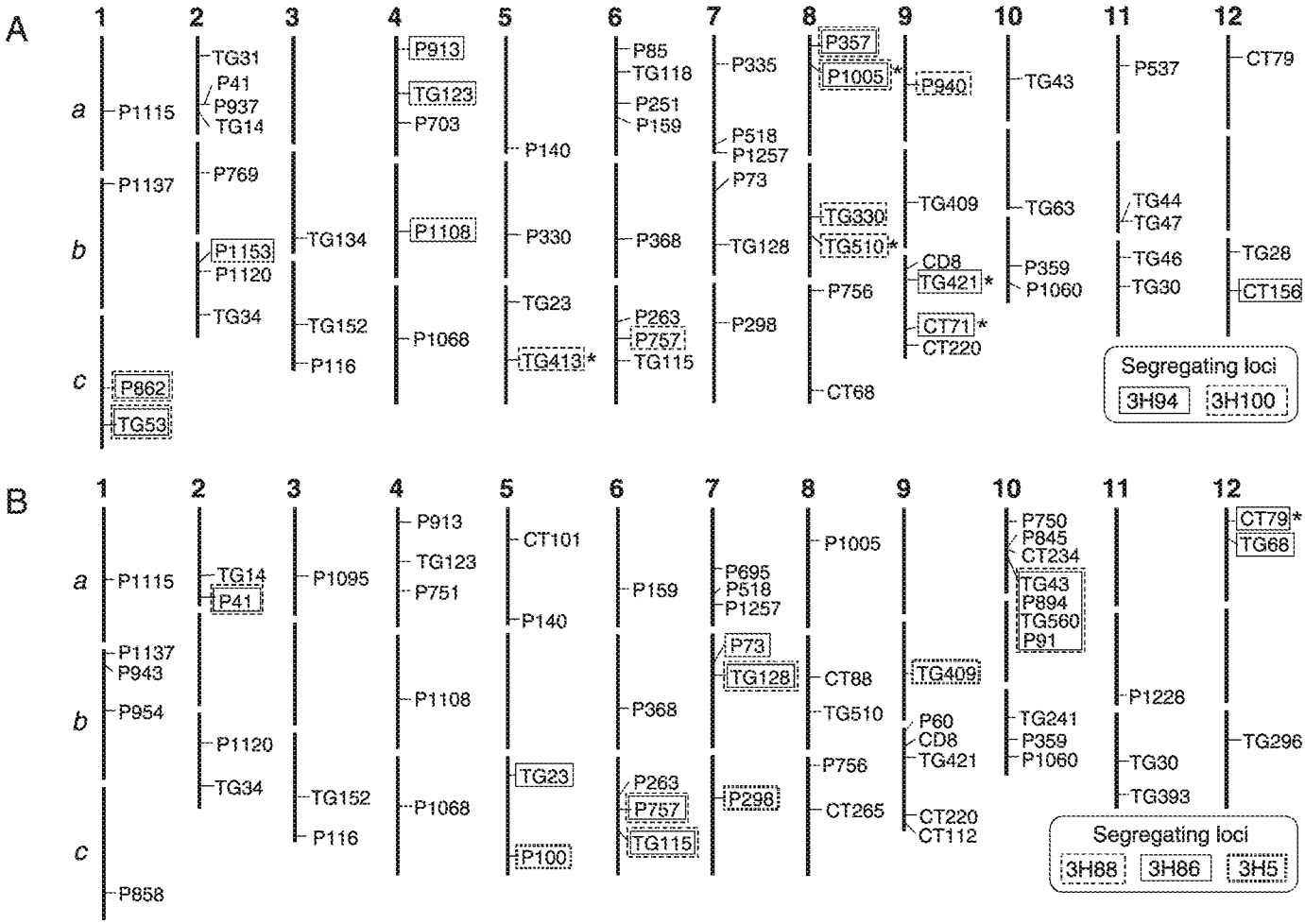
Page 0965

Table 1. Total number and percentage of plants that were self-pollinated and those that successfully produced seeds by self-pollination (= selfers). See text for details.

Generation	No. of plants grown	Self-pollinating plants	Flowers	Selfers
Inbred series A				
F_1 (S_0)	1	1 (100%)	10	1 (100%)
S_1	91	57 (62.6%)	401	47 (82.5%)
S_2	11	3 (27.3%)	15	2 (66.7%)
S_3	81	37 (45.7%)	307	20 (54.1%)
S_4	424	30 (7.1%)	132	8 (26.7%)
Inbred series B				
F_1 (S_0)	209	90 (43.1%)	578	60 (66.7%)
S_1	831	186 (22.4%)	712	67 (36.0%)
S_2	315	58 (18.4%)	231	16 (27.6%)
S_3	118	38 (32.2%)	282	15 (39.5%)
S_4	106	55 (51.9%)	727	28 (50.9%)
S_5	427	115 (26.9%)	536	43 (37.4%)

Fig. 2. Segregating RFLP loci in the most advanced selfed progenies in inbred series A and B. Asterisks indicate loci showing significant segregation distortion at the 5% level. Each chromosome linkage map was trisected into *a*, *b*, and *c*, and the chromosome section based observed percentage of heterozygosity was obtained.



ily (the mean SOPH = 30.8%) (Table 2). For the S_4 families 3H94 and 3H100, the mean LOPH values obtained were 9.6% and 8.2%, respectively, and the mean SOPH values obtained were 12.0% and 9.4%, respectively.

Eleven and 9 loci segregated in the 3H94 and 3H100 families, respectively, of which 4 loci on 2 sections (1*c* and 8*a*) were common (Fig. 2A). Four loci (TG413, TG510, TG421, and CT71) showed significant segregation distortion at the

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Page 0966

5% level in either 1 of the 2 S_4 families, while the P1005 locus was distorted in both families but differently in each family.

AFLP banding patterns were obtained using 22 AFLP primer combinations, generating a total of 304 bands segregating (present versus absent) in the S_1 family. Assuming that each band was controlled by 1 locus, AFLP loci indicated a steady decrease in LOPH as the selfed generation proceeded (Table 2). The AFLP estimates for LOPH were often much higher than those for RFLP. The real reason is unknown, but might be that heterozygous loci were sometimes doubly counted as a result of the silver-staining method that stained both strands of a denatured DNA fragment.

Heterozygosity in inbred series B

Fifty-nine of 121 RFLP loci surveyed segregated in the S_1 family (3H2), which covered 29 chromosome sections. In addition, 326 segregating AFLP bands were used to monitor the inbreeding process of inbred series B. RFLP phenotypes provided no evidence for either contamination by accidental out-crossing or new mutations, because bands present in the selfed progeny were all present in the initial S_0 plant 99H2–1.

The SOPH decreased steadily from the original 100% in 99H2–1 (S_0) to the family means of 62.4% in 3H2 (S_1), 37.9% in 3H3 (S_2), 9.9% in 2H32 or 10.4% in 2H34 (both S_4), and 6.0–12.2% in each of 3H86, 3H88, and 3H5 (all S_5) (Table 2). The S_1 parent (SOPH = 65.5%) was more heterozygous than the population mean (SOPH = 62.4%). One of S_5 families, 3H86, showed a higher SOPH (12.2%) than the mean SOPH of its parental 2H32 population (9.9%). This was because 2H32–3, a true parental clone of the 3H86 family not used for the DNA analysis, was estimated to be SOPH = 20.7%, which was distinctly higher than the SOPH estimates from any 2H32 plants used for DNA analysis.

Genetic segregation occurred in 12 loci in 3H86, 8 loci in 3H88 and 3 loci in 3H5 families, none of which were common in the 3 S_5 families (Fig. 2B). Only locus CT79 in the 3H86 family showed distorted segregation favoring one of homozygote genotypes. The LOPH estimated by AFLP bands were somewhat similar to those by RFLP, indicating the gradual decrease of heterozygosity through selfing generations (Table 2).

Comparison between inbred series A and B

For inbred series A, all available SOPH (RFLP) and LOPH (AFLP) values for each generation (Table 2) were pooled and averaged. These averages were as follows: 100% in S_0, 57.4% in S_1, 57.6% in S_2, 31.3% in S_3, and 10.7% in S_4, with an average of 38.4% reduction per generation. For inbred series B, the averages were 100% in S_0, 69.0% in S_1, 37.9% in S_2, 24.9% in S_3, 15.6% in S_4, and 8.6% in S_5, with an average of 38.5% reduction per generation. Thus, both inbred series experienced decreases in their levels of heterozygosity, with approximately the same rate by selfing, although the reduction rates fluctuated greatly among generations in series A. There was neither a heterozygous chromosome section nor a distorted locus common to all 5 of the advanced self-progeny (Fig. 2).

Table 2. Locus-based observed percentage of heterozygosity (LOPH) or chromosome section-based observed percentage of heterozygosity (SOHP) estimated using RFLP and AFLP markers. For parental clones, LOPH and SOPH were estimated by the proportion of segregating loci in their progeny, while those for the progeny were represented by the mean with the range in parentheses.

Parent						Progeny					
Generation	Clone	Expected	LOPH (RFLP)	SOPH (RFLP)	LOPH (AFLP)	Generation	Family	No.	Expected	LOPH (RFLP)	SOPH (RFLP)
Inbred series A											
S_0	97H32–6	100	100	100	100	S_1	2H21	15	50	49.6 (32.3–62.9)	57.4 (41.2–70.6)
S_2	2KH521–4	25	50.0	52.9	62.2	S_3	2H22	15	12.5	26.1 (19.4–41.9)	30.8 (20.6–44.1)
S_3	2H22–4	12.5	17.7	20.6	45.1	S_4	3H94	15	6.25	9.6 (3.2–14.5)	12.0 (2.9–17.6)
S_3	2H22–84	12.5	14.5	17.7	42.4	S_4	3H100	15	6.25	8.2 (0–14.5)	9.4 (0–17.6)
Inbred series B											
S_0	99H2–1	100	100	100	100	S_1	3H2	12	50	56.0 (39.0–76.3)	62.4 (48.3–75.9)
S_1	2KH18–3	50	59.3	65.5	79.1	S_2	3H3	14	25	29.1 (15.5–39.0)	37.9 (20.7–44.8)
S_3	1H2–8	12.5	20.3	20.7	19.9	S_4	2H32	7	6.25	7.5 (1.7–13.6)	9.9 (3.4–13.8)
S_3	1H3–6	12.5	11.9	20.7	38.3	S_4	2H34	16	6.25	5.6 (0–10.3)	10.4 (0–17.9)
S_4	2H32–3	6.25	20.3	20.7	19.3	S_5	3H86	15	3.125	10.7 (1.7–18.6)	12.2 (3.4–17.2)
S_4	2H32–5	6.25	13.6	13.8	19.0	S_5	3H88	15	3.125	6.1 (0–11.9)	7.6 (0–10.3)
S_4	2H34–4	6.25	5.1	10.3	21.2	S_5	3H5	12	3.125	3.0 (0–5.1)	6.0 (0–10.3)

Aardevo Exhibit 1010
Page 0967

Discussion

There have been various studies on the inbreeding of diploid cultivated potatoes using self-compatible variants (De Jong and Rowe 1971; Olsder and Hermsen 1976; De Jong 1977; Hermsen 1978; Birhman and Hosaka 2000; Phumichai et al. 2004). However, to our knowledge, inbreeding has never reached beyond S_4, and this is the first report that self-fertile S_5 diploid inbreds with more than 90% homozygosity were produced. This demonstrated that highly homozygous and seed-propagated diploid potatoes could be obtained using the *Sli* gene. Because of the simple procedure (crossing with a *Sli* gene donor, then continued selfings), the *Sli* gene method may be a better alternative in obtaining such inbreds. In this study, we paid little attention to tuber yield because most self-pollinations were made on seedlings. Nevertheless, some S_4 plants in inbred series A produced considerable tuber yields (data not available). Thus, these advanced inbreds may be valuable genetic stocks for studying the mechanism of heterosis and for producing hybrid variety at the diploid level.

The frequencies of plants that were self-pollinated and actually selfed decreased constantly in inbred series A. Since the parental clone 97H32–6 was considered to be a *Sli* gene homozygote (Phumichai et al. 2004), the decrease of the selfer frequency seems solely due to the reduction in general fertility, as demonstrated by increasing frequencies of plants that did not flower or produce pollen. In comparison, inbred series B maintained relatively constant frequencies of flowering plants and selfers throughout selfing generations, which might be explained partly by a strong selection pressure for fertility in the initial stage (4.1% were selected before selfings started). However, the fact that only 1 of 7 cross combinations reached S_5 generation indicates that serious loss of fertility is typical in most families. Thus, inbreeding depression, typically expressed as reduced pollen viability and tuber yield (Krantz 1924, 1946; Mendiburu and Peloquin 1977; Ross 1986; Golmirzaie et al. 1998*a*, 1998*b*), will certainly limit continued selfing of the present diploid inbreds.

Continuous selfing may eliminate undesirable recessive alleles as they become homozygous and may in turn lead to the recovery of fertility with advancing generations (Busbice 1968; Pfeiffer and Bingham 1983; Ray and Bingham 1992). This expectation is not supported by the present study, because there was no indication of fertility recovery even at the S_5 generation. Fertility-related traits, such as flowering or nonflowering, pollen shedding, pollen fertility, self-compatibility, and seed fertility appear to be under polygenic control except for self-compatibility, which is controlled by the *Sli* gene. A large population would be necessary to simultaneously select desirable gene combinations and eliminate undesirable genes. Alternatively, a slower approach to homozygosity using sib mating or composite sibbing of inbreds may permit more effective selection before complete fixation occurs (Kinman 1952).

The observed average percentage of reduction of heterozygosity per generation (38.4% or 38.5%) was lower than the theoretically expected value of 50%. The slower than expected decline in heterozygosity has been reported by Brouwer and Osborn (1997) with inbreeding diploid alfalfa and by Scotti et al. (2000) with inbreeding tetraploid alfalfa. Consequential selection for vigor and fertility during selfing has been suggested as the cause (Brouwer and Osborn 1997). We previously suggested that reproductive traits, such as flowering versus nonflowering and the degree of pollen production, were strongly correlated with heterozygosity, and selection for fertility favored the more heterozygous plants (Phumichai et al. 2004). Pollen performance might also be involved because the level of heterozygosity in the sporophyte affects the phenotypic expression of the gametophyte or pollen viability (Ortiz and Peloquin 1994; Stephenson et al. 2001). In this study, however, the levels of heterozygosity of the parental clones (selfers) were not always higher than the population means. Thus, other factors, such as seed germination ability and vegetative vigor, could have been involved in unintentional selection.

In conclusion, heterozygosity might be correlated with both fertility and viability, which agrees with numerous inbreeding studies (Krantz 1946; Wilsie 1958; Jugenheimer 1976; Ray and Bingham 1992). However, there was no apparent indication for the presence of an exclusively heterozygous locus or chromosome section, which is possibly related to plant survivability. Thus, further continuous selfings, if possible, would bring about near pure lines: 99% homozygosity at S_{10}, based on a theoretical calculation from the observed 38.4%–38.5% reduction rate in heterozygosity, or at S_7, based on the theoretical 50% reduction rate.

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Page 0969

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Page 0970

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	Attorney Docket Number	VONL:004USC1

/R.T.B/	1	Response to Non-Final Office Action regarding U.S. Application No. 14/112,747 (Atty. Dkt. No. VONL:006US), dated July 16, 2020.	☐

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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

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Name/Print	Amy Dunstan	Registration Number	61069

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Research Article Open Access

Michiel de Vries*, Menno ter Maat and Pim Lindhout

The potential of hybrid potato for East-Africa

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Abstract: Potato is an important staple crop in East Africa. Most of the seed tubers are propagated in informal systems, whereby the tubers become deteriorated and contaminated, resulting in low crop yields. Potato breeding has not resulted in significantly improved varieties that overcome these constraints. Recently, true potato hybrid breeding technology has been developed, whereby diploid hybrids are generated by crossing between homozygous inbred parent lines. The first series of experimental hybrids were pre-screened in The Netherlands and ten representative hybrids were tested in East-Africa, whereby the yield of the best hybrid was 29 ton/ha. These results show the great potential of hybrid potato for East Africa.

Keywords: Hybrid breeding, true seed, genetics, ploidy, cropping system, yield

1 Introduction

Potato is the world's third food crop for human consumption, and is grown all over the world. Breeding of new cultivars is done by national public institutes, by the international Potato Centre (CIP) or by private industries, mainly in Europe. Despite a growing worldwide number of more than 4000 cultivars (http://cipotato.org/potato/facts/), still very old varieties like Russet Burbank (introduced in 1876) in USA and Bintje (introduced in 1910) in Europe, are cultivated. The lack of genetic progress is also reflected in the lack of yield gain over the past century (Douches et al., 1996).

In East-Africa, potato is an important staple crop, with doubled production area over the last 20 years. Mainly CIP varieties and some European varieties are grown. The vast majority of potato is cultivated by millions of small scale farmers, who use farm saved seeds or acquire seed tubers from local markets (Gildemacher et al., 2009). The average yield in East African countries is low and ranges between 5 and 15 ton/ha (Gildemacher et al., 2009; Janssens et al., 2013; FAOstat, 2016). The propagation, storage and distribution of high quality seed tubers is the main constraint that limits production. Therefore, there is a great need for high quality seed tubers or true seeds.

There are several techniques to generate high quality mini-tubers from clean *in-vitro* grown potato plantlets (Tierno et al., 2014). This requires a formal potato production chain as these technologies are applied in specialised companies or public institutes and several rounds of clonal propagation are used before the seed tubers are distributed to the farmers. During these rounds of clonal propagation, the seed tubers may become contaminated with pathogenic micro-organisms. This is even more harmful when farmers keep tubers for next season as the costs of formal seed tubers may be too high for refreshment at each cultivation cycle (Kaguongo et al., 2008). This will further deteriorate the quality of the seed tubers (Thomas-Sharma et al., 2016).

Recently, Solynta has developed a true hybrid breeding system in diploid potato, whereby homozygous parent lines are developed by several rounds of inbreeding and hybrid cultivars are generated by crossing these parent lines (Lindhout et al., 2011). This method enables the creation of true seeds that are genotypically uniform. The starting material was an interspecific cross between diploid potato and the wild species *S. chacoense*, that was used to overcome the self-incompatibility (Lindhout et al., 2011). It took many rounds of crossing, selfings and selections to increase the performance of the inbred lines and to generate the first experimental hybrids (Lindhout et al., 2017). This paper describes the first trials with these experimental hybrids in tropical highlands, in Congo DR. The comparison of hybrid performance in Congo and The Netherlands is made. The results show the potential of hybrid potato for East-Africa.

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2 Materials and methods

2.1 Genetic material

Inbred lines of diploid potato were developed in a dedicated diploid breeding programme as described by Lindhout et al. (2011; 2017). In winter 2014/15 the first series of 203 hybrid crosses were made. Typically, the parents of these hybrids were obtained after several rounds of crossing and selfings followed by at least three rounds of selfing. As an example, the pedigree of hybrid SOL1-7 is shown in Figure 1. From this figure, it becomes clear that the three heterozygous diploid founders (D1, D2 and D16) were crossed with the *Sli*-donor DS, to create self-compatible populations (Lindhout et al., 2011). Subsequent generations of selfing resulted in homozygous parents of the hybrid SOLH1-7. Ten hybrids were selected as representatives for this series of 203 hybrids for further testing in DRC and designated SOLH1 - SOLH-10.

2.2 Selection of potato hybrids in The Netherlands

In 2015, the very first series of 203 experimental diploid potato hybrids were grown in The Netherlands to produce seed tubers. On May 1st, 2015, 100-200 seeds of these hybrids were sown in seed-boxes substrate in a heated greenhouse with 16 hours' day length. The seedlings were transplanted in Est, The Netherlands on June 2nd 2015, in irrigated ridges at 30 cm spacing within a ridge and 75 cm distance between the ridges in one replicate with plant numbers dependent on the number of seedlings available. Soil type was a heavy fluvial clay soil (28% clay). A typical crop management scheme was applied: fertilization and crop protection was done as recommended. Aphids were controlled by weekly chemical treatments combined with mineral oil. The first two weeks after transplanting overhead irrigation was applied two times to protect the seedling against drying out. Tubers were harvested on September 25th, two weeks after chemical defoliation of the plants by Reglone® (diquat dibromide). The tuber weight was evaluated per plot.

2.3 Field trial with potato hybrids in DRC

The experimental site was located at Nioka, Albertine Rift in Ituri Province, Democratic Republic of Congo at 1680 m.a.s.l. The site was chosen for its representativeness for the potato growing environment in the highlands of East-Africa. On January 25th, 2016, 200 seeds of the ten selected experimental hybrids, designated SOLH1-SOLH-10, were sown in seed-boxes with a substrate composed of goat manure, straw, ash and sand. The seed-boxes were placed

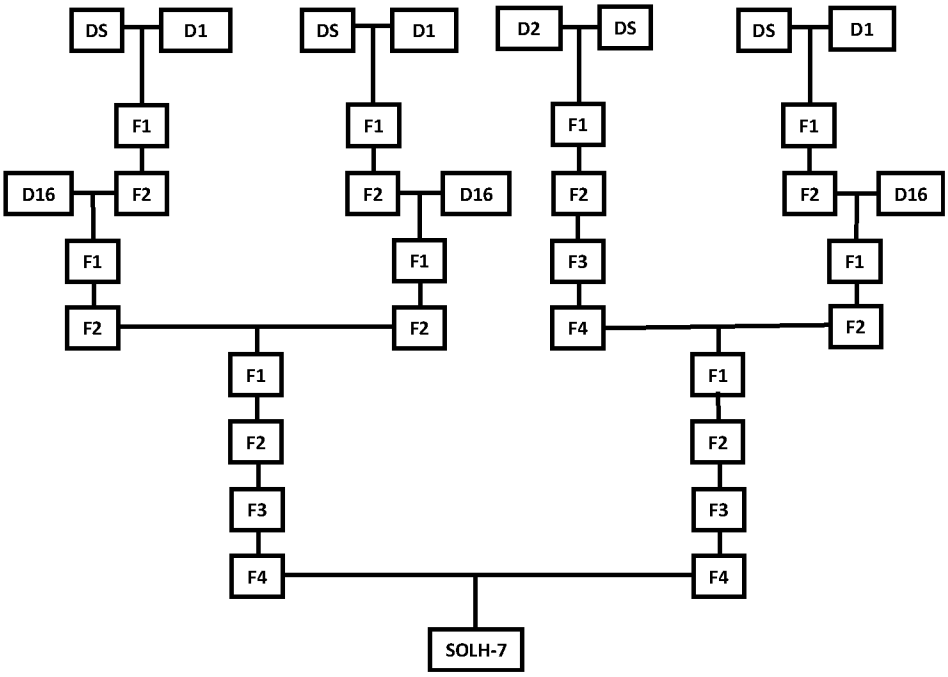

Fig 1. A typical example of an experimental diploid potato hybrid "SOLH-7", obtained by various crossing among the diploid founders D1, D2 and D16 and the Sli-donor DS (see Lindhout et al., 2011)

in an open shed, with a roof to protect against the sun and nets to protect against insects. The seedlings were transplanted on March 10th, in irrigated ridges, with 30 cm spacing within a ridge and 75 cm spacing between the ridges. The experiment was planted in three non-random replicates of 18 to 39 plants per plot, depending on the number of plants available. During the first two months after transplanting hand watering was done to protect the seedlings against drying out. Tubers were harvested on July 1st, when all plants had naturally died. The tuber weight and tuber number per plant was evaluated per plot.

3 Results

The seedlings of the first series of 203 experimental diploid potato hybrids were grown in the field in the Netherlands and evaluated for yield. The germination rate was 74 to 99%. After emergence, 6-weeks old seedlings were transplanted in the field. The growth of the field-plants was artificially stopped after 116 growing days. The tuber yield per plant varied from 0 to 580 gr, which is equivalent to 0 to 26 ton/ha (not shown). A representative set of ten hybrids was selected for trialing in DRC and designated SOLH-1 to SOLH-10.

In many regions in Africa, the potato yield, starting from seed tubers, is only 5 to 10 tons/ha. The greatest constraint is the quality of the seed tubers. True potato seeds are completely free of contaminating pathogens and may solve the lack of good potato starting materials. Therefore, a field trial starting with true seeds of the ten selected experimental hybrids was done in Nioka, Congo DR.

The germination rate after two weeks was on average 64%, ranging from 54 to 76%. After transplanting, a total of 1320 seedlings developed into plantlets, that were between 5-8 cm tall and had on average 8 true leaves at transplanting.

All available seedlings were transplanted into ridges according the standard cultivation techniques, resulting in plot sizes of 110 to 140 plants. Irrigation was performed during the first two months after transplanting but not during the last two months. Visual observation of damage was noted and 54 cases of insect damage during the growing season were noted, assessed as different types of crickets and ants. As there was no rain from 90 days after transplanting all plants were dead at the beginning of the harvest at 113 days after transplanting into the field.

The tuber yield ranged between 167 (SOLH-8) to 640 grams (SOLH-7) per plant, which is equivalent to 8 to 29 ton/ha (Fig 2). Tuber number ranged from 11 (SOLH-5) to 37 (SOLH-7) per plant, with 17 to 48 gr/tuber (not shown). SOLH-1 had the highest number of tubers in the 40-60mm class: three per plant.

The yield data of the same genetic materials at two locations allow to study the genotype x environment (GxE) interaction. Therefore, we compared the yield in gr/plant between the field trial in Democratic Republic of Congo and in The Netherlands (Fig 3.). Despite the large

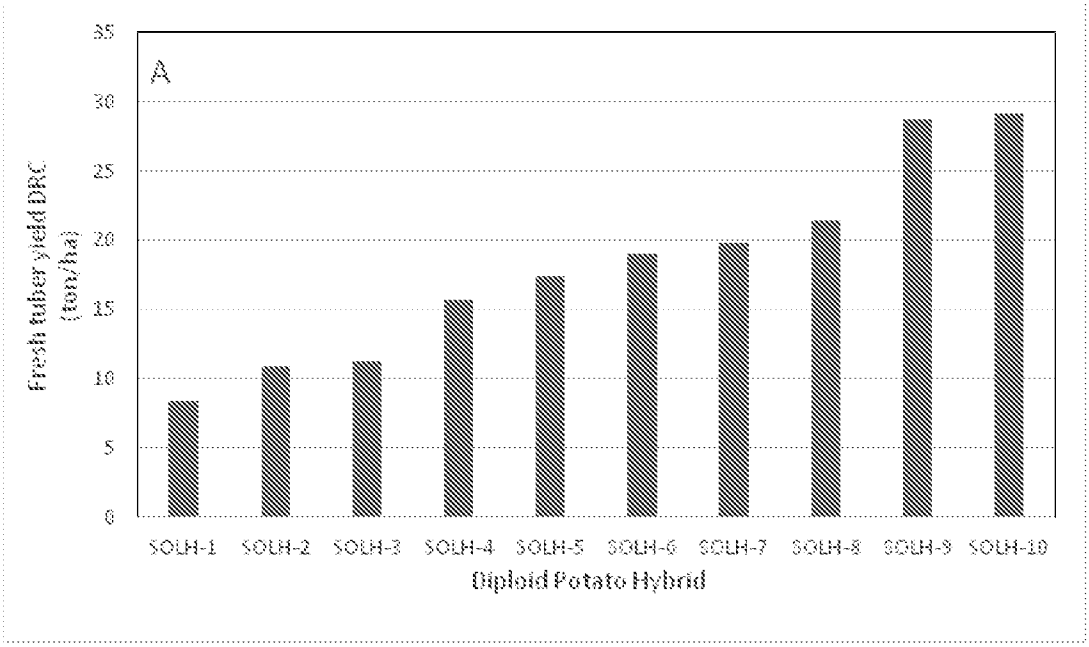

Fig 2. Yield data of ten experimental diploid potato hybrids, grown from seedlings in a field trial in Nioka, DR Congo

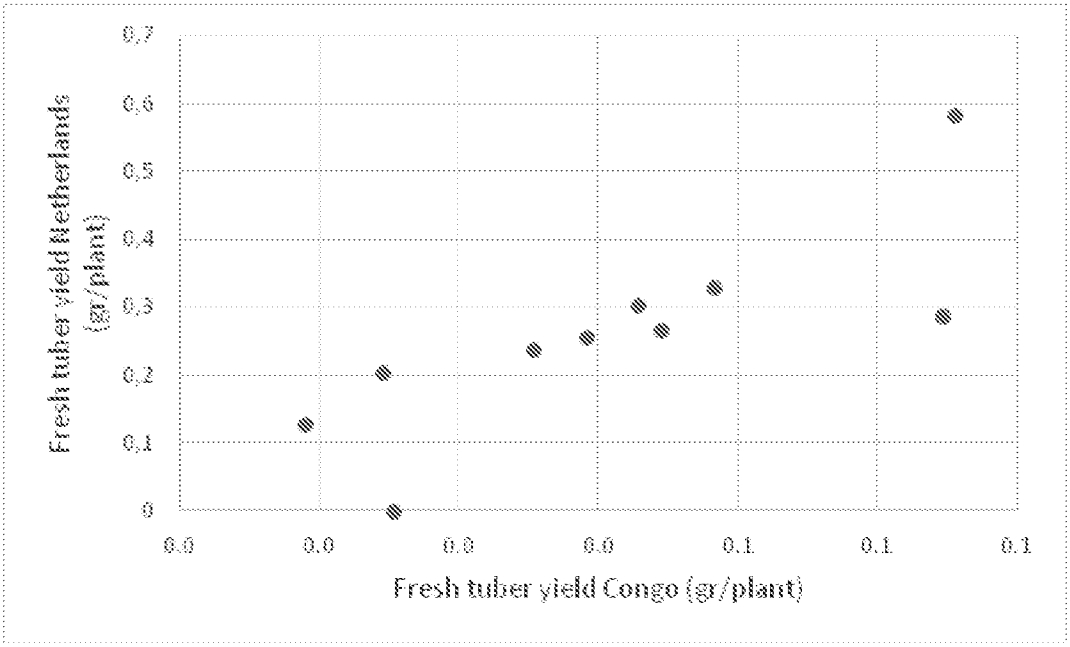

Fig 3. Correlation between the yield in gram/plant of ten diploid experimental potato hybrids in Congo DR compared to The Netherlands

difference in climatic conditions, geographical location and agricultural practices at both locations, we observed a striking correlation between both field tests, whereby the top five hybrids and the bottom five hybrids in both locations were identical.

4 Discussion

This paper describes the very first trial with the very first experimental diploid potato hybrids, generated from advanced inbred lines, reported ever. Before 2014, no dedicated crosses were made to generate experimental hybrids. One of the advantages of hybrid seeds as the number of seeds produced in the first cross as usually 50 to 250 seeds are collected and this number is sufficient to test the performance at multi-locations. In the present study two very distant locations in the world were chosen as the first testing sites for hybrids grown from seeds.

The parents of the hybrids were only selfed for three generations after the last breeding cross (Fig. 1). So, these parents may contain 10 to 20% heterozygous loci. Giving the very large genetic variation in potato (PGSC, 2011; Uitdewilligen et al., 2013; Visser et al., 2014), these parents may show phenotypic segregation for many traits. The main aim of the present study is to evaluate the yield of experimental hybrids and therefore we paid little attention to other traits that were expected to segregate.

The average yield in East Africa ranges from 5 ton/ ha in Uganda to 14 ton/ha in Kenya (FAOstat, 2016). The diploid potato hybrids yielded 8 to 29 ton/ ha in DRC, even higher than in The Netherlands with max. yield of 26 ton/ha. This higher yield in DRC is most likely due to the more favourable weather conditions at the tropical highlands, where the temperature is ideal for potato cultivation, while the harsh spring in The Netherlands is often too cold for potato plant growth.

Compared to the average yield in East Africa of 7 to 15 ton/ ha, the yield of the highest yielding potato hybrids was 29 ton/ha. This extra yield may also be due to optimisation of the cultivation conditions with such as fertilization and irrigation. The tuber number was high, up to 37, while the tuber size was small (17-48 gr/tuber), compared to 50-100 gr for usual tuber weight range (Struik & Wiersema, 2012). Extension of the growing period by irrigation could be a way to increase yield. Further research with tubers raised hybrid plants and tuber raised controls is needed to better dissect the cultivation conditions with the genetics, combined with the effect of clean seeds.

The yield of the highest yielding diploid hybrid in The Netherlands was about half of the yield of seed tuber production of 35 - 40 ton/ha (FAOstat, 2016). So, the highest yielding hybrid, grown from seedlings, showed about half the yield of a commercial tetraploid hybrid, grown from seed tubers.

It is known that seed tubers (raised from tubers) give higher yields than seedling transplants (raised from seeds) (Almekinders et al., 1996; Muthoni et al., 2014). In addition, diploids show lower yields than tetraploids

(Hutten et al., 1994), though interspecific diploid progenies may show high yields as well (Jansky et al., 2016). In this study, we introduce the first diploid potato hybrid. The genetic origin of this breeding system is an interspecific cross between *S. tuberosum* and *S. chacoense* (Lindhout et al., 2011; Fig 1.). The plants of the early selfed progenies were extremely weak (Lindhout et al., 2017) and we did not expect that the very first experimental diploid potato hybrid already have high yields. However, a yield of 50% of the best hybrid grown from seeds compared to the yield of tetraploid seed tuber starting from seed tubers is remarkably high.

The 50% lower yield is due to three confounded factors: diploid versus tetraploid, experimental hybrid versus commercial cultivar and seedlings versus seed tubers. Further tests with tuber grown plants and tetraploid experimental hybrids are needed to dissect these factors.

These results illustrate the potential of diploid hybrid potato in combination of good cultivation practises. In the present study, the cultivation period in DR Congo was restricted by the lack of rainfall. We expect that with a better irrigation system or during the rainy season the yield of these hybrids would have been even higher. In addition, these were the very first hybrids generated by crossing parents without any a prior information about general combining abilities (GCA) of the parent lines. The present results are being used to select parent lines based on high GCA values. New parent lines will also be more inbred and hence more homozygous and more uniform. As a result, the next series of hybrids, is expected to be more advanced, both in terms of tuber yield, tuber quality as well as tuber uniformity.

In addition, by using marker assisted backcrossing programmes a new series of hybrids with new traits can be obtained such as disease resistances to *Phytophthora infestans* and tuber quality (Lindhout et al, 2017). Finally, locally popular tetraploid varieties such as Kinigi in Rwanda could be exploited for a dedicated diploid hybrid potato programme for East Africa to develop a hybrid, that is optimally adapted to the climate conditions and market preferences in East Africa. However, such an effort is to start a breeding programme anew, therefore it may take ten years to have the first hybrid released to the market.

In conclusion, hybrid potato has shown a great potential and will pave the way to a prosperous potato food chain that will contribute to feed the present and future fast growing populations in East Africa.

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Aardevo Exhibit 1010
Page 0979

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Genetic Resources (Including Wild and Cultivated *Solanum* Species) and Progress in their Utilisation in Potato Breeding

J. E. Bradshaw · G. J. Bryan · G. Ramsay

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Abstract The genetic resources available for the improvement of the cultivated potato (*Solanum tuberosum*) are reviewed along with progress in their utilisation. The conclusions are as follows. The wild and cultivated species of potato have been utilised in potato breeding to good effect, but only a very small sample of the available biodiversity has been exploited. New knowledge and technology will open possibilities for much greater use of these genetic resources in breeding. The strategy for utilising the cultivars native to Latin America will either be the introgression of desirable genes or the direct use of parents from improved populations, depending on how far modern *S. tuberosum* cultivars have genetically diverged from them and the extent to which *S. tuberosum* cultivars have been improved in the process. Molecular marker-assisted selection will be used for faster introgression of desirable genes from wild species, and the possibility exists of moving genes directly from wild species to cultivated potato with transgenic methods. New cultivars will continue to come from crosses between pairs of parents with complementary features but adapted to local growing conditions. However, increasingly these parents will possess desirable genes which have been introgressed from wild species and may also be from complementary groups of cultivated germplasm to exploit hybrid vigour. Successful cultivars may be genetically modified, if consumers see benefits in the use of the technology, to introduce genes not present in cultivated potatoes and their wild relatives to achieve novel biochemistry and further desirable improvements.

Keywords breeding · genetic base broadening · genetically modified potatoes · germplasm collections · introgression · marker-assisted selection

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Aardevo Exhibit 1010
Page 0981

Introduction

Farmers and consumers of potatoes will, no doubt, judge the achievements of potato breeding by how well currently available cultivars meet their needs. Potato breeders, however, should also consider how effective they have been in utilising the biodiversity available to them in cultivated potatoes and their cross-compatible wild relatives. This paper reviews the biodiversity that has been used in the development of the modern potato and then considers the prospects for more extensive utilisation of available germplasm in the future. This is certainly going to be required to produce the new cultivars needed for sustainable increases in potato production during a period of environmental (including climate) change and human population growth. Finally, there is a brief discussion of the need to use the genetic modification of successful potato cultivars to introduce genes not present in cultivated potatoes and their wild relatives.

Domestication and Evolution of the Modern Crop

Wild tuber-bearing *Solanum* species are distributed from the south western United States (38°N) to central Argentina and adjacent Chile (41°S) and cover a great ecogeographical range (Spooner and Hijmans, 2001). They form a polyploid series from diploid ($2n = 2x = 24$) to hexaploid ($2n = 6x = 72$) in which nearly all of the diploid species are self-incompatible outbreeders and the tetraploids and hexaploids are mostly self-compatible allopolyploids that display disomic inheritance (Hawkes, 1990). Crossability groups occur, each defined by an endosperm balance number (EBN), although interspecific pollen-pistil incompatibility and nuclear-cytoplasmic male sterility can occur (Camadro et al., 2004). The taxonomy of tuber-bearing *Solanum* species is currently under revision and the number of wild species has been reduced and is likely to be further reduced in the future (Spooner and Hijmans, 2001) from the 219 recognised by Hawkes (1990), and grouped into 19 series (Fig. 1).

Simmonds (1995) concluded that just a few closely related species in series Tuberosa (e.g., *S. brevicaule*, *S. leptophyes* and *S. canasense*) were domesticated in the Andes of southern Peru and northern Bolivia about 7000 years ago. More recently, Spooner et al. (2005a) have provided evidence for a single domestication in Peru from the northern group of members of the *S. brevicaule* complex of species. The member species of this group are poorly defined and may be reduced to a single species of which *S. bukasovii* is the earliest valid name. The result of domestication was diploid *S. stenotomum*, also referred to as a form of *S. tuberosum* (Group Stenotomum), from which other cultivated species were derived, including diploid *S. phureja* (or Group Phureja), tetraploid *S. tuberosum* subsp. *andigena* (or Group Andigena) and tetraploid *S. tuberosum* subsp. *tuberosum* (or Group Tuberosum). Phureja potatoes were selected from Stenotomum for lack of tuber dormancy and faster tuber development so that up to three crops per year could be grown in the lower, warmer, eastern valleys of the Andes. Andigena potatoes became the most widely grown form in South America, presumably because Andean farmers found them superior to the diploids for yield and other traits. Tuberosum potatoes were selected from Andigena types for tuber production in long days in coastal Chile and are referred to as Chilean Tuberosum. The involvement of wild species in the

Aardevo Exhibit 1010
Page 0982

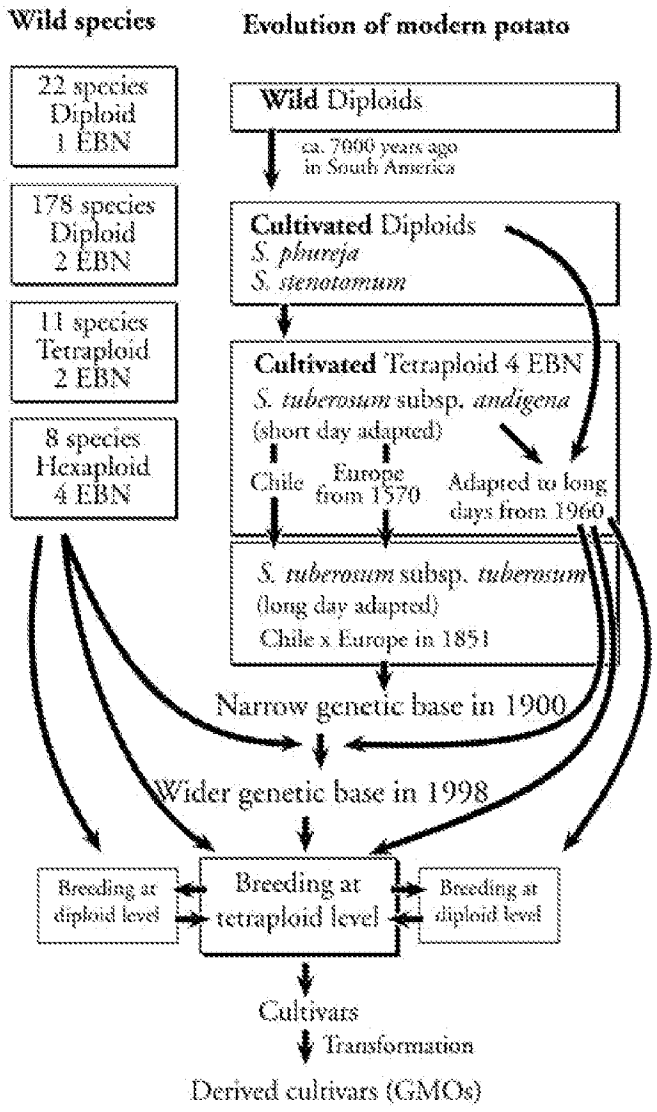

Figure 1 Genetic resources and their utilisation in potato breeding.

emergence and selection of Andigena and Chilean Tuberosum potatoes is still a matter of debate, but they are genetically distinct groups of potatoes with different cytoplasms (Raker and Spooner, 2002; Hosaka, 2004), although Huaman and Spooner (2002) regard all cultivated potatoes as one taxon.

Potatoes (*S. tuberosum*) were introduced into Europe in the 1570s, and then starting in the 17th century, they were taken from Europe and cultivated in many other parts of the world (Hawkes and Francisco-Ortega, 1993; Pandey and Kaushik, 2003). It is likely that the early introductions came from both the Andes and coastal Chile but were few in number and hence captured only some of the biodiversity present in South America (Hawkes, 1990; Spooner et al., 2005b). It is therefore remarkable that today potatoes are grown in 149 countries from latitudes 65°N to 50°S and at altitudes from sea level to 4000 m (Hijmans, 2001), and that the potato is the fourth most important food crop after wheat, maize and rice (Lang, 2001). The distribution reflects the adaptation of *S. tuberosum* first to the short summer days of the highland tropics and sub-tropics, then to the long summer days of lowland

Aardevo Exhibit 1010
Page 0983

temperate regions, and finally to the short winter days of the lowland sub-tropics and tropics. The distribution also reflects growing conditions that are neither too hot nor too cold and where there is adequate water, whether from rain or irrigation. Potatoes have also been adapted to a wide range of end uses. As well as being a staple food, the potato is grown as a vegetable for table use, is processed into French fries and crisps (chips) and is used for dried products and starch production. New uses can be anticipated for the future such as designer starches (Davies, 1998) and bioreactors for biopharmaceuticals (Sonnewald et al., 2003).

Reproductive Biology of Potato

The adaptation of the potato to a wide range of environments and end uses was helped by its reproductive biology that is ideal for creating and maintaining variation. Potatoes flower and set true seed in berries following natural pollination by insects, particularly bumble bees. Outcrossing is enforced in cultivated (and most wild) diploid species by a gametophytic self-incompatibility system (Dodds, 1965). Whilst this system does not operate in *S. tuberosum* because it is a tetraploid, 40% (range 21 to 74%) natural cross-pollination between plants was estimated to occur in subsp. *andigena* in the Andes (Brown, 1993) and 20% (range 14 to 30%) in an artificially constructed Andigena population (Glendinning, 1976). This sexual reproduction creates an abundance of diversity by recombining allelic variants of genes which arose by mutation, and overcomes the inbreeding depression which would develop on selfing. The genetically unique seedlings that grow from true seeds produce tubers that can be replanted as seed tubers, and hence distinct clones can be established and maintained by asexual vegetative reproduction. No doubt many Andean cultivars were produced by farmer selection from naturally occurring variation. Domestication must have involved selection of less bitter and hence less toxic tubers and Andean farmers certainly retained a much wider variety of tuber shapes and skin and flesh colours than seen in wild species (Simmonds, 1995). Subsequent selection for early maturity, appropriate dormancy and resistance to abiotic and biotic stresses must have occurred in many environments, both in South America and worldwide.

Today breeders normally raise seedlings from crosses between pairs of parents with complementary traits in order to find the best genotype in the best progeny to clonally (vegetatively) propagate as a new cultivar. However, there are circumstances where cultivars based on true potato seed (TPS) propagation are an attractive proposition despite being genetically variable and inferior to the best genotype that exists within the progeny. In the torrid zones of the lowland tropics and subtropics, reduced seed costs (because of much smaller amounts of planting material required), flexibility of planting time (no physiological age of seed tubers) and freedom from tuber-borne diseases (particularly viruses, but not viroids) can outweigh the difficulty of establishing the crop, later maturation and less uniformity (Golmirzaie et al., 1994). None of the current breeding methods can deliver genetic uniformity and hence all of them involve selection for acceptable phenotypic uniformity, as well as high yield and other desirable traits. Methods and progress have been reviewed by Golmirzaie et al. (1994), Almekinders et al. (1996), Ortiz (1997) and Simmonds (1997). In essence, they utilise the same genetic resources and the same kind of progenies as breeding aimed at clonal propagation.

Aardevo Exhibit 1010
Page 0984

Cultivars Native to Latin America

The cultivars native to Latin America, are both an example of the utilisation of diversity since domestication and a valuable resource for the future. Following its creation in 1971, the International Potato Centre (CIP) in Peru assembled a potato collection of more than 15,000 accessions of native potato cultivars from nine countries in Latin America (Argentina, Bolivia, Chile, Colombia, Ecuador, Guatemala, Mexico, Peru and Venezuela). CIP identified those which were duplicate accessions of the same cultivar, and selected 3527 individual cultivars among them of which 552 were diploids, 128 triploids, 2836 tetraploids (2644 subsp. *andigena*, 144 subsp. *tuberosum* and 48 hybrids) and 11 pentaploids (Huaman et al., 1997). By 1997, researchers at CIP had already conducted 46124 evaluations on the collection for the reactions of cultivars to biotic and abiotic stresses and for other desirable traits. Then to aid utilisation, Huaman et al. (2000a) used cluster analysis of morphological data to establish a core set of 306 accessions from the 2379 cultivars of subsp. *andigena* still held at CIP. The set was chosen to represent the widest morphological diversity and to maximise geographical representation. Evaluation data were taken into account in choosing the representative accession of each cluster. Isozyme analysis of the entire collection confirmed that the core collection had captured a representative sample of the alleles at nine allozyme loci with only the loss of rare alleles whose frequency was less than 0.05% (Huaman et al., 2000b). However, a simulation study revealed that a core collection size of 600 would be required to adequately represent allele frequencies and locus heterozygosity (Chandra et al., 2002). The core collection should nevertheless be a valuable base for detailed evaluation and future breeding. Sukhotu et al. (2005) used molecular markers to study the genetic diversity in Andigena potatoes and found no clear cut evidence for genetically differentiated subpopulations, although there was a geographical trend in nuclear DNA marker differentiation from north to south along the Andes. Interestingly, the Chilean Tuberosum included in the study showed the highest similarity to Andigenas from Mexico and Guatemala rather than from north Argentina. This kind of information should prove valuable in the utilisation of the CIP core collection for breeding both in South America and worldwide.

Modern Potato Breeding and Cultivars

Present day cultivars not native to Latin America are an example of progress in utilisation since the potato was introduced to Europe over 400 years ago. They are also the foundation for future breeding and utilisation. Potato breeding in the modern sense began in 1807 in England when Knight made deliberate hybridisations between varieties by artificial pollination (Knight, 1807). It flourished in Britain and elsewhere in Europe and North America during the second half of the 19th century when many new cultivars were produced by farmers, hobby breeders and seedsmen, and has continued to the present day. The South American cultivar Daber was introduced into Germany in 1830, most probably from Chile (Plaisted and Hoopes, 1989). Likewise, a single Chilean Tuberosum cultivar, Rough Purple Chili, was introduced into the USA in 1851 (Goodrich, 1863), and North America's most popular potato cultivar, Russet Burbank, was derived from it by three

Aardevo Exhibit 1010
Page 0985

generations of open pollination with selection, and released in 1914 (Ortiz, 2001). The descendents of Rough Purple Chili were widely employed as female parents in crosses with European Tuberosum at the end of the 19th century. For example, the first two cultivars to be bred at the Scottish Plant Breeding Station, the Alness and Craigs Defiance, respectively had Abundance and Epicure as a parent and these were descended from Rough Purple Chili via Early Rose. Hence Chilean Tuberosum cytoplasm predominates in modern cultivars (Provan et al., 1999), and this uniformity has been a cause for concern since the epidemics of Southern corn leaf blight on maize hybrids with T (Texas) cytoplasm in 1970–71 (Ullstrup, 1972). Although there has been a frequent exchange of germplasm between Europe and North America, Braun et al. (2004/5) were able to use molecular markers to identify Anglo-American, Central European and East European cultivars as three distinct but overlapping groups which reflect breeding history and differences in selection priorities. Modern potato breeding started later in the 1930s in China and India but these countries are now two of the leading potato producers in the world (Gaur and Pandey, 2000; Jin et al., 2004). The 2005 edition of the World Catalogue of Potato Varieties (Hils and Pieterse, 2005) is currently able to list over 4000 cultivars from more than 100 countries, a remarkable achievement, since outside of South America the genetic base of potato breeding must still be considered a relatively small sample of potatoes from the Andes and coastal Chile. Some introgressions of disease resistance genes and some base broadening during the 20th century do not alter this conclusion (Bradshaw and Mackay, 1994).

The main route to new cultivars will, no doubt, continue to be traditional breeding involving crosses between pairs of parents with complementary features. Increasingly, however, parents will have genes introgressed from wild species and they may also be from complementary groups of germplasm in order to exploit heterosis, as discussed in the next section. Furthermore, as genetical knowledge increases, parents will be chosen on the basis of their genotypes and breeders will be able to design better breeding programmes.

Use of Cultivated Species from South America in Base Broadening

During the second half of the 20th century recognition was given to the narrow genetic base of European and North American breeding programmes and CIP, on its establishment, also recognised the need to make broad-based germplasm from South America available to National Programmes in developing countries, particularly germplasm with durable resistance to late blight.

Attempts were made to widen the genetic base of European and North American potatoes through the creation of broadly based populations of long-day adapted *S. tuberosum* subsp. *andigena* (Neotuberosum) and long-day adapted *S. phureja/S. stenotomum*. Since the first experiment started by Simmonds in 1959 (Simmonds, 1969), a number of programmes worldwide have demonstrated that, through simple mass selection under northern latitude, long-day summer conditions, subsp. *andigena* will adapt and produce parents suitable for direct incorporation into modern potato breeding programmes (Bradshaw and Mackay, 1994). Furthermore, under long days, Tuberosum × long-day Andigena/Neotuberosum hybrids have shown yield advantages of 13–17% over Tuberosum × Tuberosum hybrids

Aardevo Exhibit 1010
Page 0986

(Glendinning, 1969; Cubillos and Plaisted, 1976; Tarn and Tai, 1983; Maris, 1989), but it appears that the extent of heterotic advantage is dependent on day length (Cubillos and Plaisted, 1976). Likewise, during the period 1962–1979, Carroll (1982) employed a mass selection method to produce a population of *S. phureja/S. stenotomum* adapted to long-day North European conditions. Direct hybridisation of members of this improved diploid population with tetraploid potato cultivars via unreduced pollen grains ($4x \times 2x$ crosses) resulted in tetraploid hybrids, some of which were superior to standard tetraploid cultivars in both total and marketable yield (Carroll and De Maine, 1989). Similar work has been done in North America (Plaisted and Hoopes, 1989; Haynes and Christ, 1999; Haynes and Lu, 2005). The tetraploid offspring from Tuberosum ($4x$) $\times$ Tuberosum/Phureja hybrids ($2x$) have shown yield advantages of 27% over their $4x$ parents and controls (Tai and De Jong, 1980), but in a more recent study (Buso et al., 1999), a 15% yield advantage occurred only in the higher yielding of two environments. Using molecular markers and Tuberosum, Neotuberosum and Phureja germplasm in the Cornell University potato breeding programme, Bonierbale et al. (1993) found that specific combinations of individual marker fragments were more important than maximum heterozygosity for tuber yield heterosis.

However, to date, relatively few clones of Neotuberosum and long-day *S. phureja/S. stenotomum* have been used to any extent in the breeding of modern cultivars. Part of the reason for this is that whilst adaptation to tubering in long days was quickly achieved, other problems remained. Neotuberosum clones lacked the regularity of tuber shape of intensively selected subsp. *tuberosum* clones, and long-day adapted *S. phureja* clones lacked tuber dormancy. Hence these populations need, and are receiving, selection for further improvements in order to achieve the original goal of direct use as parents in breeding finished cultivars (i.e., contributing 50% of their genes to their offspring, or 25% in backcrosses), as suggested by Tai (1994) for *S. phureja*-based $2x$ parents. The improved populations should be compared with CIP's core collections to see how many common alleles have been captured in the long day populations, and hence whether or not there is a need to create and select new long day adapted populations that capture more biodiversity. These could then be used either for further base broadening or as sources of desirable alleles for marker-assisted introgression. A question that needs answering is how inferior to modern cultivars do such populations have to be for targeted gene transfer to be a better strategy than base broadening? Either way, in the future, given the biodiversity available, crosses of subsp. *tuberosum* with Neotuberosum, subsp. *andigena* and *S. phureja/S. stenotomum* should result in higher yields, better table and processing quality, improved flavour and nutritional value and some useful disease resistances.

Probably the best known broad-based germplasm from CIP's breeders are the B populations which they have developed from 1988 onwards. The aim was to help the development of improved cultivars for a wide range of environments that possess high and stable levels of resistance to late blight in combination with resistances to viruses and suitable culinary and processing quality (Trognitz et al., 2001; Landeo, 2002). Briefly, there were three groups of material differing in their genetic base and degree of enhancement. GroupB1 was native cultivated forms of short-day-adapted *S. tuberosum* subsp. *andigena* lacking the major Rgenes which had failed to give durable resistance, GroupB2 comprised crosses of the subsp. *andigena* clones with

Aardevo Exhibit 1010
Page 0987

subsp. *tuberosum* clones that also lacked Rgenes, and GroupB3 comprised subsp. *tuberosum* germplasm with *S. demissum* derived resistance, subsp. *andigena*, Neotuberosum and complex hybrids of *S. acaule* and *S. bulbocastanum* with *S. phureja* and *S. tuberosum* subsp. *tuberosum* (ABPT's, see later). The breeding involved recurrent selection for quantitative resistance to late blight and other desirable traits together with selection in those geographical areas where the new cultivars were to be grown, for example in East Africa (Mulema et al., 2004/5). Clones with good general combining ability were also identified for use as parents in the local breeding programmes of the National Agricultural Research Systems (NARS) in developing countries.

Wild Species

Germplasm Collections

Central and South America are the centres of origin and diversity of tuber-bearing *Solanum* species, and hence the primary sources of genes for disease and pest resistances lacking in European and North America cultivars. Recognition of this resulted in numerous collecting expeditions, from those pioneered by the Russians in the 1920s (Hawkes, 1990) to the more recent ones of the 1990s (Spooner and Hijmans, 2001). These in turn led to the establishment of a number of potato germplasm collections worldwide. The main ones are the world collection at the International Potato Centre (CIP, Lima, Peru), the Commonwealth Potato Collection (CPC, Dundee, Scotland), the Dutch-German Potato Collection (CGN, Wageningen, The Netherlands), the Groß Lusewitz Potato Collection (GLKS, IPK, Groß Lusewitz, Germany), the Potato Collection of the Vavilov Institute (VIR, St Petersburg, Russia), the US Potato Genebank (NRSP-6, Sturgeon Bay, USA) and Potato Collections in Argentina, Bolivia and Peru. Together, they comprise the Association for Potato Intergenebank Collaboration, which has established an Intergenebank Potato Database (IPD) which can be accessed through the World Wide Web (www.potgenebank.org). The IPD contains 7112 different accessions of 188 taxa (species, subspecies, varieties and forms) out of the 247 tuber-bearing wild potato taxa recognized by Hawkes (Huaman et al., 2000c). Data are available for more than 33000 evaluations of wild potato accessions covering 55 traits (dry matter, starch, reducing sugar and glycoalkaloid content and resistances to fungi, bacteria, viruses, viroids, insects and environmental stresses such as frost and heat/drought). Clearly, these germplasm collections contain a lot of biodiversity, but it is pertinent to ask to what extent have they been utilised in the past and what are the prospects for their further use in the future?

Past Utilisation

Resistance to late blight has been introgressed into cultivated potato from *S. demissum* and *S. stoloniferum*, resistance to viruses from these species together with *S. chacoense* and *S. acaule*, and resistance to potato cyst nematodes has been introgressed from *S. vernei* and *S. spegazzinii*. By the end of the 1980s, these wild

Aardevo Exhibit 1010
Page 0988

species together with cultivated *S. tuberosum* subsp. *andigena* and *S. phureja*, had been used extensively in the breeding of successful cultivars in Europe, for example, through the valuable parental material developed by the Max Planck Institute in Köln, Germany, and described by Ross (1986). Likewise, the species *S. demissum*, *S. chacoense* and *S. acaule* dominated in North American cultivars (Plaisted and Hoopes, 1989). Furthermore, one of the most successful cultivars to be introduced into China by CIP, CIP-24, had *S. acaule*, *S. demissum* and *S. stoloniferum* in its pedigree (Ortiz, 2001). The genetic base of modern Indian potato selections can be traced to only 49 ancestors of which 10 from the UK account for 41% of the total genomic constitution (Gopal and Oyama, 2005). The most frequent ancestors were two clones from the UK, 2814a1 and 3069d4, which trace back to a cross between *S. rybinii* (a variant of *S. phureja*) and *S. demissum* which was made by Dr. Black in 1937 in order to introgress late blight resistance from *S. demissum*. It is therefore reasonable to conclude that the introgression of genes from wild species has been fairly limited in number and that the genetic base of modern cultivars is primarily a relatively small sample of *S. tuberosum* from the Andes and coastal Chile.

It took from three to seven backcrosses to transfer a major dominant resistance gene from a wild species into a successful cultivar, but fewer generations from cultivated potatoes. There have been mixed fortunes with the durability of resistance, clearly an important issue, given the time and resources required for introgression. The major gene resistances to PVY have proved durable and the hybrid parent MPI 61.303/34 from the Max Planck Institute in Köln has been used extensively in European breeding as the source of the *Ry* gene for extreme resistance from *S. stoloniferum*. Likewise, the major gene resistances to PVX have proved durable despite the occurrence of resistance-breaking strains (Jones, 1985). The widely used *H1* gene from a CPC accession (CPC 1673) of Andigena potatoes has remained effective against *Globodera rostochiensis* in Britain because Ro1 is still the main pathotype, but its widespread deployment has encouraged the spread of *G. pallida* (Bradshaw and Ramsay, 2005). The *S. demissum* derived R genes for resistance to late blight, in contrast, have been anything but durable and breeders have switched to selecting for high levels of quantitative field resistance, as seen in the Group B germplasm from CIP referred to above. Currently there is much interest and debate over whether or not the R genes being found in other wild species will be more durable per se or can be deployed in a more durable way such as a multiline produced by map-based cloning and stable transformation of a popular but susceptible cultivar (Huang, 2005; Smilde et al., 2005). Thus in 2004, the first potato cultivar Biogold (Van Rijn BV) was released which carried blight resistance from *S. bulbocastanum* via the ABPT bridging clones produced by Hermsen and Ramanna (1973) in the early 1970s (Huang, 2005).

Future Utilisation

Further improvements in the resistance of cultivated potatoes to abiotic and biotic stresses should come from the greater use of wild species, given the wide range of habitats in which they have evolved. The screening of germplasm collections for desirable traits will continue and so will genetic studies on their inheritance, followed by introgression of desirable alleles into Tuberosum, as described by Bradshaw and Ramsay (2005) for the CPC. These approaches will be supplemented

Aardevo Exhibit 1010
Page 0989

by an increasing amount of molecular marker and DNA sequence data. McGregor et al. (2002) analysed the entire CGN collection of 314 accessions of series *Acaulia* with two AFLP primer pairs and were able to verify taxonomic classification and to identify redundancies. However, there was no consistent relationship between the geographic distances between collection sites and genetic distance. At SCRI, a comprehensive molecular genetic analysis of the CPC is being performed to determine its overall genetic structure, a better understanding of the genome origins of the allopolyploid species and a better appreciation of between and within accession diversity. The AFLP analyses have been completed and have already provided insight into the domestication of the potato (Spooner et al., 2005a). Further analyses will inform the selection from the CPC of core collections of cultivated and wild material to be used for research purposes and will allow the integrity of genebank accessions to be established. The challenge now is to use the knowledge being gained to help breeders find desirable novelty amongst the available germplasm. This may depend on whether or not allele frequencies and distributions can be associated with features of the natural habitats of the accessions.

In future, then, we can anticipate greater use of wild species in potato breeding. By manipulation of ploidy, with due regard to Endosperm Balance Number, virtually any potato species can be utilised for the introgression of desirable genes into *S. tuberosum* (Hermsen, 1994; Ortiz, 1998, 2001). The schemes used for ploidy level manipulations exploit the facts that unreduced 2*n* gametes are common in *Solanum* species and maternal haploids (dihaploids) of tetraploid *S. tuberosum* can be extracted following crosses with pollinator clones of *S. phureja*, thus allowing breeding at the diploid level before returning to the tetraploid level for cultivar production (Jansky et al., 1990; Carputo and Barone, 2005). Furthermore, somatic fusion has allowed the production of hybrids between tetraploid 4EBN *S. tuberosum* and diploid 1EBN species, for example, the non-tuber bearing species *S. brevidens* that has tuber soft rot and early blight resistances (Tek et al., 2004) and *S. bulbocastanum* that has a major gene for broad spectrum resistance to late blight (Naess et al., 2000). Today, molecular-marker assisted introgression offers the possibility of faster progress than can be achieved by traditional back-crossing. Genetical knowledge of the trait coupled with that of chiasmata frequency and distribution and the properties of the binomial distribution, allows the estimation of the optimal combination of population sizes and number of backcross generations (Hospital, 2003). For *S. vernei*-derived PCN resistance, three backcrosses with population sizes in the hundreds is realistic and could be achieved with adequate PCN screening in 6 years, which is much less than the five generations spread over 28 to 30 years that actually occurred without the use of molecular markers (Bradshaw et al., 1995). The use of molecular markers also allows the breeder to track the fate of the wild species chromosomes. Where introgression is performed at the tetraploid level, the result may not be a genotype with 48 *S. tuberosum* chromosomes including one or more with the introgressed gene(s). This does not necessarily matter for the commercial success of the clones produced, but might affect their use as parents in future breeding. The result of the introgression from *S. brevidens* referred to above was a high yielding clone, C75-5+297, with resistances to both tuber soft rot and early blight. Using both molecular and cytogenetic approaches, Tek et al. (2004) showed that C75-5+297 had 47 chromosomes, including four copies of chromosome 8, three from potato and one from *S.*

Aardevo Exhibit 1010
Page 0990

brevidens which was the only part of the wild species genome present. In contrast, Barone et al. (2001) did obtain 48 chromosomes and evidence of recombination between *S. commersonii* (2*x* 1EBN) and *S. tuberosum* chromosomes in their molecular marker-assisted introgression of tuber soft rot resistance.

Gene Cloning

As potatoes are heterozygous outbreeders, use of the same recurrent parent during introgression would result in a self of the recurrent parent and hence inbreeding depression. This can be avoided by using different Tuberosum parents for each backcross but would result in an entirely new cultivar, which may or may not be the desired outcome. The only way to introduce a gene into a known cultivar is by the transgenic route. Hence, the molecular cloning of natural resistance genes and their transfer into well-adapted but susceptible cultivars is being pursued in a number of laboratories worldwide. The *Rx* (Andigena) gene in cv. Cara has been genetically delimited within a bacterial artificial chromosome (BAC) clone and stably introduced into the susceptible cultivar Maris Bard by *Agrobacterium*-mediated transformation (Bendahmane et al., 1999). The transgenic *Rx*-mediated resistance was indistinguishable from the *Rx*-mediated phenotype in cultivar Cara. The *R1* gene for resistance to late blight has been cloned and introduced into the susceptible cultivar Desiree and shown to give a typical hypersensitive response (HR), similar to the resistant line hosting *R1* (Ballvora et al., 2002), but *R1* has failed to give durable resistance. The *RB* and *Rpi-blb1* genes (which are allelic) from *S. bulbocastanum* have been cloned and introduced into the susceptible cultivars Katahdin and Impala, respectively, and shown to confer broad spectrum resistance to late blight (Song et al., 2003; Van der Vossen et al., 2003). Most recently, Paal et al. (2004) have cloned the *Gro1* gene for resistance to *G. rostochiensis* from *S. spegazzinii* and introduced it into the susceptible cultivar Désirée and shown that it confers resistance to pathotype Ro1.

Base Broadening versus Introgression

Most of the evolutionarily more advanced tuber-bearing wild species are diploid with an EBN of 2, the same as haploids (dihaploids) of *S. tuberosum* with which they will readily cross. These wild species are sources of genetic diversity in general, as well as genetic resistances to diseases, pests and abiotic stresses, but they do not tuber in long days. In contrast, Jansky et al. (2004) found that in 125 out of 154 families of haploid-wild species crosses, more than 50% of the plants did tuberise in long days, and hence could be selected for tuber characteristics. Hence, if they also produce 2*n* gametes by FDR, the genetic diversity of the wild species can be efficiently transferred to the tetraploid cultivated form in $4x \times 2x$ crosses and result in about 25% of the wild species genes in the final product. This led Peloquin and his co-workers (Hermundstad and Peloquin, 1987; Jansky et al., 1990) to propose such $4x \times 2x$ crosses as a novel breeding strategy designed to both introgress specific characteristics and to broaden the genetic base, in a way similar to that envisaged by Chase (1963) in his analytic breeding scheme. Likewise in Europe, since the 1960s tetraploid breeding programmes have benefited from parental lines produced from $4x \times 2x$ crosses by organisations such as PRI in the Netherlands, IHAR in Poland,

Aardevo Exhibit 1010
Page 0991

BAZ in Germany and INRA in France. However, Tai (1994) concluded that haploid-wild species hybrids need to be improved before they are used in $4x \times 2x$ crosses, for example, through population improvement by recurrent selection (Rousselle-Bourgeois and Rousselle, 1992). In summary, the issue is whether or not wild species contain sufficient desirable alleles to warrant base broadening as opposed to the introgression of just a few desirable genes. Tentative answers could be obtained from comparing the offspring of $4x \times 2x$ crosses with those from $4x \times 4x$ crosses among Tuberosum cultivars.

Genetically Modified Potatoes

Genetic modification of successful potato cultivars offers the possibility of further targeted improvements which can be brought about using two types of genetic resource, the allelic and genic diversity already present in cultivated potatoes and their cross-compatible relatives, or, more controversially, the diversity present in all living organisms. There is tremendous potential for the targeted improvement of potatoes using genes from wild relatives, and the improving availability of tools for such work as the science of genomics matures means that the examples given in the preceding section on gene cloning represent only the beginning of the application of this approach. It is likely that using genetic modification to move genes efficiently from cross-compatible relatives to cultivated potato will gain acceptance from sceptical consumers more readily than other applications of the technology. However, exotic sources of genes have clear value by permitting the introduction of traits not found in cross-compatible relatives. The genes used to date have mainly been ones that code for proteins which are toxic to pests and pathogens, ones whose expression interferes with virus multiplication in host cells and ones that code for key enzymes in biochemical pathways in other organisms, often, but not always other plant species. Genes which encode 'Cry' proteins from the bacterium *Bacillus thuringiensis* confer resistance to Colorado potato beetle (CPB, *Leptinotarsa decemlineata* Say) and potato tuber moth (*Phthorimaea operculella*) (Mohammed et al., 2000; Duncan et al., 2002; Davidson et al., 2004) and genes which encode cysteine proteinase inhibitors (cystatins) confer resistance to potato cyst nematodes (*Globodera rostochiensis* and *G. pallida*) (Urwin et al., 2003). Replicase and coat protein genes from Potato Leafroll Luteovirus and Potato Y Potyvirus, respectively, provide resistance to these viruses (Duncan et al., 2002). The gene *chly* encoding a chicken lysozyme enzyme enhances resistance to blackleg and soft rot caused by infection with *Erwinia carotovora* subsp. *atroseptica* (Serrano et al., 2000). Expression of a gene for a derivative of the antimicrobial peptide dermaseptin B1, from the arboreal frog *Phyllomedusa bicolor*, has been shown to increase resistance to diseases such as late blight, dry rot and pink rot and to markedly extend the shelf life of tubers (Osusky et al., 2005). Protein content can be increased and amino acid composition improved by expression of a non-allergenic seed albumin gene (*AmA1*) from *Amaranthus hypochondriacus* (Chakraborty et al., 2000) and carbohydrate composition can be improved by inulin production from the expression of fructosyltransferases from globe artichoke (*Cynara scolymus*) (Hellwege et al., 2000). Inulins enrich food with dietary fibre and replace sugar and fat. The conversion of sucrose to glucose and fructose, and hence cold sweetening (the cause

Aardevo Exhibit 1010
Page 0992

of dark and bitter tasting fry products), can be minimised by expressing a putative vacuolar invertase inhibitor protein from tobacco (Greiner et al., 1999).

Where the control of biochemical pathways of interest is understood, down regulation of gene expression using antisense technology has proved useful. Bruise resistant potatoes can be produced by down regulation of polyphenol oxidase (Bachem et al., 1994), high carotenoid (pigments with health promoting attributes) potatoes by down regulation of zeaxanthin epoxidase (Romer et al., 2002), high amylopectin starch by down regulation of granule bound starch synthase (Visser et al., 1991) and high amylose starch by down regulating two starch branching enzymes, A and B (Schwall et al., 2000).

Despite these many potential benefits, the demonstration of economic, environmental and health benefits will be crucial in convincing sceptical consumers in some countries of the value of genetically modified potatoes.

Conclusions

1. A wide range of germplasm is held in genebanks and by breeders, and many evaluations for economically important traits have been done, yet many more remain and the wild and cultivated species of potato have not been utilised as well as might have been expected. This should change in the future.
2. The strategy for utilising the cultivars native to Latin America will either be introgression of desirable genes or direct use of parents from improved populations, depending on how far modern *S. tuberosum* cultivars have genetically diverged from them and the extent to which *S. tuberosum* cultivars have been improved in the process. Direct use of parents requires modern cultivars and the populations derived from Latin American potatoes to have diverged genetically and to complement each other for many desirable alleles so that their offspring display yield heterosis and other desirable combinations of traits, but not the deleterious effects of germplasm unadapted to modern usage and the target environment. Hence, it is important to determine the global genetical population structure of cultivated germplasm. This latter objective can be achieved by the coordinated use of common molecular markers in different cultivated genebanks.
3. Molecular marker-assisted selection should be used for faster introgression of desirable genes from wild species.
4. New cultivars will continue to come from crosses between pairs of parents with complementary features but adapted to local growing conditions. However, increasingly these parents should possess desirable genes that have been introgressed from wild species and may also be from complementary groups of cultivated germplasm to exploit hybrid vigour. Furthermore, as genetical knowledge accumulates, these parents should be chosen so that one or both of them have desired major genes and alleles of large effect at quantitative trait loci.
5. Successful cultivars may be genetically modified if consumers see benefits in the use of the technology to introduce genes not present in cultivated potatoes and their wild relatives, and hence introduce novel biochemistry to achieve further desirable improvements.

Aardevo Exhibit 1010
Page 0993

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Aardevo Exhibit 1010
Page 0996

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Aardevo Exhibit 1010
Page 0997

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Art Unit	1663
Examiner Name	Russell T. Boggs
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/R.T.B/	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.~~	☐
/R.T.B/	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.	☐
/R.T.B/	4	JANSKY et al., "The Evolution of Potato Breeding", Plant Breeding Reviews, Vol. 41, pp. 169-214, 2018.	☐
/R.T.B/	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.	☐
/R.T.B/	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	☐

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Examiner Name	Russell T. Boggs
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Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

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Inbreeding Process of an Outbreeding Diploid Potato Monitored Using DNA Markers

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Abstract

The inbreeding coefficient (*F*) predicts 50% reduction of heterozygosity by selfing. This theoretical expectation has rarely been examined particularly in absolute outbreeding species. We developed S_1 and S_2 inbred progenies from an outbreeding diploid potato by introducing an *S*-locus inhibitor gene (*Sli*). The reduction rates of observed percentage heterozygosity were 49.8% from S_0 to S_1 and 51.7% from S_2 to S_3. However, the observed percentage heterozygosity in the S_2 plant that produced the S_3 progeny was 49.1% estimated by RFLP analysis or 57.7% by AFLP analysis, both being much greater than expected (25.0%). Thus, it is suggested that reproductive traits were strongly correlated with heterozygosity and selection for fertility favored the more heterozygous plants.

Key Words: *Sli* gene, self-fertilization, RFLP, AFLP, diploid potato inbreds

1. Introduction

The inbreeding coefficient (*F*) measures the probable degree of heterozygosity and reduces at a rate of 50% in each self-fertilization [1]. The derivation of *F* assumes no mutation, no gene flow, or no selection during inbreeding. However, gametic selection such as pollen competition or zygotic selection such as full or partial lethality and sterility by recessive homozygotes may occur, which can modify the genotypic frequencies, particularly in favor of the more heterozygous individuals [4]. Inbreeding often decreases vigor and fecundity, the phenomenon being known as inbreeding depression. It is well known as classical examples that upon continuous selfing height of stalk, grain yield and plant height rapidly decrease in maize, and yields in alfalfa and onions [1].

In potato (*Solanum tuberosum* L. ssp. *tuberosum*, 2*n*=4*x*=48), serious tuber yield reduction on selfing has been known [6, 10]. Tetraploidy and inbreeding depression have hampered advancement of potato breeding and genetics studies. Although use of closely related diploid species or dihaploid potatoes can simplify genetic analysis, gametophytic self-incompatibility system controlled by multi-allelic *S* genes prevents self-fertilization in diploid potatoes [5, 11, 12]. Recently, we have found a dominant allele, named an *S* locus inhibitor gene (*Sli*), from a self-compatible variant in a wild diploid species *S. chacoense*, which

can alter self-incompatible plants to self-compatible ones [7]. Using RFLP and RAPD markers, the *Sli* gene was mapped at a distal end of the potato chromosome 12 [8].

To develop diploid inbred lines of cultivated potatoes, the *Sli* gene was incorporated by crossing into cultivated diploid potatoes of diverse origins and S_2 inbreds were established, although serious inbreeding depression associated with selfing occurred in each generation [3]. The genotypes of S_2 plants were investigated using *S. chacoense*-specific RFLP markers, among which markers on chromosome 12 were all over-represented in the S_2 plants probably because these markers were linked in coupling with the *Sli* gene essential for selfing [3].

Self-fertilization of diploid potatoes has become possible by using the *Sli* gene [3]. However, the actual process to homozygosity during selfing is unknown and may differ from that predicted by the inbreeding coefficient (F). Brouwer and Osborn [4] reported that the observed percentage heterozygosity, calculated as the percentage of the 40 RFLP loci that remained heterozygous in each plant of alfalfa upon selfing, declined less than expected.

The S_2 inbreds developed by Birhman and Hosaka [3] were, later, accidentally pollinated with bulked pollen grains from S_2 plants, deriving a new line of inbred progenies (Fig. 1). In this study, molecular markers were used to follow the decline in heterozygosity over three generations of selfing from one diploid potato genotype 97H32-6. The specific objectives were: (1) to compare the observed percentage heterozygosity calculated from segregating RFLP and AFLP markers with the expected percentage heterozygosity based on F, (2) to investigate correlation between reproductive traits and heterozygosity, and (3) to estimate the *Sli* genotype of the parental clone 97H32-6.

2. Materials and methods

2.1 Plant materials and reproductive trait evaluation

The diploid clone 97H32-6 was derived by crossing of 96H14-10 (an S_2 inbred described in [3]) with S_2 bulked pollen. 97H32-6 was selfed using a function of *Sli* gene originally derived from a self-compatible variant of *S. chacoense*. The selfed seeds from 97H32-6 were grown twice in 1998 (98H23 family) and 2002 (2H21 family); the former family was used to evaluate reproductive traits and the latter to conduct DNA analysis. One of the plants in 98H23 family (98H23-21) was selfed, and S_2 plants were obtained (2KH521 family), in which 2KH521-4 was selfed to generate S_3 plants (2H22 family) (Fig. 1).

Reproductive traits were evaluated for 91 S_1 plants (family 98H23) and 81 S_3 plants (family 2H22) by pollinating with self or bulked pollen and by checking pollen stainability, as basically described in Birhman and Hosaka [3].

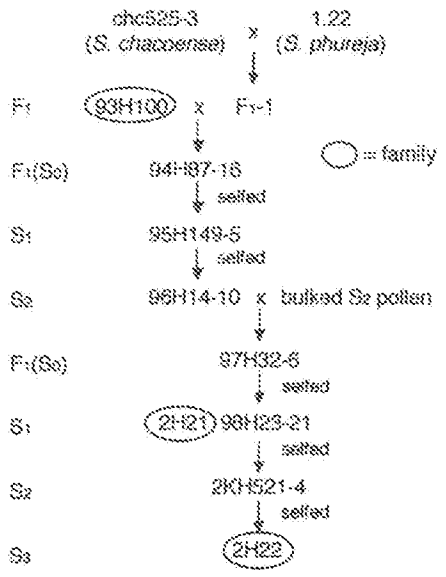

Fig. 1 Pedigree of diploid potato inbreds

2.2 RFLP analysis

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Page 1004

Total DNA was isolated from 97H32-6 and 15 plants each from the 2H21 (S_1) and 2H22 (S_3) families by the method of Hosaka and Hanneman [8]. Total DNA from S_1 plants was digested with a restriction endonuclease *Eco*R1, *Hin*cII or *Hin*dIII and surveyed in order to obtain segregating loci. Probe and enzyme combinations that showed segregation among S_1 plants were used to analyze S_3 plants. RFLP probes used were either single-copy tomato probes obtained from Dr. S. D. Tanksley, Cornell University [14] or potato probes [9]. RFLP detection procedures were described in Hosaka and Hanneman [8]

2.3 AFLP analysis

The above sets of DNA samples were used for AFLP analysis using procedures essentially similar to the protocols of AFLP® Analysis System (Invitrogen). AFLP amplification products were mixed with 10 μl of loading dye (95% formamide, 0.25% bromophenol blue and 0.25% xylene cyanol), heated at 95 °C for 3 min and immediately placed on ice. Five μl of the sample were separated by electrophoresis in 4.5% denaturing polyacrylamide gels (Sequi-Gen® GT Nucleic Acid Electrophoresis Cell, Bio-Rad) at 40 W constant power for 2 hr and visualized by silver staining [2].

2.4 Data analysis

Observed percentage heterozygosity was estimated by the proportion of heterozygous loci for each individual plant (estimated by RFLP markers) or by the proportion of segregating loci in the selfed progeny for the parental clone (estimated by RFLP and AFLP markers). Segregation ratios of genotypes (AA+BB:AB) and of alternate alleles (A:B) in S_1 and S_3 plants were tested against a 1:1 segregation ratio using Chi-square test.

3. Results

3.1 Reproductive traits

Ninety-one S_1 plants were evaluated for reproductive traits, in which 57 (62.6%) flowered, and were self-pollinated. A total of 401 self-pollinations, ranging from 2 to 14 pollinations per plant, produced 166 berries from 47 plants (82.5%). Ten plants did not set berries by self-pollination, of which two showed good pollen stainability (>79.3%) and reasonable female fertility. The other plants that did not set berries were possibly due to female sterility (three plants), pollen sterility (one plant), or unknown reasons (four plants).

Of the 81 S_3 plants grown, 27 (33.3%) flowered, and were self-pollinated. A total of 138 self-pollinations, ranging from 1 to 19 pollinations per plant, produced 85 berries from 15 plants (55.6%). For all 12 unsuccessful plants, only three or less number of self-pollinations were made and failed. Further characterization for female and male fertility was not carried out because of lack of flowers.

3.2 Heterozygosity estimated by RFLP markers

Fifty-seven single-copy RFLP probes (Fig. 4) were selected from a total of 123 RFLP probes, which were segregating among 15 S_1 plants, or heterozygous in the S_0 plant 97H32-6. The RFLP phenotypes provided no evidence for contamination by accidental outcrossing or for new mutations, because bands present in the selfed progenies were all present in the initial S_0 plant 97H32-6.

For all RFLP loci, genotypes (AA, AB or BB) in each of 15 S_1 plants were estimated (Fig. 2). The

observed percentage heterozygosity in each S_1 plant ranged from 35.7 to 60.7% with an average of 50.2% (Fig. 5). Likewise, genotypes in each of 15 S_3 plants were characterized for the same set of RFLP loci. Twenty-nine out of 57 markers showed no segregation, or genetically fixed to homozygosity among 15 S_3 plants, indicating 49.1% heterozygosity in the S_2 parent 2KH521-4. The observed percentage heterozygosity in each S_3 plant ranged from 14.0 to 33.3% with an average of 23.7% (Fig. 5).

3.3 Heterozygosity estimated by AFLP markers

AFLP banding patterns from 22 AFLP primer combinations were compared between S_1 and S_3 plants using the same sets of DNA samples as those used in RFLP analysis. A total of 310 bands, or an average of 14.1 bands per primer combination, were segregating (present or absent) among 15 S_1 plants, of which 179 bands were still segregating among 15 S_3 plants (Fig. 3). Assuming each segregating band resulted from one independent locus, the observed percentage heterozygosity in the S_0 plant 97H32-6 and the S_2 plant 2KH521-4 were estimated to be 100% and 57.7%, respectively.

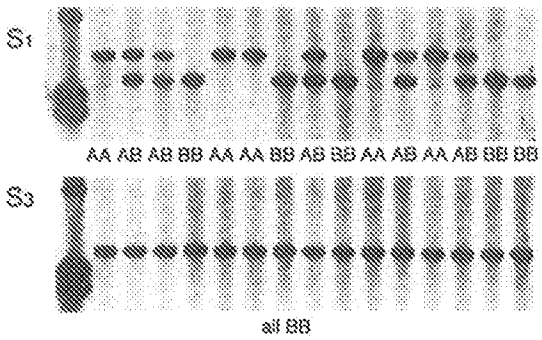

Fig. 2 RFLP banding patterns and the proposed genotypes in S_1 and S_3 progenies. *Hin*dIII-digested genomic DNAs were probed with TG63. The left lane contains *Hin*dIII-digested lambda DNA

3.4 Genetic distortion of RFLP alleles and its localization

The ratio of homozygous (AA and BB) to heterozygous plants (AB) was tested against a 1:1 ratio for each of all 57 loci among 15 S_1 plants or 28 segregating loci among 15 S_3 plants. In the S_1 plants, five of 57 loci (8.8%) showed significant deviation at 5% level; two loci (TG34 and P140) significantly favored heterozygotes, while three loci (P263, P359 and P1060) favored homozygotes. In the S_3 plants, four of 28 loci (14.3%) showed distorted segregation; one locus (P215) favored heterozygotes, while three loci (CD8, TG409 and P697) favored homozygotes (Fig. 4).

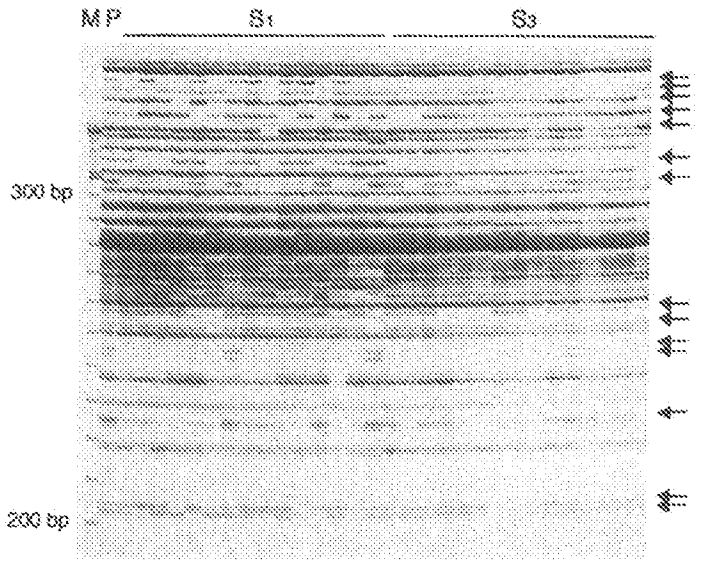

Fig. 3 AFLP banding patterns of the parental clone 97H32-6 (P) and its S_1 and S_3 progenies. Segregating bands among S_1 plants are indicated by arrows. The left lane contains 10 bp ladder markers

Aardevo Exhibit 1010
Page 1006

The ratio of alternate alleles in each segregating RFLP locus among 15 S_1 and among 15 S_3 plants were tested against a 1:1 ratio. Alleles in five loci (TG31, P215, TG23, TG413 and TG47) (8.8%) in the S_1 plants and three loci (TG14, P937 and P697) (10.7%) in the S_3 plants showed significant deviation from a 1:1 ratio. The P697 locus segregated to 13AA:2AB:0BB among 15 S_3 plants, resulting in the most extreme distortion in both ratios of homozygotes vs. heterozygotes and A allele vs. B allele.

Approximate locations of the segregating loci among S_3 plants were shown in Fig. 4. Most of them were clustered into several locations on chromosomes 1, 2, 4, 6, 8 and 9. All the loci surveyed on chromosomes 10 and 11 were already fixed to homozygosity. Two (CT79 and TG28) of three loci on chromosome 12 were fixed and the remaining P697 tended to be fixed as described above. By comparison with our previous data, it was indicated that CT79 was fixed to homozygosity of *S. chacoense*-allele, whereas TG28 was fixed to the other allele. P697 could not be compared because the previous data was unavailable.

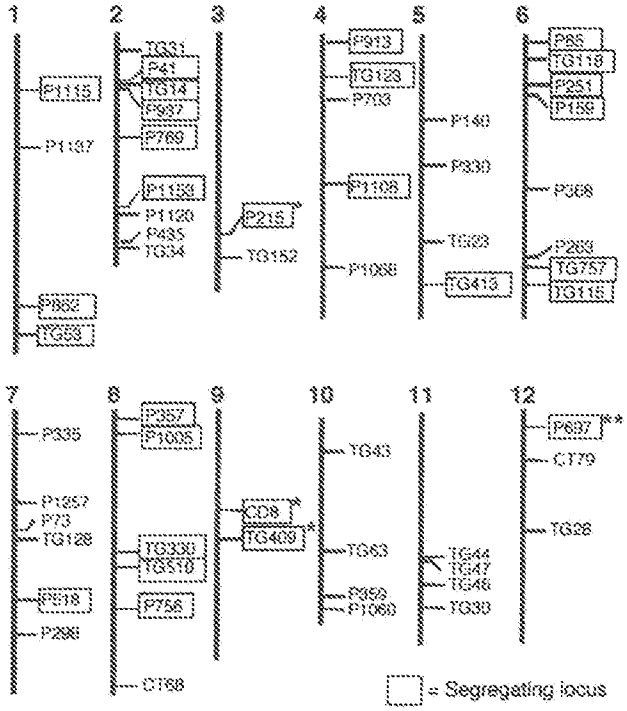

Fig. 4 RFLP linkage map showing approximate locations of segregating loci among S_3 plants. The ratio of heterozygotes vs. homozygotes was significantly deviated at 5% (*) or 1% level (**) from an expected 1:1 segregation

4. Discussion

Continuous selfing in this study resulted in reduced fertility, fixation of marker alleles, and decreased heterozygosity at marker loci. Direct selection was avoided throughout the selfing process, although selection for vigor and self-fertility was unavoidable. A high degree of inbreeding depression was manifested by the frequencies of non-flowering plants (37.4% and 66.6% in the S_1 and S_3 progenies, respectively). This is concordant with our previous observation in the earlier selfing generations of the starting clone of this study [3] and observation made by De Jong and Rowe [6] for selfed progenies from hybrids between cultivated diploids and dihaploids of *S. tuberosum* ssp. *tuberosum*. However, the percentage of self-compatible plants was 82.5% in S_1 progeny and 55.6% in S_3 progeny, which were notably higher than those of the earlier selfing generations; 18.3% in S_0, 30.3% in S_1 and 20.9% in S_2 progenies [3]. The increase in self-fertility might be partly due to improvement of male and female fertility by eliminating partial sterility genes through the previous selfing generations, or due to heterotic effect caused by hybridization with bulked S_2 pollen (Fig. 1).

The observed percentage heterozygosity reduced from 100% in S_0 to 50.2% in S_1 and from 49.1% in S_2 to 23.7% in S_3 (Fig. 5). The reduction rates of observed percentage heterozygosity by selfing were 49.8% and 51.7%, respectively, which fit the expected 50% reduction based on *F*. Both allelic and zygotic frequencies in the S_1 and S_3 progenies mostly fit an expected frequency of 50%. These suggest that both gametic and zygotic selection did not act significantly until the stage used for DNA extraction. However, observed percentage heterozygosity in the S_2 clone 2KH521-4 was 49.1% estimated by RFLP markers or 57.7% by AFLP markers, which were much higher than the expected 25% based on *F* for S_2 plants. This indicates that preferential selection occurred after the DNA extraction stage or during a period between flowering and seed germination in a following progeny. 2KH521-4 was probably selected inadvertently from the more heterozygous S_2 plants and its parental clone 98H23-21 from the more heterozygous S_1 plants. Slower declines of heterozygosity than expected have been reported by Brouwer and Osborn [4] in inbreeding of diploid alfalfa and by Scotti et al. [13] in inbreeding of tetraploid alfalfa. Unavoidable selection for vigor and fertility during selfing has been suggested for the cause [4]. Considering high percentage of non-flowering plants in the present selfed progenies, it is suggested that reproductive traits were strongly correlated with heterozygosity and selection for fertility favored the more heterozygous plants, although direct evidence was not available in this study because plant individuals used for DNA analysis and those evaluated for reproductive traits were not identical.

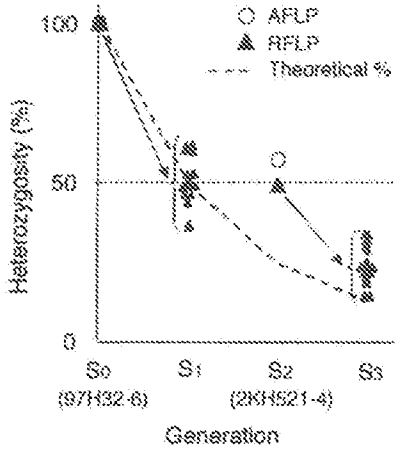

Fig. 5 Observed and expected percentage heterozygosity

We previously proposed that the *Sli* gene could be maintained only in a heterozygous condition probably because of tight linkage in coupling with a recessive lethal gene [7]. Thus, every selfed progenies would show a ratio of 1 self-incompatible : 2 self-compatible (=*Sli* heterozygotes). However, only two of 57 S_1 plants that flowered were confirmed as self-incompatible in this study. In S_2 plants of the earlier selfing generations, all RFLP loci analyzed for chromosome 12 were over-represented by *S. chacoense*-alleles because of the linkage with *Sli* gene [3]. In the present S_3 progeny, although most of the RFLP loci on chromosome 12 were fixed to homozygosity, the locus TG28 closest to the *Sli* gene in this study, was fixed to the none-*S. chacoense*-allele. In the present S_1 progeny, ratios of homozygotes to heterozygotes on the RFLP loci of chromosome 12 were all normal, indicating no evidence for elimination of recessive homozygotes due to lethality. These results suggest that the *Sli* gene was already fixed in the S_0 plant 97H32-6, and the linkage with the presumed lethality gene was broken by recombination that occurred through the earlier selfing generations.

Acknowledgment

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Aardevo Exhibit 1010
Page 1008

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(Submitted Only via EFS-Web)

Application Number	16/541,071	Filing Date	2019-08-14	Docket Number (if applicable)	VONL:004USC1	Art Unit	1663
First Named Inventor	Willem Hendrik Lindhout			Examiner Name	Russell T. Boggs		

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.
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- ☐ Information Disclosure Statement (IDS)
- ☐ Affidavit(s)/ Declaration(s)
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(Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

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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

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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).

The present Supplemental Information Disclosure Statement is being filed in response to the Final Office Action issued on November 9, 2020 in which the Examiner identified a

typographical error in the title of reference "2" of the non-patent literature documents citations. In response thereto, Applicants hereby submit the attached SB/08 identifying the correct reference as "1" of the non-patent literature documents citations. A substantive response to the Final Office Action is also being filed concurrently herewith.

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.

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Respectfully submitted,

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Date: February 9, 2021

PTO/SB/08a (02-18)
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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					

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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
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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							☐

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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)		
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	Examiner Name	Russell T. Boggs
	Attorney Docket Number	VONL:004USC1

	1	DE VRIES et al., "The potential of hybrid potato for East-Africa," Open Agriculture 1:151–156, 2016.	☐
	2	STOKSTAD, "The New Potato," Science 363(6427):574–577, 2019.	☐

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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

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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

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☐ 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)	2021-02-09
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.**

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THE NEW POTATO

Breeders seek a breakthrough to help farmers facing an uncertain future

By **Erik Stokstad**, *in the Sacred Valley of the Incas in Peru*

On a bleak, brown hill here, David Ellis examines a test plot of potato plants and shakes his head. "They're dead, dead, dead," he says. Pests and lack of rain have laid waste to all 17 varieties that researchers had planted.

It is a worrying sign for Ellis, the now-retired director of the gene bank at the International Potato Center (CIP) in Lima. People have grown potatoes in this rugged stretch of the Andes for thousands of years. In recent years, that task has gotten tougher, in part because of climate change. Drought and frost are striking more often. The rains come later, shortening the growing season. And warmer temperatures have allowed moths and weevils to encroach from lower elevations.

To find potatoes that can cope with those challenges, researchers and Peruvian farmers are testing dozens of the 4350 locally cultivated varieties, or landraces, kept in CIP's refrigerated storage. The plants in this plot fell short. "Native landraces evolved over time," Ellis says. But, he says, climate change is happening "too fast for these varieties to adapt."

In Peru and around the world, enhancing the potato has become a high priority. It is the most important food crop after wheat and rice. Potatoes are already a staple for 1.3 billion people, and the nutritious tubers are becoming increasingly popular in the developing world. Keeping up with the demand means adapting the potato to various soils and climates. It must also resist new threats from pests, disease, heat, and drought.

Unlike other major crops, however, the potato has not had a breeding breakthrough of the kind that helped dramatically boost yields during the Green Revolution of the 1950s and 1960s. The reason is that creating a new potato variety is slow and difficult, even by the patient standards of plant breeders. Commercial varieties carry four copies of each chromosome, which forces breeders to create and test hundreds of thousands of seedlings to find just one with the desired combination of traits.

Diverse potatoes, such as these from Peru, will help breeders create resilient new varieties.

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Aardevo Exhibit 1010
Page 1018

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Readying a new variety for farm fields can take a decade or more.

Many countries continue to plant popular potato varieties that have remained essentially unchanged for decades. But new approaches, including genetic engineering, promise to add more options. Potato breeders are particularly excited about a radical new way of creating better varieties. This system, called hybrid diploid breeding, could cut the time required by more than half, make it easier to combine traits in one variety, and allow farmers to plant seeds instead of bulky chunks of tuber. "It will change the world tremendously," says Paul Struik, an agronomist at Wageningen University in the Netherlands.

To breed a better potato, it helps to have plenty of genetic raw material on hand. But the world's gene banks aren't fully stocked with the richest source of valuable genes: the 107 potato species that grow in the wild. Habitat loss threatens many populations of those plants. In a bid to preserve that wild diversity before it vanishes, collectors have made their biggest push ever, part of a $50 million program coordinated by the Crop Trust, a charity based in Bonn, Germany.

The collectors and breeders are racing against warming, drying, and the proliferation of pests. "Because of climate change," says Nigel Maxted, a conservation biologist at the University of Birmingham in the United Kingdom, "we require higher levels of diversity than ever before."

THE CLOSEST ANCESTORS of cultivated potatoes evolved in the Andes, where people domesticated the plant at least 7000 years ago. After the Spanish brought the tuber to Europe in the 16th century, it remained a botanical curiosity and was mostly fed to livestock. Europeans began to eat potatoes in earnest only in the 1800s, during the famines of the Napoleonic Wars.

Once the potato caught on, there was no turning back. The plant can grow in cold climates and poor soil, and in some places yields several crops per season. Once harvested, the energy-rich tubers, packed with vitamin C, can be stored for months and cooked in many ways. A hectare of potatoes can provide up to four times the calories of a grain crop.

Like rice and wheat, the potato was a target for improvement during the Green Revolution. Yields increased thanks to fertilizer and improved farming techniques, but they didn't skyrocket. Potato breeders achieved no genetic gains such as the one that produced wheat with short, sturdy stalks that can bear more grain.

Still, global potato production has steadily grown (see graph, p. 576). China has doubled its harvests over the past 20 years. It now grows more than twice as many potatoes as India, the next-biggest producer. Uzbekistan and Bangladesh, among other nations, have come to depend on the potato for food security. In 2005, developing countries for the first time grew more potatoes than the developed world. Many African countries are aiming to boost production.

To reap bigger harvests, farmers will have to manage many risks, including disease.

Climate change threatens yields in Potato Park, a farming association near Cuzco, Peru.

The potato's greatest scourge is the funguslike pathogen *Phytophthora infestans*, which causes a disease called late blight. The pathogen unleashed the Irish potato famine in the mid-19th century, and plant breeders have struggled ever since to rein it in. "*Phytophthora* is always evolving and overcoming resistance," says Jadwiga Śliwka of the Plant Breeding and Acclimatization Institute in Młochów, Poland. Rich countries use fungicides to minimize the devastating losses from late blight. But in developing countries, 15% to 30% of the crop is ruined.

Then there is heat and drought, which climate change is exacerbating. In some parts of the world, farmers are planting their crop earlier so that it matures before the nights get too hot, which prevents tubers from forming. But eventually farmers will need hardier plants. "We focus on developing a robust potato that will perform better in a stressful environment," says Thiago Mendes, a potato breeder with CIP's regional office in Nairobi. "Our target is food security."

The key to that robust potato may be waiting in the wild species that grow from southwestern North America through Central and South America. Wild potatoes from Mexico, for example, evolved in the presence of *P. infestans* and can resist many strains. Many other wild species have yet to be thoroughly collected or studied.

IN JUNE 2018, beside a cattle pasture in southern Brazil, botanist Gustavo Heiden strode along an embankment, his eyes fixed on the long grass. Then, he dropped to his knees and jabbed a trowel into the soil. "Aha! Look at this," said Heiden, who works with the Brazilian Agricultural Research Corporation (EMBRAPA) in Pelotas. He pulled up a short plant with small tubers dangling from its roots. It was *Solanum commersonii*, one of three wild relatives of the potato known in Brazil.

Brazil is far from the potato's center of origin in the Andes. But the ranges of wild relatives extend into the state of Rio Grande do Sul, where the climate shifts from temperate to tropical. Plants in this transition zone have evolved to survive occasionally harsh winters and hot, dry summers. "The wild potatoes here are probably pretty adapted to the extreme weather that will be happening more frequently with climate change," Heiden says.

Heiden's collecting trip was just one element of the Crop Trust's effort to collect, conserve, and breed the wild relatives of 29 crops, which began in 2011. Plant collectors used to travel the world on such expeditions. But they became much less frequent after governments began to adopt the Convention on Biological Diversity in the 1990s. Intended to prevent unfair exploitation of biodiversity, the convention made it harder to get collecting permits and to exchange plant material. An international seed treaty established in 2004 eased swaps for crops and wild relatives, but collecting remained stagnant because of lack of funding and expertise. "We didn't have any experience on how to collect wild potatoes or how to conserve them," says Cinthya Zorrilla, subdirector of genetic resources at the National Institute of Agricultural Innovation in Lima.

The Crop Trust has provided grants and training to collectors around the world. The effort on wild potatoes, which wraps up this month, has yielded a collection representing 39 species from six nations: Peru, Brazil, Ecuador, Guatemala, Costa Rica, and Chile. Zorrilla's team alone found 31 species in Peru, including one for which no seeds had ever been collected. They plan to continue to search for four other species still missing from gene banks. "We will not stop," she says. The plants are being stored in each nation's gene bank, CIP, and the Millennium Seed Bank at the Royal Botanic Gardens, Kew, in

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Aardevo Exhibit 1010
Page 1019
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the United Kingdom. The stored seeds will be available to potato breeders worldwide.

THE HARDEST PART comes next: getting desirable genes from wild species into cultivated potatoes. In the past, breeders acquired traits such as disease resistance from a dozen wild species. Those victories were hard-won, some taking decades to achieve. That's largely because wild relatives also carry many unwanted traits, which combine with those of cultivated potatoes and vastly lower a breeder's chances of finding a good variety.

Even without wild species, potato breeding is a crapshoot. Because breeding lines have four copies of their 12 chromosomes, the traits of the two parents show up in the next generation in largely unpredictable combinations. As experts say, the current varieties don't breed true, which is why farmers plant bits of "seed tuber," which yield genetically identical plants, rather than seeds. Compounding the headache, breeders select for many traits at once, further lowering the probability of finding a winner. "The numbers get really hard, really fast," says Laura Shannon, a potato breeder at the University of Minnesota in St. Paul.

Genetic markers linked to specific genes have sped up the process. To find out whether seedlings have inherited a trait such as disease resistance, breeders can quickly test for the marker rather than wait for the plants to mature and then expose them to the disease. Even with this tool, a potato breeder must screen up to 100,000 offspring per year. It can take 15 years or longer to find one with the right traits, fully test it, and generate enough seed tubers to distribute to farmers.

Another frustration is that potato breeders can't easily improve existing varieties. Once a potato variety is established, introducing new traits while retaining all of its favored characteristics is practically impossible. That's why classic, widely grown varieties, such as the russet burbank, still dominate the market many decades after their debuts.

Patient breeders using traditional methods can nevertheless achieve impressive results. In 2017, for example, CIP released four new varieties in Kenya, the result of crosses from established breeding lines. In field trials, the new potato plants maintained yields with 20% less rainfall and temperatures higher by 3°C.

Such success shows there is still genetic diversity to be tapped in existing breeding lines. But researchers fear that gene pool may not be deep enough to adapt the potato to future climates or enable other improvements. Wild potatoes, however, hold valuable, untapped genetic diversity. One trait from those wild plants, Mendes says, "could save our life."

THE SEARCH for vital traits is already underway. Last year, at an EMBRAPA research station near Pelotas, technicians in lab coats leaned over the wild species Heiden had collected. They gently daubed their faintly purple flowers with yellow powder from a plastic tube, fertilizing them with pollen from domesticated potatoes.

In a nearby greenhouse, tables were lined with the offspring of previous crosses. Researchers have evaluated thousands of those seedlings for health and yield, among other traits. They screened older plants for drought resistance by limiting the water in plastic-lined troughs. In a temperature-controlled walk-in chamber, researchers tested the ability of other plants to withstand heat; the yellowed plants appeared to be sweltering.

Such expansive testing is aimed at moving wild genes into traditional breeding programs as quickly as possible. It's part of EMBRAPA's larger effort to help Brazil expand production of potato, the country's most important vegetable crop.

In Lima, the Crop Trust has funded CIP to test wild varieties for promising traits even before any breeding begins. In 2013, center researchers started to characterize 12 wild species collected 30 years ago. Records suggested those species might tolerate drought and resist diseases such as bacterial wilt, a serious problem for developing countries. Merideth Bonierbale and colleagues planted seeds and have tested the plants in greenhouses at CIP's main facility. Mendes is now expanding the work to Kenya.

Other researchers are skirting the limitations of traditional breeding by using genetic engineering. CIP's Marc Ghislain and colleagues, for example, have directly added genes to already successful potato varieties without altering the plants in any other way—an approach not possible with traditional breeding. They took three genes for resistance to late blight from wild relatives and added them to varieties of potato popular in East Africa. The engineered varieties have proved successful in 3 years of field tests in Uganda and are undergoing final studies for regulators. Transgenic potatoes that resist late blight have already been commercialized in the United States and Canada.

Biotech approaches have their own limits. They have succeeded with traits controlled by single genes, such as disease resistance and tolerance of bruising. But improving complex physiological traits governed by many genes, such as water-use efficiency, requires traditional breeding, however cumbersome.

IN WAGENINGEN, Pim Lindhout has been plotting a revolution that would do away with much of that tedium and complexity. As head of R&D for Solynta, a startup company founded in 2006, he and his colleagues have been developing a new way to breed potatoes: creating hybrid offspring from true-breeding parent lines. "Everyone was convinced it's impossible," he says. "Many people thought I was crazy."

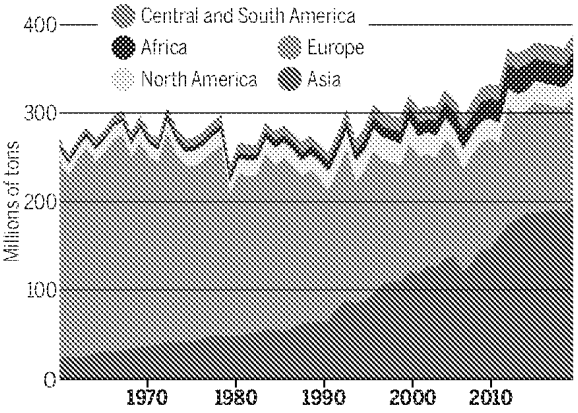
Hybrid breeding revolutionized maize production in the 20th century. It enabled breeders to quickly create high-yielding varieties that have what's known as hybrid vigor. The first step is to make inbred parent lines, which have identical alleles on all chromosome copies; the offspring of those true-breeding parents then inherit a predictable set of traits. Making the inbred lines requires repeated self-pollination over many generations. That process tends to impair the health of the plants, but when breeders cross two inbred lines, the first-generation offspring are healthy and have beneficial traits from both parents.

Potato breeders doubted the approach was possible for tubers. "I was trained to believe that potatoes can't be inbred," says Shelley Jansky, a potato breeder with the U.S. Department of Agriculture in Madison. One big obstacle is that many potato species cannot fertilize themselves. In 1998, researchers discovered a gene that somehow allows one wild species of potato to self-fertilize. When that gene is bred into other species, it lets them self-fertilize as well. But the resulting plants are frail and produce puny tubers.

The next step is to inbreed those weaklings by self-fertilizing them, generation after generation. Don't bother trying it at home: Success with cultivated potatoes would likely

Growing appetite

Potato production has grown in Asia, particularly in China and India, while falling in Europe.



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Aardevo Exhibit 1010
Page 1020
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Wild species have traits that could improve cultivated potatoes. Alberto Salas of the International Potato Center in Lima prepares a sample of *Solanum contumazaense* (above); its hairs defend against insects.

take decades because of the small odds of getting the same allele on all four copies of their chromosomes. Breeders reduce the complexity either by using species with only two sets of chromosomes (known as diploids) or by manipulating domesticated potatoes to cut the number of chromosomes in half. With persistence, diploid potatoes can be inbred. In 2011, Lindhout published the first report of inbred diploid lines that are vigorous and productive. More recently, Jansky and colleagues also created inbred diploid lines.

Such diploid inbred plants are at the heart of Solynta's strategy to revolutionize potato breeding. They will make it possible to combine traits in commercial varieties with unprecedented certainty, ease, and speed. And the plants will simplify efforts to add desirable traits directly from wild relatives while eliminating their many drawbacks, such as small tubers or poor flavor. Undesirable traits can be bred out of the descendants of a diploid cross through a standard technique called backcrossing.

In 2016, Solynta conducted its first field trials of hybrid seedlings in the Democratic Republic of the Congo and in 17 locations across Europe. The plants did well, yielding large tubers over a typical growing season. The company has not yet commercialized a variety. Within a few years, it hopes to create customized potatoes for European and African markets. Other firms, including large seed companies, are also working to develop hybrid potatoes. HZPC in Joure, the Netherlands, has begun field trials in Tanzania and in several countries in Asia.

Hybrid breeding "could be a real game changer," says geneticist Glenn Bryan, head of the Potato Genetics and Breeding group at the James Hutton Institute in Dundee, U.K. "It will definitely make breeding more agile."

Basic research could benefit from the work. "Having diploid potatoes will drastically increase our understanding of the potato genome," Shannon says. Although firms typically keep their inbred plants secret, Solynta plans to release a line, dubbed Solynthus, so that scientists can study its genetics. Jansky, for one, hopes further research could reveal genes that control yield, which might then be tapped to boost harvests.

Hybrids could also change how potatoes are planted, giving farmers the option of sowing fields with true seeds, because these are genetically identical in hybrids. Another benefit is logistical; planting 10 hectares, for instance, takes just 200 grams of easily transported seeds, compared with 25 tons of bulky tubers. In the developing world, where quality seed tubers are rare, seeds could also make obtaining superior plants easier for farmers. And in perhaps the biggest advantage over tubers for poor farmers, seeds transmit no major diseases.

Hybrid potato seeds aren't a panacea. Young plants grow more quickly and vigorously from tubers than from seeds, putting seeds at a disadvantage in some climates. And depending on how complete the inbreeding, hybrid potatoes could have less uniformly shaped tubers than those of traditional plants, a problem for farmers who supply food-processing companies. Such complexities have prompted the Dutch government to commission a study of the potential socioeconomic impacts of hybrid potatoes.

With collectors amassing genetic diversity and new techniques promising to overcome the complexities of the potato genome, researchers are optimistic that they can make significant improvements. "That's what gets me up in the morning," Jansky says. "There's no better time to be a breeder—and especially a potato breeder."

UNTIL HYBRID BREEDING and other strategies produce more resilient potatoes, farmers will have to work with the resources at hand. Here in Peru's Sacred Valley, Ellis and others from CIP have teamed with small-scale farmers who belong to an association known as Potato Park, which is dedicated to preserving hundreds of local potato varieties. The group has been planting those colorful potatoes in test plots.

Some succumb to pests or drought, like those that Ellis found dead, whereas others survive. In May 2018, as part of their search for more resilient tubers, Potato Park farmers neatly piled red, yellow, and brown tubers harvested from some of Ellis's experimental plots on rows of sacks, scoring each variety for yield and health. Local farmers had abandoned many of those landraces generations ago, as villages faded and exchange fewer plants.

Bringing some of that ancient diversity back into cultivation could hedge against environmental change. In Potato Park, farmers have already tried to escape the pests and disease that thrive in warmer temperatures by moving their cultivation 200 meters higher over the past 30 years. But René Gomez, CIP's curator of cultivated potatoes, warns that arable land is scarcer at higher elevations.

Pedro Condori Quispe, one of the park's growers, is optimistic that the communities will find a way to keep growing potatoes here. Potato farmers, he says with a smile, "are used to challenges." ※

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Aardevo Exhibit 1010
Page 1021
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Research Article Open Access

Michiel de Vries*, Menno ter Maat and Pim Lindhout

The potential of hybrid potato for East-Africa

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Abstract: Potato is an important staple crop in East Africa. Most of the seed tubers are propagated in informal systems, whereby the tubers become deteriorated and contaminated, resulting in low crop yields. Potato breeding has not resulted in significantly improved varieties that overcome these constraints. Recently, true potato hybrid breeding technology has been developed, whereby diploid hybrids are generated by crossing between homozygous inbred parent lines. The first series of experimental hybrids were pre-screened in The Netherlands and ten representative hybrids were tested in East-Africa, whereby the yield of the best hybrid was 29 ton/ha. These results show the great potential of hybrid potato for East Africa.

Keywords: Hybrid breeding, true seed, genetics, ploidy, cropping system, yield

1 Introduction

Potato is the world's third food crop for human consumption, and is grown all over the world. Breeding of new cultivars is done by national public institutes, by the international Potato Centre (CIP) or by private industries, mainly in Europe. Despite a growing worldwide number of more than 4000 cultivars (http://cipotato.org/potato/facts/), still very old varieties like Russet Burbank (introduced in 1876) in USA and Bintje (introduced in 1910) in Europe, are cultivated. The lack of genetic progress is also reflected in the lack of yield gain over the past century (Douches et al., 1996).

In East-Africa, potato is an important staple crop, with doubled production area over the last 20 years. Mainly CIP varieties and some European varieties are grown. The vast majority of potato is cultivated by millions of small scale farmers, who use farm saved seeds or acquire seed tubers from local markets (Gildemacher et al., 2009). The average yield in East African countries is low and ranges between 5 and 15 ton/ha (Gildemacher et al., 2009; Janssens et al., 2013; FAOstat, 2016). The propagation, storage and distribution of high quality seed tubers is the main constraint that limits production. Therefore, there is a great need for high quality seed tubers or true seeds.

There are several techniques to generate high quality mini-tubers from clean *in-vitro* grown potato plantlets (Tierno et al., 2014). This requires a formal potato production chain as these technologies are applied in specialised companies or public institutes and several rounds of clonal propagation are used before the seed tubers are distributed to the farmers. During these rounds of clonal propagation, the seed tubers may become contaminated with pathogenic micro-organisms. This is even more harmful when farmers keep tubers for next season as the costs of formal seed tubers may be too high for refreshment at each cultivation cycle (Kaguongo et al., 2008). This will further deteriorate the quality of the seed tubers (Thomas-Sharma et al., 2016).

Recently, Solynta has developed a true hybrid breeding system in diploid potato, whereby homozygous parent lines are developed by several rounds of inbreeding and hybrid cultivars are generated by crossing these parent lines (Lindhout et al., 2011). This method enables the creation of true seeds that are genotypically uniform. The starting material was an interspecific cross between diploid potato and the wild species *S. chacoense*, that was used to overcome the self-incompatibility (Lindhout et al., 2011). It took many rounds of crossing, selfings and selections to increase the performance of the inbred lines and to generate the first experimental hybrids (Lindhout et al., 2017). This paper describes the first trials with these experimental hybrids in tropical highlands, in Congo DR. The comparison of hybrid performance in Congo and The Netherlands is made. The results show the potential of hybrid potato for East-Africa.

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2 Materials and methods

2.1 Genetic material

Inbred lines of diploid potato were developed in a dedicated diploid breeding programme as described by Lindhout et al. (2011; 2017). In winter 2014/15 the first series of 203 hybrid crosses were made. Typically, the parents of these hybrids were obtained after several rounds of crossing and selfings followed by at least three rounds of selfing. As an example, the pedigree of hybrid SOL1-7 is shown in Figure 1. From this figure, it becomes clear that the three heterozygous diploid founders (D1, D2 and D16) were crossed with the *Sli*-donor DS, to create self-compatible populations (Lindhout et al., 2011). Subsequent generations of selfing resulted in homozygous parents of the hybrid SOLH1-7. Ten hybrids were selected as representatives for this series of 203 hybrids for further testing in DRC and designated SOLH1 - SOLH-10.

2.2 Selection of potato hybrids in The Netherlands

In 2015, the very first series of 203 experimental diploid potato hybrids were grown in The Netherlands to produce seed tubers. On May 1st, 2015, 100-200 seeds of these hybrids were sown in seed-boxes substrate in a heated greenhouse with 16 hours' day length. The seedlings were transplanted in Est, The Netherlands on June 2nd 2015, in irrigated ridges at 30 cm spacing within a ridge and 75 cm distance between the ridges in one replicate with plant numbers dependent on the number of seedlings available. Soil type was a heavy fluvial clay soil (28% clay). A typical crop management scheme was applied: fertilization and crop protection was done as recommended. Aphids were controlled by weekly chemical treatments combined with mineral oil. The first two weeks after transplanting overhead irrigation was applied two times to protect the seedling against drying out. Tubers were harvested on September 25th, two weeks after chemical defoliation of the plants by Reglone® (diquat dibromide). The tuber weight was evaluated per plot.

2.3 Field trial with potato hybrids in DRC

The experimental site was located at Nioka, Albertine Rift in Ituri Province, Democratic Republic of Congo at 1680 m.a.s.l. The site was chosen for its representativeness for the potato growing environment in the highlands of East-Africa. On January 25th, 2016, 200 seeds of the ten selected experimental hybrids, designated SOLH1-SOLH-10, were sown in seed-boxes with a substrate composed of goat manure, straw, ash and sand. The seed-boxes were placed

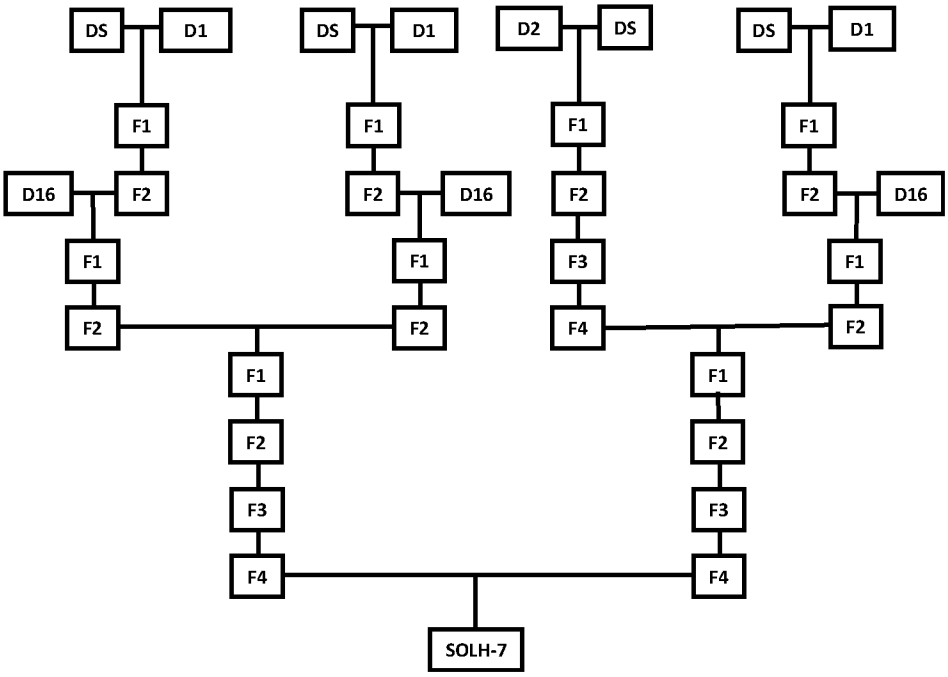

Fig 1. A typical example of an experimental diploid potato hybrid "SOLH-7", obtained by various crossing among the diploid founders D1, D2 and D16 and the Sli-donor DS (see Lindhout et al., 2011)

in an open shed, with a roof to protect against the sun and nets to protect against insects. The seedlings were transplanted on March 10th, in irrigated ridges, with 30 cm spacing within a ridge and 75 cm spacing between the ridges. The experiment was planted in three non-random replicates of 18 to 39 plants per plot, depending on the number of plants available. During the first two months after transplanting hand watering was done to protect the seedlings against drying out. Tubers were harvested on July 1st, when all plants had naturally died. The tuber weight and tuber number per plant was evaluated per plot.

3 Results

The seedlings of the first series of 203 experimental diploid potato hybrids were grown in the field in the Netherlands and evaluated for yield. The germination rate was 74 to 99%. After emergence, 6-weeks old seedlings were transplanted in the field. The growth of the field-plants was artificially stopped after 116 growing days. The tuber yield per plant varied from 0 to 580 gr, which is equivalent to 0 to 26 ton/ha (not shown). A representative set of ten hybrids was selected for trialing in DRC and designated SOLH-1 to SOLH-10.

In many regions in Africa, the potato yield, starting from seed tubers, is only 5 to 10 tons/ha. The greatest constraint is the quality of the seed tubers. True potato seeds are completely free of contaminating pathogens and may solve the lack of good potato starting materials. Therefore, a field trial starting with true seeds of the ten selected experimental hybrids was done in Nioka, Congo DR.

The germination rate after two weeks was on average 64%, ranging from 54 to 76%. After transplanting, a total of 1320 seedlings developed into plantlets, that were between 5-8 cm tall and had on average 8 true leaves at transplanting.

All available seedlings were transplanted into ridges according the standard cultivation techniques, resulting in plot sizes of 110 to 140 plants. Irrigation was performed during the first two months after transplanting but not during the last two months. Visual observation of damage was noted and 54 cases of insect damage during the growing season were noted, assessed as different types of crickets and ants. As there was no rain from 90 days after transplanting all plants were dead at the beginning of the harvest at 113 days after transplanting into the field.

The tuber yield ranged between 167 (SOLH-8) to 640 grams (SOLH-7) per plant, which is equivalent to 8 to 29 ton/ha (Fig 2). Tuber number ranged from 11 (SOLH-5) to 37 (SOLH-7) per plant, with 17 to 48 gr/tuber (not shown). SOLH-1 had the highest number of tubers in the 40-60mm class: three per plant.

The yield data of the same genetic materials at two locations allow to study the genotype x environment (GxE) interaction. Therefore, we compared the yield in gr/plant between the field trial in Democratic Republic of Congo and in The Netherlands (Fig 3.). Despite the large

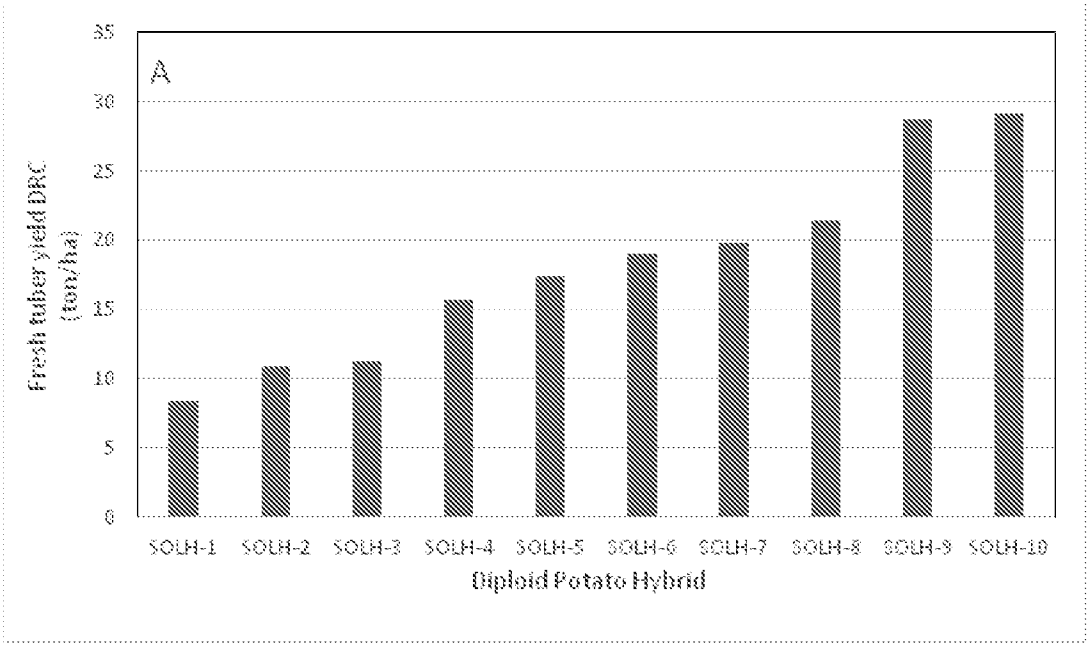

Fig 2. Yield data of ten experimental diploid potato hybrids, grown from seedlings in a field trial in Nioka, DR Congo

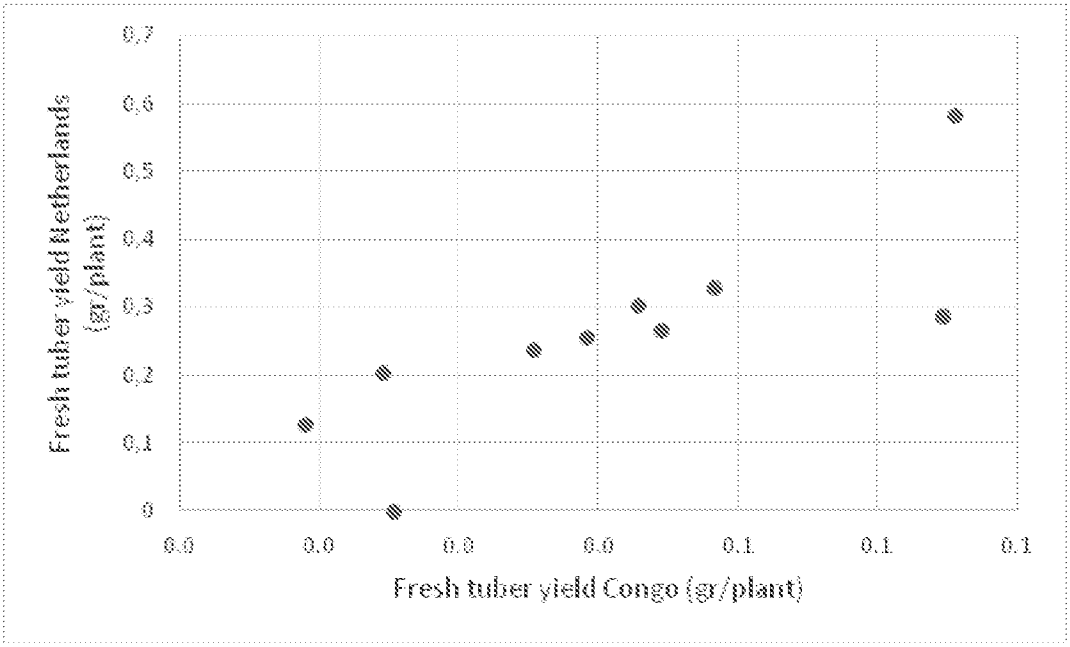

Fig 3. Correlation between the yield in gram/plant of ten diploid experimental potato hybrids in Congo DR compared to The Netherlands

difference in climatic conditions, geographical location and agricultural practices at both locations, we observed a striking correlation between both field tests, whereby the top five hybrids and the bottom five hybrids in both locations were identical.

4 Discussion

This paper describes the very first trial with the very first experimental diploid potato hybrids, generated from advanced inbred lines, reported ever. Before 2014, no dedicated crosses were made to generate experimental hybrids. One of the advantages of hybrid seeds as the number of seeds produced in the first cross as usually 50 to 250 seeds are collected and this number is sufficient to test the performance at multi-locations. In the present study two very distant locations in the world were chosen as the first testing sites for hybrids grown from seeds.

The parents of the hybrids were only selfed for three generations after the last breeding cross (Fig. 1). So, these parents may contain 10 to 20% heterozygous loci. Giving the very large genetic variation in potato (PGSC, 2011; Uitdewilligen et al., 2013; Visser et al., 2014), these parents may show phenotypic segregation for many traits. The main aim of the present study is to evaluate the yield of experimental hybrids and therefore we paid little attention to other traits that were expected to segregate.

The average yield in East Africa ranges from 5 ton/ ha in Uganda to 14 ton/ha in Kenya (FAOstat, 2016). The diploid potato hybrids yielded 8 to 29 ton/ ha in DRC, even higher than in The Netherlands with max. yield of 26 ton/ha. This higher yield in DRC is most likely due to the more favourable weather conditions at the tropical highlands, where the temperature is ideal for potato cultivation, while the harsh spring in The Netherlands is often too cold for potato plant growth.

Compared to the average yield in East Africa of 7 to 15 ton/ ha, the yield of the highest yielding potato hybrids was 29 ton/ha. This extra yield may also be due to optimisation of the cultivation conditions with such as fertilization and irrigation. The tuber number was high, up to 37, while the tuber size was small (17-48 gr/tuber), compared to 50-100 gr for usual tuber weight range (Struik & Wiersema, 2012). Extension of the growing period by irrigation could be a way to increase yield. Further research with tubers raised hybrid plants and tuber raised controls is needed to better dissect the cultivation conditions with the genetics, combined with the effect of clean seeds.

The yield of the highest yielding diploid hybrid in The Netherlands was about half of the yield of seed tuber production of 35 - 40 ton/ha (FAOstat, 2016). So, the highest yielding hybrid, grown from seedlings, showed about half the yield of a commercial tetraploid hybrid, grown from seed tubers.

It is known that seed tubers (raised from tubers) give higher yields than seedling transplants (raised from seeds) (Almekinders et al., 1996; Muthoni et al., 2014). In addition, diploids show lower yields than tetraploids

(Hutten et al., 1994), though interspecific diploid progenies may show high yields as well (Jansky et al., 2016). In this study, we introduce the first diploid potato hybrid. The genetic origin of this breeding system is an interspecific cross between *S. tuberosum* and *S. chacoense* (Lindhout et al., 2011; Fig 1.). The plants of the early selfed progenies were extremely weak (Lindhout et al., 2017) and we did not expect that the very first experimental diploid potato hybrid already have high yields. However, a yield of 50% of the best hybrid grown from seeds compared to the yield of tetraploid seed tuber starting from seed tubers is remarkably high.

The 50% lower yield is due to three confounded factors: diploid versus tetraploid, experimental hybrid versus commercial cultivar and seedlings versus seed tubers. Further tests with tuber grown plants and tetraploid experimental hybrids are needed to dissect these factors.

These results illustrate the potential of diploid hybrid potato in combination of good cultivation practises. In the present study, the cultivation period in DR Congo was restricted by the lack of rainfall. We expect that with a better irrigation system or during the rainy season the yield of these hybrids would have been even higher. In addition, these were the very first hybrids generated by crossing parents without any a prior information about general combining abilities (GCA) of the parent lines. The present results are being used to select parent lines based on high GCA values. New parent lines will also be more inbred and hence more homozygous and more uniform. As a result, the next series of hybrids, is expected to be more advanced, both in terms of tuber yield, tuber quality as well as tuber uniformity.

In addition, by using marker assisted backcrossing programmes a new series of hybrids with new traits can be obtained such as disease resistances to *Phytophthora infestans* and tuber quality (Lindhout et al, 2017). Finally, locally popular tetraploid varieties such as Kinigi in Rwanda could be exploited for a dedicated diploid hybrid potato programme for East Africa to develop a hybrid, that is optimally adapted to the climate conditions and market preferences in East Africa. However, such an effort is to start a breeding programme anew, therefore it may take ten years to have the first hybrid released to the market.

In conclusion, hybrid potato has shown a great potential and will pave the way to a prosperous potato food chain that will contribute to feed the present and future fast growing populations in East Africa.

Acknowledgements: Lake Albert Investment is greatly appreciated for the reliable testing of the hybrids at their farm in Nioka, DRC. The Food & Business Knowledge Platform provided financial support to visit East Africa by MdV and PL. Sadrach Talahatu (Solynta) is acknowledged for preparing Fig 1.

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Aardevo Exhibit 1010
Page 1027

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	Attorney Docket No.: VONL:004USC1
For: Hybrid Seed Potato Breeding	Confirmation No. 4348

RESPONSE TO FINAL OFFICE ACTION DATED NOVEMBER 9, 2020

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

Commissioner:

This paper is submitted in response to the Final Office Action dated November 9, 2020, for which the deadline for response is February 9, 2021. A Request for Continued Examination and fees and a Supplemental Information Disclosure Statement are also filed herewith. No additional fees are believed due in connection with this submission. However, the Commissioner is authorized to deduct any fees that may become due for this case under 37 C.F.R. §§ 1.16 to 1.21 from Dentons US LLP's Deposit Account No.: 19-3140/VONL:004USC1.

Amendments to the Claims begin on page 2.

Telephonic Interview Summary begins on page 6.

Remarks begin on page 7.

Aardevo Exhibit 1010
Page 1028

AMENDMENTS TO THE CLAIMS

Please amend the claims as follows:

1-34. (Cancelled)

35. (Currently Amended) A plant of a potato line, wherein said potato line is of the species *Solanum tuberosum* optionally comprising introgression segments of other tuber bearing *Solanum* species crossable to *Solanum tuberosum*, wherein said potato line is diploid, fertile, self-compatible, produces at least 200 grams fresh weight of tubers per plant, and at least [[50]]75% of the genomic loci in said potato 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 potato plant of claim 35, wherein said plant ~~further~~ comprises at least one agronomically desirable trait 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 resistance.

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

39. (Cancelled)

40. (Previously Presented) A seed or tuber of the plant of claim 35.

41. (Currently Amended) A method of producing a plant of a potato line, wherein said potato line is diploid, fertile, self-compatible, produces at least 200 grams fresh weight of tubers per plant, and at least [[50]]75% 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 according to claim 35;

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

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

(d) growing ~~a multiplicity of~~ said hybrid offspring seeds to provide[[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 said self-compatible potato plant ~~to a potato plant of said line of said first potato plant,~~ for between 1 to 8 selfings to thereby increase the degree of homozygosity in each successive generation; and

(f) selecting within each generation of inbreeding for a fertile offspring plant, and providing a diploid, fertile, self-compatible potato plant, wherein at least [[50]]75% of the genomic loci in said plant are homozygous.

42. (Cancelled)

43. (Previously Presented) The method of claim 41, wherein said second potato plant is a donor for an 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.

44. (Previously Presented) A potato plant produced by the method of claim 41.

45. (Currently Amended) A method of producing a uniform diploid hybrid *Solanum tuberosum* potato seed, which seed, when sown produces a diploid, fertile, self-compatible and essentially heterozygous potato plant having a tuber yield expressed in grams of fresh weight of at least 200 grams and wherein said self-compatibility trait is due to the presence of the *Sli* gene, said method comprising:

(a) providing a first potato plant, wherein said first potato plant is a plant of a first potato line, wherein said first potato line is of the species *Solanum tuberosum* optionally comprising introgression segments of other tuber bearing *Solanum* species crossable to *Solanum tuberosum*, wherein said first potato line is diploid, fertile, self-compatible and wherein at least [[50]]75% of the genomic loci in said first potato line are homozygous, 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, and wherein said first potato plant has a tuber yield expressed in grams of fresh weight of at least 200 grams;

(b) providing a second potato plant, wherein said second potato plant is a plant of a second potato line, wherein said second potato line is of the species *Solanum tuberosum* optionally comprising introgression segments of other tuber bearing *Solanum* species crossable to *Solanum tuberosum*, wherein said second potato line is diploid fertile, self-compatible and wherein at least [[50]]75% of the genomic loci in said second potato line are homozygous, 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, wherein said second potato plant has a tuber yield expressed in grams of fresh weight of at least 200 grams, and wherein said 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 said second potato plant[[s]] and allowing ~~one of~~ said potato plant[[s]] to produce berries with seeds; and

(d) collecting said seeds from said berries to provide [[a]]hybrid potato seeds.

46. (Cancelled)

47. (Currently Amended) The method according to claim 45, wherein at least one of said first and said second potato plant[[s]] 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.

48. (Previously Presented) A uniform hybrid potato seed produced according to the method of claim 45.

Aardevo Exhibit 1010
Page 1032

TELEPHONIC INTERVIEW SUMMARY

On February 9, 2021, Applicants' undersigned representative, Sheila Walker, Reg. No. 71,087, spoke telephonically with Examiner Boggs to discuss submission of a color version of the figure shown in Exhibit B of the present response. The Examiner indicated that the color figure could be sent to him via email. In view of this, Applicants have sent the figure to the Examiner via email on February 9, 2021 accordingly.

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

Aardevo Exhibit 1010
Page 1033

REMARKS

A. Status of the Claims

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

B. Information Disclosure Statement

The Action states that the title listed for reference #2 of the non-patent literature documents (de Vries *et al.*, 2016) in the information disclosure statement submitted May 5, 2020 is inconsistent with the journal citation in the IDS and no copy from that journal citation was provided, but notes that the Examiner obtained the reference associated with the listed journal citation and has been considered. Applicants thank the Examiner for consideration of the reference and have submitted herewith a supplemental IDS listing the title of the reference accordingly.

C. Claims Objections

The Action objects to claims 35, 41, and 45 due to informalities. Applicants note that claims 35, 41, and 45 are amended herein in the interest of compact prosecution. The objection is thus believed to be moot and withdrawal thereof respectfully requested.

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

The Action rejects claims 35, 37-38, 40-41, and 43-44 under 35 U.S.C. §112 (pre-AIA), 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, fall within the scope of the claims or the breeding history of the deposited lines. The Action further asserts that one of skill in the art would not

Aardevo Exhibit 1010
Page 1034

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. The Action acknowledges the 132 Declaration of Dr. Lindhout that was submitted with the Response to Office Action filed on May 5, 2020, but states that the arguments made based on the Declaration were found to be unpersuasive. Specifically, the Action asserts that the crux of the previous arguments relies on the opinions of Dr. Lindhout, who is the first named inventor of the application, and Dr. Jones, who is allegedly recanting his previous statements due to their misinterpretation without explaining how the statements were misinterpreted or inaccurate. The Action also notes that the 2012 letter from Dr. Koornneef and the previously cited Jansky reference (*Crop Sci*. 56:1412-22, 2016; "Jansky 2016") remain un-contradicted on the record. Applicants respectfully traverse.

The Action asserts that in his Declaration dated August 15, 2016, Dr. Lindhout argued that success of the present invention relied not only on use of the *Sli* gene but also on the potato line used as the starting material. Specifically, it is asserted that the unique nature of the deposited potato lines is emphasized by statements made by Dr. Lindhout in the 2016 Declaration at, for example, p. 4 of the document and by statements made by Applicants in the EPO proceedings for the parent application of the present case, which characterize the chances of successfully obtaining the plant of the of the invention as "close to zero" and "impossible" due to inbreeding depression in potato that is mainly caused by the presence of deleterious or lethal alleles. The Action further asserts that one of ordinary skill in the art is given no criteria in the claims to distinguish an "impossible" plant from a "possible" plant, such as those found in the deposited lines. Applicants note that the statements cited by the Examiner are in no way an admission that the plants of the present invention are "impossible" plants to generate, as such statements are made with reference

to the state of the art at the time the invention was made and not as to a person of skill in the art in possession of the present specification teaching. The statements made in the EPO proceedings indicate that, in order to arrive at the invention, the important hurdle of inbreeding depression had to be overcome. The inventors were able to overcome inbreeding depression and attain the claimed plants through a novel method *as described in the application*: by introgressing the *Sli* gene into *S. tuberosum* germplasm, and increasing homozygosity by inbreeding (selfing) while selecting for fertile offspring.

Indeed, the chances of obtaining the claimed plants are very small when using methods other than those disclosed in the present application, such as those described in the prior art cited by the Examiner. Contrary to the Action's assertion, Jansky 2009, Phumichai *et al.* 2005, and Jansky 2016 *do not* describe the approach of the present invention. For example, Jansky 2009 does not describe introducing the *Sli* gene into *S. tuberosum*, followed by selfing to obtain homozygous inbreds. As described in Stokstad 2019 (**EXHIBIT A**), Jansky appears to believe that potatoes cannot be inbred (p. 576, right hand column of the reference). Phumichai *et al.* 2005 proposes an inbreeding technique for obtaining homozygous potatoes, but this technique is not performed with *S. tuberosum*. Moreover, Phumichai *et al.* 2005 proposes inbreeding followed by somatic hybridization, which is a technique involving *in vitro* fusion of isolated protoplasts to form a hybrid cell and its subsequent development to form a hybrid plant. This technique is aimed at producing, and will result in, tetraploid plants, not diploid plants, as contemplated by the present inventors and as claimed. Jansky 2016 is not a scientific paper and thus cannot be taken as an objective scientific indication of the progress and achievement of the inventors. The statement made by the Examiner on p. 12 of the Action that Jansky 2016 demonstrates that "approximately seven years after the filing date of the instant application, the problem of creating vigorous inbred

diploid *S. tuberosum* plants apparently still exists," disregards the teaching and contribution of the present invention as described in the application and as published by Lindhout *et al.* in 2011, which is now widely adopted by the potato breeding industry. With regard to the assertion that Jansky 2016 remains un-contradicted on the record, this is incorrect as the Declaration of Dr. Lindhout under 37 C.F.R. § 1.132, submitted on May 5, 2020, addresses Jansky 2016 in paragraphs 30-37 of the document.

The Action asserts that Dr. Jones' new position is unpersuasive on the basis that he recanted his statement from his 2012 letter without specific reference to the previous letter itself and that his comments are presented in a letter and not in a Declaration. Therefore, submitted herewith is the Declaration of Prof. Jonathan D. G. Jones under 37 C.F.R. § 1.132. With regard to the assertion made in the Action that the previous letter written by Dr. Jones in 2012 "speaks for itself," (p. 18 of the Action), Dr. Jones provides his comments regarding the portion of the letter cited in the present Action in paragraphs 10 and 11 of the Declaration. Furthermore, at paragraph 11 of the Declaration, Dr. Jones clearly states that he does not believe that the claimed methods are limited to lines AGVD1, AGVD2, AGVD3, and AGVD17.

The Action proceeds to state at p. 18 that "[E]ven if Dr. Jones was persuasive in contradicting his earlier letter, his new position is irrelevant to the letter from Dr. Koornneef as seen below." At p. 19 of the Action, a portion of the letter dated December 18, 2012 from Dr. Koornneef is reproduced as evidence that the present invention is not fully described. The Action asserts in this regard that the letter of Dr. Koornneef remains un-contradicted on the record. In view of this, submitted herewith is the Declaration of Prof. Maarten Koornneef under 37 C.F.R. § 1.132. At paragraph 8 of the Declaration, Dr. Koornneef acknowledges the portion of his 2012 letter cited in the present Action and states that he disagrees with the Examiner's interpretation of

the contents of the letter and the Examiner's assertions regarding the claimed invention. At paragraph 10 of the Declaration, Dr. Koornneef states that the present application allows breeders to generate essentially homozygous diploid potato lines starting from a *wide array of diploid potato germplasm*, especially diploid *S. tuberosum* germplasm and that the claimed methods are not unique to lines AGVD1, AGVD2, AGVD3, and AGVD17.

With regard to the breeding histories, and thus the genetic backgrounds, of the deposited lines, Applicants note that Figure 1 (**EXHIBIT B**) from the 132 Declaration of Dr. Lindhout filed on May 5, 2020 is submitted herewith. As discussed in the Response to Office Action filed on May 5, 2020, in his Declaration, Dr. Lindhout extensively discusses the genetic variation in lines D1, D2, and D3. This variation demonstrates that D1-D3 are germplasm representative of the entire range of genomic diversity in the *S. tuberosum* germplasm that is currently available. This therefore demonstrates that the specification 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. Thus, the invention is not limited to a specific potato line, 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.

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

The Action rejects claims 35, 37-38, 40-41, and 43-44 under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement in view of the conditions of the seed deposit. Applicants hereby affirm that a deposit of at least 2,500 seeds each of potato lines AGVD1, AGVD2, AGVD3, and AGVD17, was made with the NCIMB in accordance with the

Budapest Treaty requirements and with 37 C.F.R. §1.801-1.809 and all restrictions upon availability to the public will be irrevocably removed upon granting of the patent. Applicants note that the Specification and claims as filed recite the deposit information. 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 - Enablement

The Action rejects claims 35, 37-38, 40-41, and 43-44 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, while such guidance is allegedly not provided in the application. The Action acknowledges the 132 Declaration of Dr. Lindhout that was submitted with the Response to Office Action filed on May 5, 2020, but states that the arguments made based on the Declaration were found to be unpersuasive. Specifically, the Action asserts that the crux of the previous arguments relies on the opinions of Dr. Lindhout, who is the first named inventor of the application, and Dr. Jones, who is recanting his previous statements due to their misinterpretation without explaining how the statements were misinterpreted or inaccurate. The Action also notes that the 2012 letter from Dr. Koornneef and the previously cited Jansky reference (*Crop Sci.* 56:1412-22, 2016; "Jansky 2016") remain un-contradicted on the record. Applicants respectfully traverse.

The Action asserts that in his Declaration dated August 15, 2016, Dr. Lindhout argued that success of the present invention relied not only on use of the *Sli* gene but also on the potato line used as the starting material. Specifically, it is asserted that the unique nature of the deposited

Aardevo Exhibit 1010
Page 1039

potato lines is emphasized by statements made by Dr. Lindhout in the 2016 Declaration at, for example, p. 4 of the document and by statements made by Applicants in the EPO proceedings for the parent application of the present case, which characterize the chances of successfully obtaining the plant of the of the invention as "close to zero" and "impossible" due to inbreeding depression in potato that is mainly caused by the presence of deleterious or lethal alleles. The Action further asserts that one of ordinary skill in the art is given no criteria in the claims to distinguish an "impossible" plant from a "possible" plant, such as those found in the deposited lines. Applicants note that the statements cited by the Examiner are in no way an admission that the plants of the present invention are "impossible" to generate. The statements made in the EPO proceedings were made in reference to the state of the art prior to the invention and indicate that in order to arrive at the invention, the important hurdle of inbreeding depression had to be overcome. The inventors were able to overcome inbreeding depression and attain the claimed plants through a novel method as described in the application: by introgressing the *Sli* gene into *S. tuberosum* germplasm, and increasing homozygosity by inbreeding (selfing) while selecting for fertile offspring.

Indeed, the chances of obtaining the claimed plants are very small when using methods other than those disclosed in the present application, such as those described in the prior art cited by the Examiner. Contrary to the Examiner's assertion, Jansky 2009, Phumichai *et al.* 2005, and Jansky 2016 do not describe the approach of the present invention. For example, Jansky 2009 does not describe introducing the *Sli* gene into *S. tuberosum*, followed by selfing to obtain homozygous inbreds. As described in Stokstad 2019 (**EXHIBIT A**), Jansky appears to believe that potatoes cannot be inbred (p. 576, right hand column of the reference). Phumichai *et al.* 2005 proposes an inbreeding technique for obtaining homozygous potatoes but this technique is not performed with *S. tuberosum*. Moreover, Phumichai *et al.* 2005 proposes inbreeding followed by

somatic hybridization, which is a technique involving *in vitro* fusion of isolated protoplasts to form a hybrid cell and its subsequent development to form a hybrid plant. This technique is aimed at producing, and will result in, tetraploid plants, not diploid plants, as contemplated by the present inventors and as claimed. Jansky 2016 is not a scientific paper and thus cannot be taken as an objective scientific indication of the progress and achievement of the inventors. The statement made by the Examiner on p. 12 of the Action that Jansky 2016 demonstrates that "approximately seven years after the filing date of the instant application, the problem of creating vigorous inbred diploid *S. tuberosum* plants apparently still exists," disregards the teaching and contribution of the present invention as described in the application and as published by Lindhout *et al.* in 2011, which is now widely adopted by the potato breeding industry. With regard to the assertion that Jansky 2016 remains un-contradicted on the record, Applicants note that the Declaration of Dr. Lindhout under 37 C.F.R. § 1.132, submitted on May 5, 2020, also addresses Jansky 2016, in paragraphs 30-37 of the document.

The Action asserts that Dr. Jones' new position is unpersuasive on the basis that he recanted his statement from his 2012 letter without specific reference to the previous letter itself and that his comments are presented in a letter and not in a Declaration. Therefore, submitted herewith is the Declaration of Prof. Jonathan D. G. Jones under 37 C.F.R. § 1.132. With regard to the assertion made in the Action that the previous letter written by Dr. Jones in 2012 "speaks for itself," (p. 18 of the Action), Dr. Jones provides his comments regarding the portion of the letter cited in the present Action in paragraphs 10 and 11 of the Declaration. Furthermore, at paragraph 11 of the Declaration, Dr. Jones clearly states that he does not believe that the claimed methods are unique to lines AGVD1, AGVD2, AGVD3, and AGVD17.

Aardevo Exhibit 1010
Page 1041

The Action proceeds to state at p. 18 that "[E]ven if Dr. Jones was persuasive in contradicting his earlier letter, his new position is irrelevant to the letter from Dr. Koornneef as seen below." At p. 19 of the Action, a portion of the letter dated December 18, 2012 from Dr. Koornneef is reproduced as evidence that present invention is not fully described. The Action asserts that the letter of Dr. Koornneef remains un-contradicted on the record. In view of this, submitted herewith is the Declaration of Prof. Maarten Koornneef under 37 C.F.R. § 1.132. At paragraph 8 of the Declaration, Dr. Koornneef acknowledges the portion of his 2012 letter cited in the present Action and states that he disagrees with the Examiner's interpretation of the contents of the letter and the Examiner's assertions regarding the claimed invention. At paragraph 10 of the Declaration, Dr. Koornneef states that the present application allows breeders to generate essentially homozygous diploid potato lines starting from a wide array of diploid potato germplasm, especially diploid *S. tuberosum* germplasm and that the claimed methods are not unique to lines AGVD1, AGVD2, AGVD3, and AGVD17.

With regard to the breeding histories, and thus the genetic backgrounds, of the deposited lines, Applicants note that Figure 1 (**EXHIBIT B**) from the 132 Declaration of Dr. Lindhout filed on May 5, 2020 is submitted herewith. As discussed in the Response to Office Action filed on May 5, 2020, in his Declaration, Dr. Lindhout extensively discusses the genetic variation in lines D1, D2, and D3. This variation demonstrates that D1-D3 are germplasm that are representative of the entire range of genomic diversity in the *S. tuberosum* germplasm that is currently available. Furthermore, the inventors found that all of D1-D3 could be rendered self-compatible, and that selfing was possible and resulted in self-compatible pants with sufficient seedset, as shown in Table 3 of the specification. In some selfed populations, fertility and self-compatibility was decreased relative to other populations, but it was found that in all *S. tuberosum* "backgrounds"

Aardevo Exhibit 1010
Page 1042

that were tested, fertile and self-compatible plants could be observed in selfed populations. This demonstrates that the invention is enabled over the full scope of the present claims.

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.

As demonstrated above, 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. 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 to remove the term "further" as suggested in the Action and in the interest of compact prosecution. The rejection is thus believed to be moot and withdrawal thereof is respectfully requested.

H. Rejection Under 35 U.S.C. § 103

The Action rejects claims 45 and 48 under 35 U.S.C. § 103 as being unpatentable over Phumichai and Hosaka (*Euphytica* 149:251-258, 2006; "Phumichai") in view of Birhman and Hosaka (*Genome* 43:495-502; 2000, "Birhman"). The Action also rejects claim 47 under 35

Aardevo Exhibit 1010
Page 1043

U.S.C. § 103 as being unpatentable over Phumichai in view of Birhman further in view of Bradshaw *et al.* (*Potato Res* 49:49-65, 2006; "Bradshaw"). The Action concedes that none of the references explicitly teach the required limitations for the claims, but asserts that Phumichai teaches selfing diploid potato clones using the *Sli* gene for obtaining self-compatible potato lines and at least one of these potato lines have 33 out of 62 loci as homozygous and that this would encompass the limitation of, "at least 50% of the genomic loci are homozygous." Birhman is asserted to teach the use of different potato lines to add traits to a reference line with the *Sli* gene and that the combination of this reference with the Phumichai reference renders claims 45 and 48 as obvious. It is also noted that there is no mention of tuber yield in claim 45. Claim 47 is asserted to also be obvious in view of the combination of Phumichai and Birhman, for the foregoing reasons, and Bradshaw, which is asserted to teach potato plants having disease resistance that were obtained through crossing.

Applicants respectfully traverse but note that claim 45 is amended herein to recite that that first and second potato lines have "at least 75% of the genomic loci are homozygous" and that the first and second potato plants have a tuber yield expressed in grams of fresh weight of at least 200 grams. The rejection is thus believed to be moot and withdrawal thereof is respectfully requested.

I. Double Patenting

The Action provisionally rejects claims 35, 37-38, 40-41, 43-45, and 47-48 on the ground of nonstatutory double patenting as being unpatentable over claims 1-19 of copending Application Ser. No. 13/504,156, which has now issued as U.S. Patent No. 10,524,436. Applicants respectfully

Aardevo Exhibit 1010
Page 1044

traverse but note that a Terminal Disclaimer will be submitted upon indication of otherwise allowable subject matter in order to expedite prosecution.

J. Conclusion

Applicants submit, in light of the foregoing, that the present case is in condition for allowance and such favorable action is respectfully requested. The Examiner is invited to contact the Applicants' undersigned representative at (214) 259-1890 with any questions, comments or suggestions relating to the referenced patent application.

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Respectfully submitted,

/Sheila Walker/

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

Date: February 9, 2021

Aardevo Exhibit 1010
Page 1045

EXHIBIT A

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THE NEW POTATO

Breeders seek a breakthrough to help farmers facing an uncertain future

By **Erik Stokstad**, *in the Sacred Valley of the Incas in Peru*

On a bleak, brown hill here, David Ellis examines a test plot of potato plants and shakes his head. "They're dead, dead, dead," he says. Pests and lack of rain have laid waste to all 17 varieties that researchers had planted.

It is a worrying sign for Ellis, the now-retired director of the gene bank at the International Potato Center (CIP) in Lima. People have grown potatoes in this rugged stretch of the Andes for thousands of years. In recent years, that task has gotten tougher, in part because of climate change. Drought and frost are striking more often. The rains come later, shortening the growing season. And warmer temperatures have allowed moths and weevils to encroach from lower elevations.

To find potatoes that can cope with those challenges, researchers and Peruvian farmers are testing dozens of the 4350 locally cultivated varieties, or landraces, kept in CIP's refrigerated storage. The plants in this plot fell short. "Native landraces evolved over time," Ellis says. But, he says, climate change is happening "too fast for these varieties to adapt."

In Peru and around the world, enhancing the potato has become a high priority. It is the most important food crop after wheat and rice. Potatoes are already a staple for 1.3 billion people, and the nutritious tubers are becoming increasingly popular in the developing world. Keeping up with the demand means adapting the potato to various soils and climates. It must also resist new threats from pests, disease, heat, and drought.

Unlike other major crops, however, the potato has not had a breeding breakthrough of the kind that helped dramatically boost yields during the Green Revolution of the 1950s and 1960s. The reason is that creating a new potato variety is slow and difficult, even by the patient standards of plant breeders. Commercial varieties carry four copies of each chromosome, which forces breeders to create and test hundreds of thousands of seedlings to find just one with the desired combination of traits.

Diverse potatoes, such as these from Peru, will help breeders create resilient new varieties.

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Aardevo Exhibit 1010
Page 1047

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Readying a new variety for farm fields can take a decade or more.

Many countries continue to plant popular potato varieties that have remained essentially unchanged for decades. But new approaches, including genetic engineering, promise to add more options. Potato breeders are particularly excited about a radical new way of creating better varieties. This system, called hybrid diploid breeding, could cut the time required by more than half, make it easier to combine traits in one variety, and allow farmers to plant seeds instead of bulky chunks of tuber. "It will change the world tremendously," says Paul Struik, an agronomist at Wageningen University in the Netherlands.

To breed a better potato, it helps to have plenty of genetic raw material on hand. But the world's gene banks aren't fully stocked with the richest source of valuable genes: the 107 potato species that grow in the wild. Habitat loss threatens many populations of those plants. In a bid to preserve that wild diversity before it vanishes, collectors have made their biggest push ever, part of a $50 million program coordinated by the Crop Trust, a charity based in Bonn, Germany.

The collectors and breeders are racing against warming, drying, and the proliferation of pests. "Because of climate change," says Nigel Maxted, a conservation biologist at the University of Birmingham in the United Kingdom, "we require higher levels of diversity than ever before."

THE CLOSEST ANCESTORS of cultivated potatoes evolved in the Andes, where people domesticated the plant at least 7000 years ago. After the Spanish brought the tuber to Europe in the 16th century, it remained a botanical curiosity and was mostly fed to livestock. Europeans began to eat potatoes in earnest only in the 1800s, during the famines of the Napoleonic Wars.

Once the potato caught on, there was no turning back. The plant can grow in cold climates and poor soil, and in some places yields several crops per season. Once harvested, the energy-rich tubers, packed with vitamin C, can be stored for months and cooked in many ways. A hectare of potatoes can provide up to four times the calories of a grain crop.

Like rice and wheat, the potato was a target for improvement during the Green Revolution. Yields increased thanks to fertilizer and improved farming techniques, but they didn't skyrocket. Potato breeders achieved no genetic gains such as the one that produced wheat with short, sturdy stalks that can bear more grain.

Still, global potato production has steadily grown (see graph, p. 576). China has doubled its harvests over the past 20 years. It now grows more than twice as many potatoes as India, the next-biggest producer. Uzbekistan and Bangladesh, among other nations, have come to depend on the potato for food security. In 2005, developing countries for the first time grew more potatoes than the developed world. Many African countries are aiming to boost production.

To reap bigger harvests, farmers will have to manage many risks, including disease.

Climate change threatens yields in Potato Park, a farming association near Cuzco, Peru.

The potato's greatest scourge is the fungus-like pathogen *Phytophthora infestans*, which causes a disease called late blight. The pathogen unleashed the Irish potato famine in the mid-19th century, and plant breeders have struggled ever since to rein it in. "*Phytophthora* is always evolving and overcoming resistance," says Jadwiga Śliwka of the Plant Breeding and Acclimatization Institute in Młochów, Poland. Rich countries use fungicides to minimize the devastating losses from late blight. But in developing countries, 15% to 30% of the crop is ruined.

Then there is heat and drought, which climate change is exacerbating. In some parts of the world, farmers are planting their crop earlier so that it matures before the nights get too hot, which prevents tubers from forming. But eventually farmers will need hardier plants. "We focus on developing a robust potato that will perform better in a stressful environment," says Thiago Mendes, a potato breeder with CIP's regional office in Nairobi. "Our target is food security."

The key to that robust potato may be waiting in the wild species that grow from southwestern North America through Central and South America. Wild potatoes from Mexico, for example, evolved in the presence of *P. infestans* and can resist many strains. Many other wild species have yet to be thoroughly collected or studied.

IN JUNE 2018, beside a cattle pasture in southern Brazil, botanist Gustavo Heiden strode along an embankment, his eyes fixed on the long grass. Then, he dropped to his knees and jabbed a trowel into the soil. "Aha! Look at this," said Heiden, who works with the Brazilian Agricultural Research Corporation (EMBRAPA) in Pelotas. He pulled up a short plant with small tubers dangling from its roots. It was *Solanum commersonii*, one of three wild relatives of the potato known in Brazil.

Brazil is far from the potato's center of origin in the Andes. But the ranges of wild relatives extend into the state of Rio Grande do Sul, where the climate shifts from temperate to tropical. Plants in this transition zone have evolved to survive occasionally harsh winters and hot, dry summers. "The wild potatoes here are probably pretty adapted to the extreme weather that will be happening more frequently with climate change," Heiden says.

Heiden's collecting trip was just one element of the Crop Trust's effort to collect, conserve, and breed the wild relatives of 29 crops, which began in 2011. Plant collectors used to travel the world on such expeditions. But they became much less frequent after governments began to adopt the Convention on Biological Diversity in the 1990s. Intended to prevent unfair exploitation of biodiversity, the convention made it harder to get collecting permits and to exchange plant material. An international seed treaty established in 2004 eased swaps for crops and wild relatives, but collecting remained stagnant because of lack of funding and expertise. "We didn't have any experience on how to collect wild potatoes or how to conserve them," says Cinthya Zorrilla, subdirector of genetic resources at the National Institute of Agricultural Innovation in Lima.

The Crop Trust has provided grants and training to collectors around the world. The effort on wild potatoes, which wraps up this month, has yielded a collection representing 39 species from six nations: Peru, Brazil, Ecuador, Guatemala, Costa Rica, and Chile. Zorrilla's team alone found 31 species in Peru, including one for which no seeds had ever been collected. They plan to continue to search for four other species still missing from gene banks. "We will not stop," she says. The plants are being stored in each nation's gene bank, CIP, and the Millennium Seed Bank at the Royal Botanic Gardens, Kew, in

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Aardevo Exhibit 1010
Page 1048
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the United Kingdom. The stored seeds will be available to potato breeders worldwide.

THE HARDEST PART comes next: getting desirable genes from wild species into cultivated potatoes. In the past, breeders acquired traits such as disease resistance from a dozen wild species. Those victories were hard-won, some taking decades to achieve. That's largely because wild relatives also carry many unwanted traits, which combine with those of cultivated potatoes and vastly lower a breeder's chances of finding a good variety.

Even without wild species, potato breeding is a crapshoot. Because breeding lines have four copies of their 12 chromosomes, the traits of the two parents show up in the next generation in largely unpredictable combinations. As experts say, the current varieties don't breed true, which is why farmers plant bits of "seed tuber," which yield genetically identical plants, rather than seeds. Compounding the headache, breeders select for many traits at once, further lowering the probability of finding a winner. "The numbers get really hard, really fast," says Laura Shannon, a potato breeder at the University of Minnesota in St. Paul.

Genetic markers linked to specific genes have sped up the process. To find out whether seedlings have inherited a trait such as disease resistance, breeders can quickly test for the marker rather than wait for the plants to mature and then expose them to the disease. Even with this tool, a potato breeder must screen up to 100,000 offspring per year. It can take 15 years or longer to find one with the right traits, fully test it, and generate enough seed tubers to distribute to farmers.

Another frustration is that potato breeders can't easily improve existing varieties. Once a potato variety is established, introducing new traits while retaining all of its favored characteristics is practically impossible. That's why classic, widely grown varieties, such as the russet burbank, still dominate the market many decades after their debuts.

Patient breeders using traditional methods can nevertheless achieve impressive results. In 2017, for example, CIP released four new varieties in Kenya, the result of crosses from established breeding lines. In field trials, the new potato plants maintained yields with 20% less rainfall and temperatures higher by 3°C.

Such success shows there is still genetic diversity to be tapped in existing breeding lines. But researchers fear that gene pool may not be deep enough to adapt the potato to future climates or enable other improvements. Wild potatoes, however, hold valuable, untapped genetic diversity. One trait from those wild plants, Mendes says, "could save our life."

THE SEARCH for vital traits is already underway. Last year, at an EMBRAPA research station near Pelotas, technicians in lab coats leaned over the wild species Heiden had collected. They gently daubed their faintly purple flowers with yellow powder from a plastic tube, fertilizing them with pollen from domesticated potatoes.

In a nearby greenhouse, tables were lined with the offspring of previous crosses. Researchers have evaluated thousands of those seedlings for health and yield, among other traits. They screened older plants for drought resistance by limiting the water in plastic-lined troughs. In a temperature-controlled walk-in chamber, researchers tested the ability of other plants to withstand heat; the yellowed plants appeared to be sweltering.

Such expansive testing is aimed at moving wild genes into traditional breeding programs as quickly as possible. It's part of EMBRAPA's larger effort to help Brazil expand production of potato, the country's most important vegetable crop.

In Lima, the Crop Trust has funded CIP to test wild varieties for promising traits even before any breeding begins. In 2013, center researchers started to characterize 12 wild species collected 30 years ago. Records suggested those species might tolerate drought and resist diseases such as bacterial wilt, a serious problem for developing countries. Merideth Bonierbale and colleagues planted seeds and have tested the plants in greenhouses at CIP's main facility. Mendes is now expanding the work to Kenya.

Other researchers are skirting the limitations of traditional breeding by using genetic engineering. CIP's Marc Ghislain and colleagues, for example, have directly added genes to already successful potato varieties without altering the plants in any other way—an approach not possible with traditional breeding. They took three genes for resistance to late blight from wild relatives and added them to varieties of potato popular in East Africa. The engineered varieties have proved successful in 3 years of field tests in Uganda and are undergoing final studies for regulators. Transgenic potatoes that resist late blight have already been commercialized in the United States and Canada.

Biotech approaches have their own limits. They have succeeded with traits controlled by single genes, such as disease resistance and tolerance of bruising. But improving complex physiological traits governed by many genes, such as water-use efficiency, requires traditional breeding, however cumbersome.

IN WAGENINGEN, Pim Lindhout has been plotting a revolution that would do away with much of that tedium and complexity. As head of R&D for Solynta, a startup company founded in 2006, he and his colleagues have been developing a new way to breed potatoes: creating hybrid offspring from true-breeding parent lines. "Everyone was convinced it's impossible," he says. "Many people thought I was crazy."

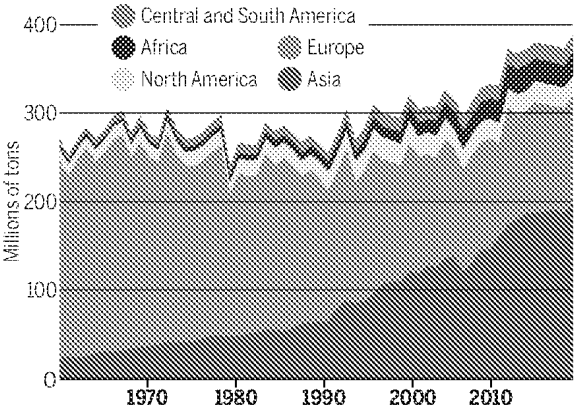
Hybrid breeding revolutionized maize production in the 20th century. It enabled breeders to quickly create high-yielding varieties that have what's known as hybrid vigor. The first step is to make inbred parent lines, which have identical alleles on all chromosome copies; the offspring of those true-breeding parents then inherit a predictable set of traits. Making the inbred lines requires repeated self-pollination over many generations. That process tends to impair the health of the plants, but when breeders cross two inbred lines, the first-generation offspring are healthy and have beneficial traits from both parents.

Potato breeders doubted the approach was possible for tubers. "I was trained to believe that potatoes can't be inbred," says Shelley Jansky, a potato breeder with the U.S. Department of Agriculture in Madison. One big obstacle is that many potato species cannot fertilize themselves. In 1998, researchers discovered a gene that somehow allows one wild species of potato to self-fertilize. When that gene is bred into other species, it lets them self-fertilize as well. But the resulting plants are frail and produce puny tubers.

The next step is to inbreed those weaklings by self-fertilizing them, generation after generation. Don't bother trying it at home: Success with cultivated potatoes would likely

Growing appetite

Potato production has grown in Asia, particularly in China and India, while falling in Europe.



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Aardevo Exhibit 1010
Page 1049
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Wild species have traits that could improve cultivated potatoes. Alberto Salas of the International Potato Center in Lima prepares a sample of *Solanum contumazaense* (above); its hairs defend against insects.

PHOTO: SARA PAJARDO/CIP

take decades because of the small odds of getting the same allele on all four copies of their chromosomes. Breeders reduce the complexity either by using species with only two sets of chromosomes (known as diploids) or by manipulating domesticated potatoes to cut the number of chromosomes in half. With persistence, diploid potatoes can be inbred. In 2011, Lindhout published the first report of inbred diploid lines that are vigorous and productive. More recently, Jansky and colleagues also created inbred diploid lines.

Such diploid inbred plants are at the heart of Solynta's strategy to revolutionize potato breeding. They will make it possible to combine traits in commercial varieties with unprecedented certainty, ease, and speed. And the plants will simplify efforts to add desirable traits directly from wild relatives while eliminating their many drawbacks, such as small tubers or poor flavor. Undesirable traits can be bred out of the descendants of a diploid cross through a standard technique called backcrossing.

In 2016, Solynta conducted its first field trials of hybrid seedlings in the Democratic Republic of the Congo and in 17 locations across Europe. The plants did well, yielding large tubers over a typical growing season. The company has not yet commercialized a variety. Within a few years, it hopes to create customized potatoes for European and African markets. Other firms, including large seed companies, are also working to develop hybrid potatoes. HZPC in Joure, the Netherlands, has begun field trials in Tanzania and in several countries in Asia.

Hybrid breeding "could be a real game changer," says geneticist Glenn Bryan, head of the Potato Genetics and Breeding group at the James Hutton Institute in Dundee, U.K. "It will definitely make breeding more agile."

Basic research could benefit from the work. "Having diploid potatoes will drastically increase our understanding of the potato genome," Shannon says. Although firms typically keep their inbred plants secret, Solynta plans to release a line, dubbed Solynthus, so that scientists can study its genetics. Jansky, for one, hopes further research could reveal genes that control yield, which might then be tapped to boost harvests.

Hybrids could also change how potatoes are planted, giving farmers the option of sowing fields with true seeds, because these are genetically identical in hybrids. Another benefit is logistical; planting 10 hectares, for instance, takes just 200 grams of easily transported seeds, compared with 25 tons of bulky tubers. In the developing world, where quality seed tubers are rare, seeds could also make obtaining superior plants easier for farmers. And in perhaps the biggest advantage over tubers for poor farmers, seeds transmit no major diseases.

Hybrid potato seeds aren't a panacea. Young plants grow more quickly and vigorously from tubers than from seeds, putting seeds at a disadvantage in some climates. And depending on how complete the inbreeding, hybrid potatoes could have less uniformly shaped tubers than those of traditional plants, a problem for farmers who supply food-processing companies. Such complexities have prompted the Dutch government to commission a study of the potential socioeconomic impacts of hybrid potatoes.

With collectors amassing genetic diversity and new techniques promising to overcome the complexities of the potato genome, researchers are optimistic that they can make significant improvements. "That's what gets me up in the morning," Jansky says. "There's no better time to be a breeder—and especially a potato breeder."

UNTIL HYBRID BREEDING and other strategies produce more resilient potatoes, farmers will have to work with the resources at hand. Here in Peru's Sacred Valley, Ellis and others from CIP have teamed with small-scale farmers who belong to an association known as Potato Park, which is dedicated to preserving hundreds of local potato varieties. The group has been planting those colorful potatoes in test plots.

Some succumb to pests or drought, like those that Ellis found dead, whereas others survive. In May 2018, as part of their search for more resilient tubers, Potato Park farmers neatly piled red, yellow, and brown tubers harvested from some of Ellis's experimental plots on rows of sacks, scoring each variety for yield and health. Local farmers had abandoned many of those landraces generations ago, as villages faded and exchange fewer plants.

Bringing some of that ancient diversity back into cultivation could hedge against environmental change. In Potato Park, farmers have already tried to escape the pests and disease that thrive in warmer temperatures by moving their cultivation 200 meters higher over the past 30 years. But René Gomez, CIP's curator of cultivated potatoes, warns that arable land is scarcer at higher elevations.

Pedro Condori Quispe, one of the park's growers, is optimistic that the communities will find a way to keep growing potatoes here. Potato farmers, he says with a smile, "are used to challenges." ※

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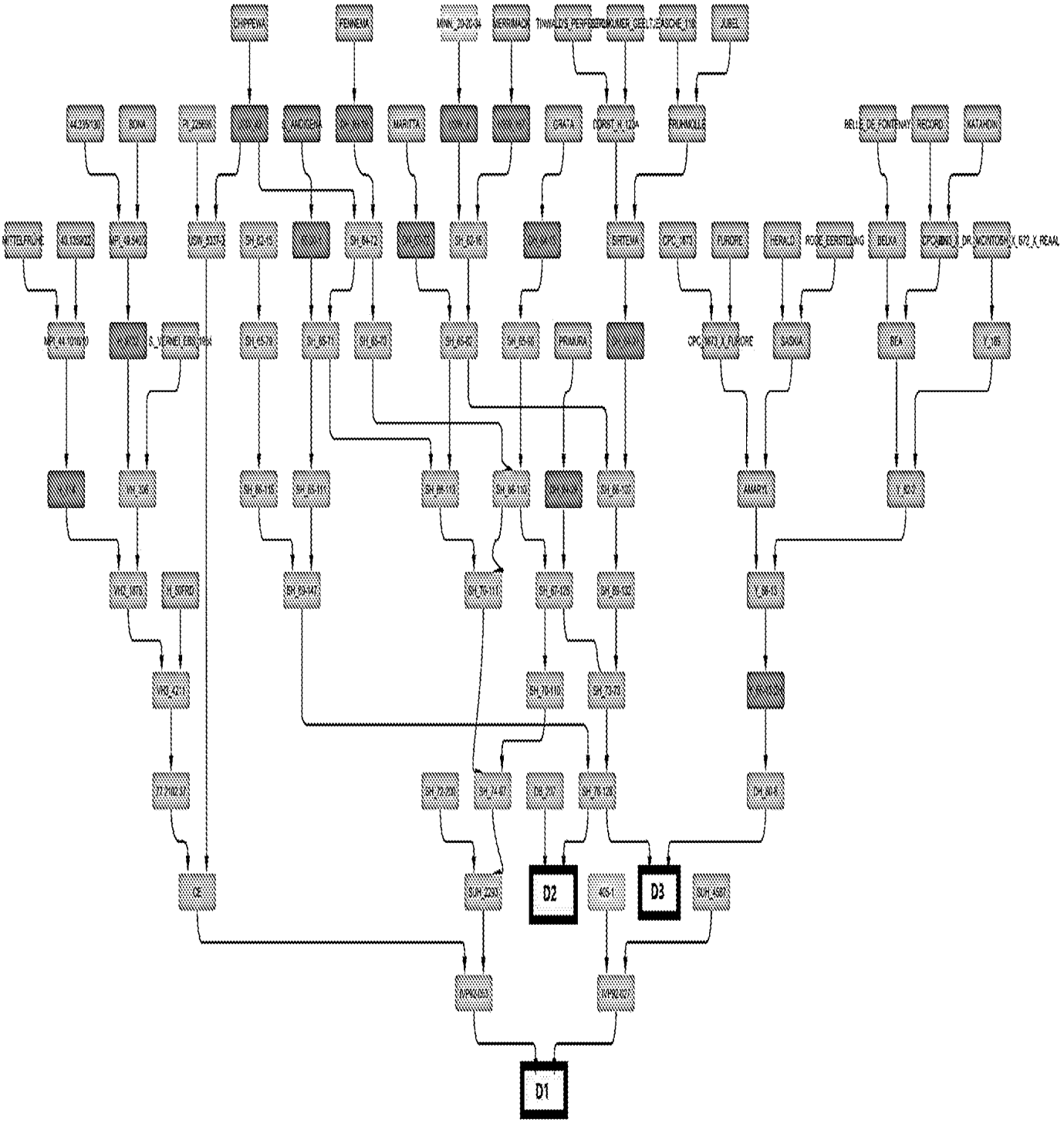
Aardevo Exhibit 1010
Page 1050
Published by AAAS

EXHIBIT B


D1
D2
D3

TSL The Sainsbury Laboratory
Plant Health Discovery for a Sustainable Future

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Recipient: Pim Lindhout, Solynta 3 February 2021

P87313US20
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
In re Application of:
Willem Hendrik Lindhout *et al.,*
Serial No.: 16/541,071
Filing or 371 (c) date: 14 August 2019
For: HYBRID SEED POTATO BREEDING
Group Art Unit: 1663
Examiner: Boggs, Russell T
Atty. Dkt. No.: VONL:004USC1
Confirmation no.: 4348

DECLARATION OF PROF. JONATHAN D. G. JONES FRS UNDER 37 C.F.R. §1.132.

I, JONATHAN DALLAS GEORGE JONES, hereby declare as follows:

I. I am a citizen of the United Kingdom, residing in Norwich, England.

2. I am a senior scientist at the Sainsbury Laboratory and a Professor at the University of East Anglia. I was educated at the University of Cambridge, and received my PhD in 1980. I performed postdoctoral research at Harvard University in Frederick M. Ausubel's lab.

3. I have been a Fellow of the Royal Society (FRS) since 2003 and a member of the US National Academy of Sciences since 2015. I also hold an EMBO Membership since 1998.

4. I have been a Board Member of the International Society of Plant Molecular Biology, and was editor of journals Plant Cell and Genome Biology. I am still editor of the journal Current Opinion in Plant Biology (COPB).

5. I am co-founder of Mendel Biotechnology, Inc., a privately held company with headquarters in Hayward, California. The R&D operations and assets relating to the discovery and development of agricultural biological products are now part of Plant Response Inc., residing in Durham, North Carolina. Mendel Biotechnology, Inc. currently operates as an IP holding company for assets relating to genetic traits in crops.

6. I am familiar with the contents of the patent application identified above. I am also familiar with the work of Dr. Willem Hendrik Lindhout who is one of the inventors listed on the referenced patent application, and Solynta B.V., the company that was founded by Dr. Lindhout. I am aware that the primary goal of Solynta B.V. and of the referenced application is production of true diploid hybrid potato seed.

7. I understand that the United States Patent and Trademark Office Examiner is in charge of assessing the patentability of the referenced patent application and has rejected the claims as failing to comply with the written description and enablement requirements. In particular, I understand that the Examiner takes the position that the application only supports claims that encompass the diploid potato lines AGVD1, AGVD2, AGVD3, and AGVD17 and asserts that the unique nature of these lines is the reason for arriving at the claimed invention. I understand that the Examiner appears to use statements from a letter that I sent to Dr. Lindhout, dated 11 December 2012, regarding my own attempts at producing hybrid potato plants, to support his assertions. I understand that this letter was previously filed as evidence supporting the non-

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Executive Director: Professor Nick Talbot FRS

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obviousness of the invention claimed in now issued U.S. Patent No. 10,524,436, which is the parent case of the referenced patent application.

8. I am submitting the present Declaration to provide clarification regarding specific statements from my letter to Dr. Lindhout that are cited in the Office Action.

9. As stated in the Office Action, the following comments were made in my letter to Dr. Lindhout, dated December 11, 2012:

> "[I]n the mid 2000s we attempted to produce hybrid potato ourselves…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)

The Examiner considers that my foregoing comments lend support to the assertion that only very specific *Solanum* germplasm, *i.e.* potato lines AGVD1, AGVD2, AGVD3, and AGVD17, can successfully be generated using the recurrent inbreeding methods disclosed in the present application. I disagree with the Examiner's interpretation of my comments and his assertions regarding the claimed invention.

10. Specifically, I note that the portion of my comments emphasized by the Examiner by underlining in the Office Action and above refer to our attempts to cross the *Sli* gene in *Solanum phureja*. As stated above, these attempts were unsuccessful. 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. While our objective in improving hybrid potato breeding was similar to that of the present inventors, we were unable to successfully achieve it for several reasons. At the time of our hybrid potato experiments, we drew no specific conclusions regarding our inability to obtain the desired starting material, however we only speculated that the nature of the germplasm we used in our program may have contributed to our results. I would add that at the time, our growth conditions for potato cultivation in greenhouses were suboptimal, and we might have been able to take our populations through further generations had our growth conditions been more suitable. By 11 December 2012, however, I had determined that the nature of the germplasm was not the cause and that we would have likely achieved favorable results with the *Solanum* lines we had used previously, had we persisted with inbreeding and selecting plants with seed carrying berries. This conclusion was drawn from my personal communications with others in the *Solanum* breeding community, and from the scientific publication in Potato Research (2011) Vol. 54: 301–312.

11. The crux of the success of Solynta resides in the selection of fertile progeny, and the perseverance upon inbreeding depression to continue breeding, even with hardly viable plants, both of which were unique for the potato breeding community. I have no reason to believe that the method is not widely applicable to potato. The inventors of the present application have certainly convinced me that the method is widely applicable to *S. tuberosum*. Therefore, I do not believe that the claimed methods are unique to lines AGVD1, AGVD2, AGVD3, and AGVD17. More specifically, I am aware of scientific groups, such as the group of Dr. S. H. Jansky in Madison, Wisconsin, and of international seed potato companies, that currently are working on the development of true diploid, hybrid *S. tuberosum* potato seed.

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Executive Director: Professor Nick Talbot FRS

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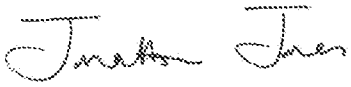
12. I hereby declare that all statements made of my own knowledge are true and all statements made on information are believed to be true and further that the statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardize the validity of this application or any patent issued thereon. I also declare that I am not being compensated for providing this declaration, and that I do not benefit from a positive prosecution outcome whatsoever.



Prof Jonathan D G Jones FRS

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Executive Director: Professor Nick Talbot FRS

Max-Planck-Institut für Pflanzenzüchtungsforschung
Max Planck Institute for Plant Breeding Research

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22-01-2021

P87313US20
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
In re Application of:
Willem Hendrik Lindhout *et al.,*
Serial No.: 16/541,071
Filing or 371 (c) date: 14 August 2019
For: HYBRID SEED POTATO BREEDING
Group Art Unit: 1663
Examiner: Boggs, Russell T
Atty. Dkt. No.: VONL:004USC1
Confirmation no.: 4348

DECLARATION OF PROF. MAARTEN KOORNEEF UNDER 37 C.F.R. §1.132.

I, PROF.DR.IR. MAARTEN KOORNNEEF, hereby declare as follows:

1. I am a citizen of the Netherlands, residing in Wageningen, the Netherlands.

2. I held a personal chair in Plant Genetics at the laboratory of Genetics at Wageningen University from 1992 until my retirement in 2015. I was Director at the Max Planck Institute for Plant Breeding Research and Head of the Department of Plant Breeding and Genetics from 2004 until 2016. I was an Honorary Professor at the Institute of Botany, University of Cologne, Germany.

3. I have been an international member of the U.S. National Academy of Sciences since 1998, a member of The Royal Netherlands Academy of Arts and Sciences, a member of the Leopoldina, the German National Academy of Science and a member of the Academia Europaea (European Academy of Science). I obtained a Silver Medal Award of the IPGSA (International Plant Growth Substances Association), and received a Corresponding Membership Award of the American Society of Plant Physiologists.

4. My research is believed to be one of the reasons why *Arabidopsis thaliana* became the model plant for research by geneticists, molecular biologists, and plant breeders. I was member of the first Multinational Arabidopsis Steering Committee (MASC).

5. I am familiar with the contents of the patent application identified above. I am also familiar with the work of Dr. Willem Hendrik Lindhout who is one of the inventors listed on the referenced patent application, and Solynta B.V., the company that was founded by Dr. Lindhout. I am aware that the

primary goal of the referenced application is production of true diploid hybrid potato seed.

6. I understand that the United States Patent and Trademark Office Examiner is in charge of assessing the patentability of the referenced patent application and has rejected the claims as failing to comply with the written description and enablement requirements. In particular, I understand that the Examiner is of the position that the application only supports claims that encompass potato lines AGVD1, AGVD2, AGVD3, and AGVD17 and asserts that the unique nature of these lines is the basis for arriving at the claimed invention. I understand that the Examiner appears to use statements from a letter that I sent to Dr. Lindhout, dated 18 December 2012, to support his assertions. I understand that this letter was previously filed as evidence supporting the non-obviousness of the invention claimed in now issued U.S. Patent No. 10,524,436, which is the parent case of the referenced patent application.

7. I am submitting the present Declaration to provide clarification regarding specific statements from my letter to Dr. Lindhout that are cited in the Office Action, as I believe the Examiner has misinterpreted the intent of my statements.

8. As stated in the Office Action, the following comments were made in my letter to Dr. Lindhout, dated 18 December 2012:

> "Ever since Lindhout and his co-workers published their results in Potato Research volume 54 of 2011, showing high yielding hybrids from 87% homozygous F3 inbreds, I have been positively surprised that Solynta has managed to challenge the dogma. Solynta could successfully obtain essentially homozygous inbred lines by making use of genotypes that are selfcompatible and by choosing the right starting material that apparently is heterozygous for a lower number of deleterious alleles. This provides the basis of generating useful inbred lines that can become parents of F1 hybrids." (underlining added by Examiner)

The Examiner appears to believe that the foregoing comments lend support to the assertion that the invention of the present application, as broadly as claimed, is not fully described. In particular, that only very specific *Solanum* germplasm, *i.e.* potato lines AGVD1, AGVD2, AGVD3, and AGVD17, can successfully be generated using the recurrent inbreeding methods disclosed in the present application. I disagree with the Examiner's interpretation of my comments and his assertions regarding the claimed invention.

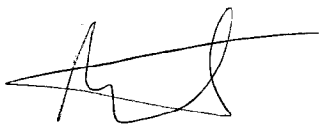
9. Specifically, I note that the portion of my comments emphasized by the Examiner by underlining in the Office Action, as is presented herein above, refers to a side note and only reflects speculation on my part regarding a reason for Solynta's success in obtaining high-yielding essentially homozygous diploid potato lines. While the choice of starting material could be an explanation for their success in being the first to generate such homozygous diploid potato

lines, my comment was not intended to be a statement of fact. Indeed, based on the teachings in Potato Research volume 54 of 2011, I have no specific reason to assume that the starting material is the primary explanation for Solynta's results. The purpose of my letter to Dr. Lindhout was to show my appreciation for Solynta's desire to challenge the dogma amongst potato geneticists and breeders regarding inbreeding depression in potato and applaud their success in obtaining high-yielding essentially homozygous diploid potato lines. My comments, such as "a project aiming at developing a good performing, homozygous diploid potato plant for the purpose of hybrid production is a daring undertaking with very little chance of success," were intended to emphasize the difficulty of the task Dr. Lindhout and his colleagues took on and accordingly show the value of the results they obtained.

10. The crux of the success of Solynta resides in the selection of fertile progeny, and the perseverance upon inbreeding depression to continue breeding, even with hardly viable plants, both of which were unique for the potato breeding community. I do not doubt that the pioneering work shown in the present application allows breeders to generate essentially homozygous diploid potato lines starting from a wide array of diploid potato germplasm, especially diploid *S. tuberosum* germplasm. Therefore, I do not believe that the claimed methods are unique to lines AGVD1, AGVD2, AGVD3, and AGVD17, and I am aware of international seed potato companies that currently are working on the development of true diploid, hybrid *S. tuberosum* potato seed.

11. I hereby declare that all statements made of my own knowledge are true and all statements made on information are believed to be true and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereon. I also declare that I am not being compensated for providing this declaration, and that I do not benefit from a positive prosecution outcome whatsoever.

Yours sincerely,



Prof Dr Maarten Koornneef
Emeritus director at the Max Planck Institute for Plant Breeding Research, Cologne and emeritus professor of Plant Genetics at Wageningen University in The Netherlands

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
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($)
Miscellaneous:				
RCE- 1ST REQUEST	2801	1	680	680
SUBMISSION- INFORMATION DISCLOSURE STMT	2806	1	130	130
		Total in USD ($)		**810**

Electronic Acknowledgement Receipt

EFS ID:	41880059
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:	09-FEB-2021
Filing Date:	14-AUG-2019
Time Stamp:	17:06:33
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$810
RAM confirmation Number	E202129H07283515
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	Request for Continued Examination (RCE)	VONL_004USC1_RCE.pdf	71104 ac99fffcae75e96cdffa79a9b8e6e6c3e142a31e	no	2
Warnings:					
This is not a USPTO supplied RCE SB30 form.					
Information:					
2		VONL_004USC1_IDS.pdf	81438 1cdedfaf8dd127bb0ecb8695ebc0dad030355d2b	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:					
3	Non Patent Literature	Stokstad_2019.pdf	1809531 66306135561d6892b3e52d3a7f50923bfb75ea34	no	4
Warnings:					
Information:					
4	Non Patent Literature	de_Vries_2016.pdf	383827 8c1c425aadb9a4aaa95acd79c7a31363ae268fee	no	6
Warnings:					
Information:					
5		VONL_004USC1_Response.pdf	2593754 0f196f07f03c38da722b5b665338ba56ab0a6176	yes	25

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Response After Final Action	1	1
	Claims	2	5
	Applicant summary of interview with examiner	6	6
	Applicant Arguments/Remarks Made in an Amendment	7	25

Warnings:					
Information:					
6	Affidavit-traversing rejectns or objectns rule 132	VONL_004USC1_Jones_Dec.pdf	125004 528d87374d51f5b426b30591cfe49b303fb01315	no	3
Warnings:					
Information:					
7	Affidavit-traversing rejectns or objectns rule 132	VONL_004USC1_Koornneef_Dec.pdf	206523 8d86751eccfda2e8e6cefa00e86f038079f52584	no	3
Warnings:					
Information:					
8	Fee Worksheet (SB06)	fee-info.pdf	32160 7c9ae964488ae1f4c599ab0b64e24e58e90b7e81	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	5303341		

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

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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 02/12/2021

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

EXAMINER	
BOGGS, RUSSELL T	

ART UNIT	PAPER NUMBER
1663	

NOTIFICATION DATE	DELIVERY MODE
02/12/2021	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 (First Inventor to File) Status** No	**Page** 1 of 1

All Participants (applicant, applicants representative, PTO personnel)	**Title**	**Type**
RUSSELL T BOGGS	Primary Examiner	Telephonic
SHEILA WALKER	Attorney	

Date of Interview: 09 February 2021

Issues Discussed:

Other

The parties discussed how to provide a color exhibit attached to a declaration to the examiner. The parties agreed to use e-mail.

/RUSSELL T BOGGS/ Examiner, Art Unit 1663	

Applicant is reminded that a complete written statement as to the substance of the interview must be made of record in the application file. It is the applicants responsibility to provide the written statement, unless the interview was initiated by the Examiner and the Examiner has indicated that a written summary will be provided. See MPEP 713.04
Please further see:
MPEP 713.04
Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews, paragraph (b)
37 CFR § 1.2 Business to be transacted in writing

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.

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).

The present Supplemental Information Disclosure Statement is being filed in response to the Final Office Action issued on November 9, 2020 in which the Examiner identified a

typographical error in the title of reference "2" of the non-patent literature documents citations. In response thereto, Applicants hereby submit the attached SB/08 identifying the correct reference as "1" of the non-patent literature documents citations. A substantive response to the Final Office Action is also being filed concurrently herewith.

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: February 9, 2021

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
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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 $100 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $460 =	
☐ 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	02/09/2021	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 $100 =	0
	Independent (37 CFR 1.16(h))	* 3	Minus	*** 3	= 0		x $480 =	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".	/CAROLYN E THOMAS/
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The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

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Application	Document	Mailroom Date	Attorney Docket No.
16541071	EXIN	02/12/2021	VONL:004USC1

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(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

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Examiner Name	Russell T. Boggs
	Attorney Docket Number	VONL:004USC1

	1	Rakosy-Tican et al., "Introgression of Two Broad-Spectrum Late Blight Resistance Genes, Rpi-B1b1 and Rpi-B1b3, From Solanum bulbocastanum Dun Plus Race-Specific R Genes Into Potato Pre-breeding Lines," Front. Plant Sci. 11:699, 2020. doi: 10.3389/fpls.2020.00699.	☐
	2	Rauscher et al. (2006) "Characterization and mapping of RPi-ber, a novel potato late blight resistance gene from Solanum berthaultii", Theor. Appl. Genet. 112:674-687.	☐
	3	Ritte et al. (2002) "The starch-related R1 protein is an α-glucan, water dikinase", Proc. Nat. Acad. Sci. USA. 99:7166-7171.	☐
	4	Ritter et al. (1991) "RFLP mapping on potato chromosomes of two genes controlling extreme resistance to potato virus X (PVX)", Mol. Gen. Genet. 227:81-85.	☐
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	7	Rommens et al. (2006) "Improving potato storage and processing characteristics through all-native DNA transformation-J", Agric. Food Chem 54:9882-9887.	☐
	8	Rouppe van der Voort et al. (1993) "A QTL for broad-spectrum resistance to cyst nematode species (Globodera spp.) maps to a resistance gene cluster in potato", Theor. Appl. Genet. 96:654-661.	☐
	9	Rouppe van der Voort et al. (2000) "Two additive QTLs conferring broad-spectrum resistance in potato to Globodera pallida are localized on resistance gene clusters", Theor. Appl Genet 101:1122-1130.	☐
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	11	Salanoubat et al. (1987) "Molecular cloning and sequencing of sucrose synthase cDNA from potato (Solanum tuberosum L.): preliminary characterization of sucrose synthase mRNA distribution", Gene. 60: 47-56.	☐

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	First Named Inventor	Willem Hendrik Lindhout
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	Attorney Docket Number	VONL:004USC1

	12	Sattarzadeh et al. (2006) "Single nucleotide polymorphism (SNP) genotyping as basis for developing a PCR-based marker highly diagnostic for potato varieties with high resistance to Globodera pallida pathotype Pa2/3", Molec. Breed. 18:301-312.	☐
	13	Song et al. (2003) "Gene RB cloned from Solanum bulbocastanum confers broad spectrum resistance to potato late blight", Proc. Nat. Acad. Sci., USA 100:9128-9133.	☐
	14	Song et al. (2008) "Development of STS markers for selection of extreme resistance (RY sto) to PVY and maternal pedigree analysis of extremely resistant cultivars", Amer. J. Potato Res. 85: 159-170.	☐
	15	Song et al. (2005) "Mapping of extreme resistance to PVY (Ry sto) on chromosome XII using another-culture derived primary dihaploid potato lines", Theor. Appl, Genet. 111:879-887.	☐
	16	Sorri et al., "Predicted kinase-3a motif of a resistance gene analogue as a unique marker for virus resistance", Theor. Appl. Genet. 99:164-170, (1999).	☐
	17	Tan et al. (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. Mol. Pl. Micr. Interact. 21:909-918.	☐
	18	Tan et al. (2010) "The effect of pyramiding Phytophthora infestans resistance genes RPi-mcd1 and RPi-ber in potato", Theor. Appl. Genet. 121: 117-125.	☐
	19	Tan et al. (2009) "GpaXI Itar originating from Solanum tarijense is a major resistance locus to Globodera pallida and is localised on chromosome 11 of potato" Theor. Appl Genet. 119:1477-1487.	☐
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	21	Trognitz et al., "Occurrence of the R1 allele conferring resistance to late blight in potato R-gene differentials and cultivars", Plant Pathology 56:150-155, 2007.	☐
	22	USPTO: Non-Final Office Action dated March 14, 2016, regarding U.S. Application No. 14/112,747 (Atty. Dkt. No. VONL:006US).	☐

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	Examiner Name	Russell T. Boggs
	Attorney Docket Number	VONL:004USC1

	23	USPTO: Response to Non-Final Office Action dated March 14, 2016, filed September 14, 2016, regarding U.S. Application No. 14/112,747 (Atty. Dkt. No. VONL:006US).	☐
	24	USPTO: Final Office Action dated December 15, 2016, regarding U.S. Application No. 14/112,747 (Atty. Dkt. No. VONL:006US).	☐
	25	USPTO: Response to Final Office Action dated December 15, 2016, filed June 15, 2017, regarding U.S. Application No. 14/112,747 (Atty. Dkt. No. VONL:006US).	☐
	26	USPTO: Non-Final Office Action dated December 22, 2017, regarding U.S. Application No. 14/112,747 (Atty. Dkt. No. VONL:006US).	☐
	27	USPTO: Response to Non-Final Office Action dated December 22, 2017, filed April 23, 2018, regarding U.S. Application No. 14/112,747 (Atty. Dkt. No. VONL:006US).	☐
	28	USPTO: Final Office Action dated August 7, 2018, regarding U.S. Application No. 14/112,747 (Atty. Dkt. No. VONL:006US).	☐
	29	USPTO: Response to Final Office Action dated August 7, 2018, filed February 7, 2019, regarding U.S. Application No. 14/112,747 (Atty. Dkt. No. VONL:006US).	☐
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	First Named Inventor	Willem Hendrik Lindhout
	Art Unit	1663
	Examiner Name	Russell T. Boggs
	Attorney Docket Number	VONL:004USC1

	34	Vleeshouwers et al., "Understanding and Exploiting Late Blight Resistance in the Age of Effectors," Annu. Rev. 49:507-531, 2011.	☐

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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

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Name/Print	Amy Dunstan	Registration Number	61,069

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EFS ID:	42080540
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
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Attorney Docket Number:	VONL:004USC1
Receipt Date:	04-MAR-2021
Filing Date:	14-AUG-2019
Time Stamp:	13:24:43
Application Type:	Utility under 35 USC 111(a)

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35	Transmittal Letter	VONL004USC1_IDSTransmittal. pdf	21862 5b12c0d05232bd113e8c47657c7ba09e0d 4e1810	no	2
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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.

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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

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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

Aardevo Exhibit 1010
Page 1084

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 1085

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 1086

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 1087

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}
	83/9/11	*Rpi-blb1, -blb3, R3a, R3b*	HR(9)	HR(9)	HR(9)	HR(9)	1.00 ± 0.00	-0.124_{13}; -0.405_{14}
	83/9/12	*Rpi-blb1, -blb3*	nd	nd	nd	nd	1.14 ± 0.15	-0.196_{14}; -0.234_{15}
	83/9/26	*Rpi-blb1, -blb3, R3a, R3b*	HR(9)	HR(9)	HR(9)	HR(9)	1.00 ± 0.00	-0.124_{13}; -0.405_{14}
	83/9/39	*Rpi-blb3, R3a, R3b*	0(9)	HR(9)	HR(8)	HR(9)	1.60 ± 0.59	0.133_{14}
	83/9/50	*Rpi-blb1, -blb3, R3a, R3b*	HR(9)	HR(9)	HR(9)	HR(9)	1.00 ± 0.00	-0.124_{13}; -0.405_{14}
	83/9/51	*Rpi-blb1, -blb3, R3a, R3b*	HR(9)	HR(9)	HR(9)	HR(9)	1.00 ± 0.00	-0.124_{13}; -0.405_{14}
	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 1088

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_{13}
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 1089

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 1090

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 1091

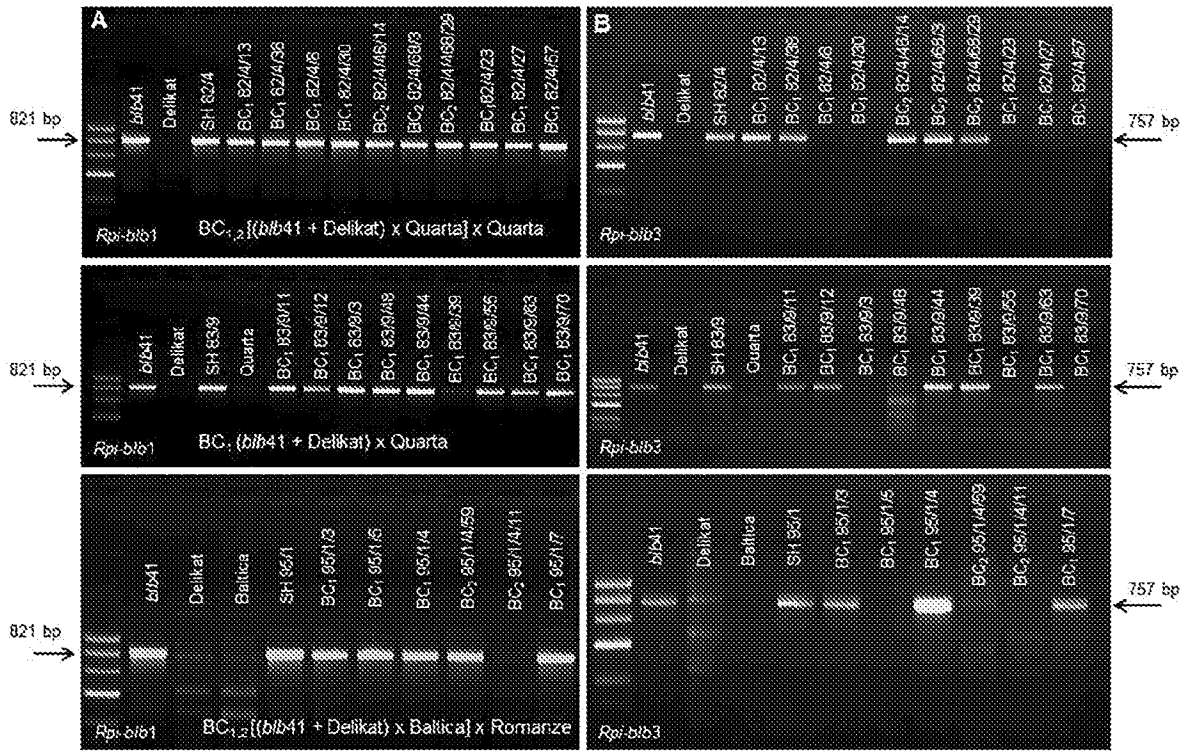

FIGURE 1 | Molecular analysis using gene specific markers to determine the presence of **(A)** *Rpi-blb1* and **(B)** *Rpi-blb3* genes in selected somatic hybrids, their parental lines *S. bulbocastanum* GLKS 33741 (*blb*41), cv. 'Delikat' and different segregating BC_1 progenies after back-crossing 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 1092

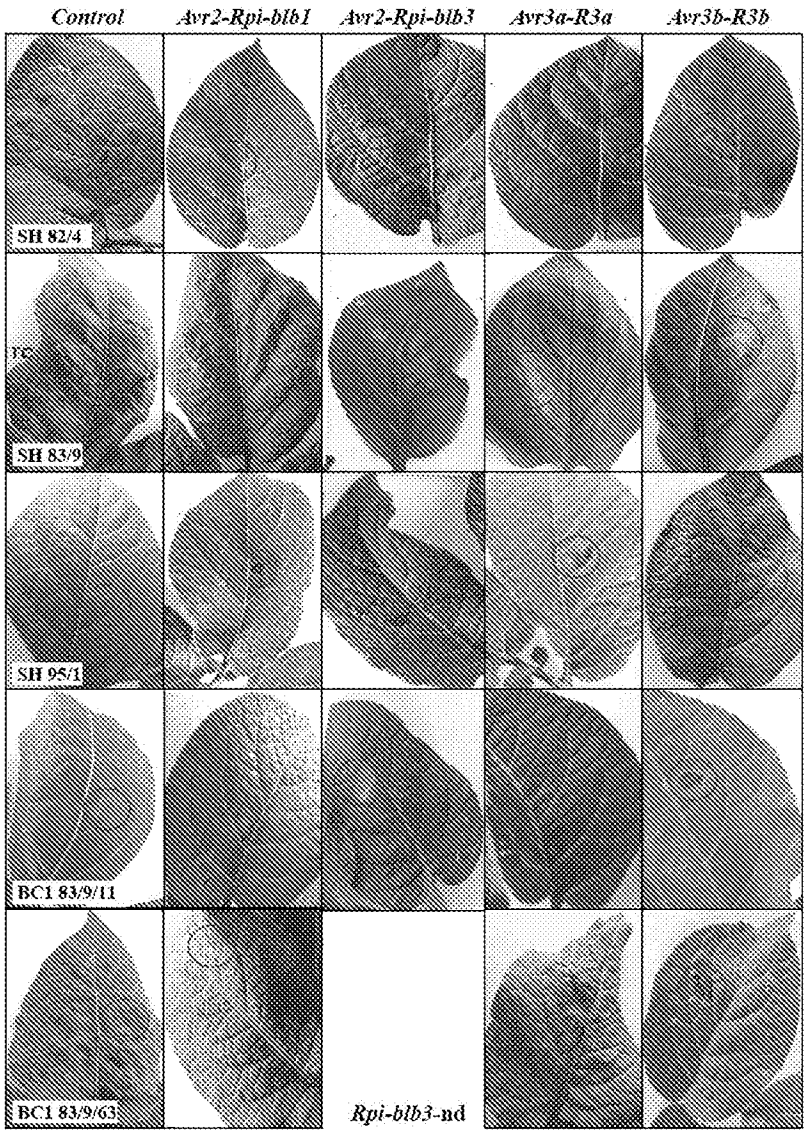

FIGURE 2 | Hypersensitive reaction of leaves of *Solanum bulbocastanum* (GLKS 33741, *blb*41) (+) cv. 'Delikat somatic hybrids (SHs) and selected back-cross progenies after agro-infiltration of *Avr*-effectors at 4*dpi*, which reveals the activity of corresponding resistance genes: *Rpi-blb1*, *Rpi-blb2*, *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 1093

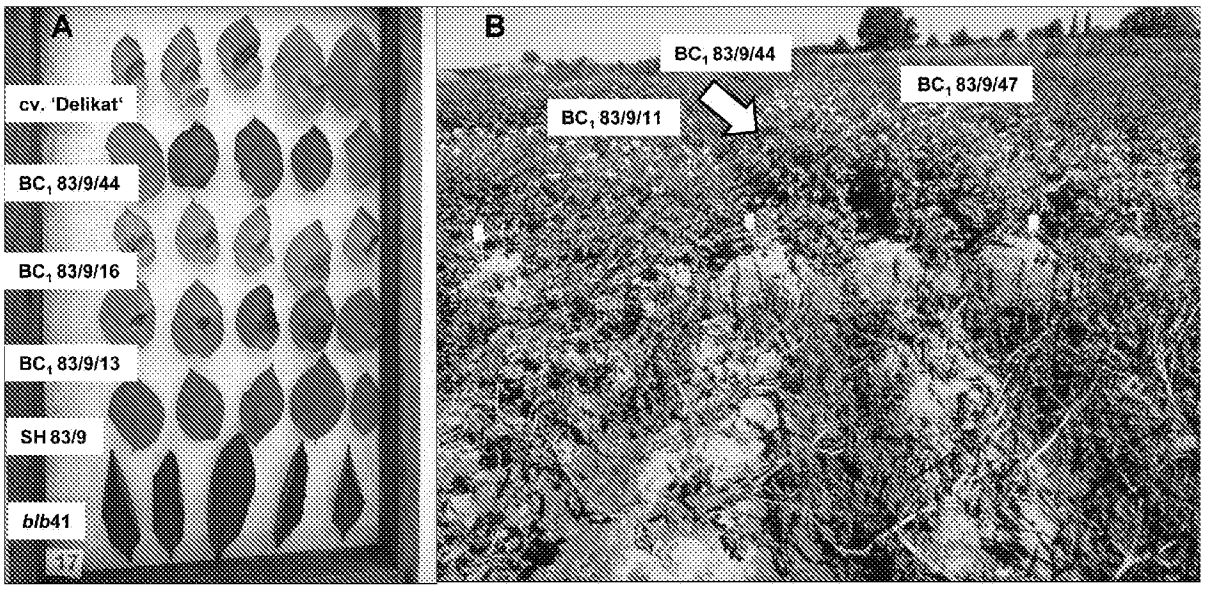

FIGURE 3 | Evaluation of resistance to late blight in a greenhouse and the field **(A)** Detached leaf assay (DLA) showing resistant *S. bulbocastanum* GLKS 33741 (*blb*41), a resistant somatic hybrid, two susceptible and one resistant BC_1 clones and the susceptible cv. 'Delikat' after infection with *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 1094

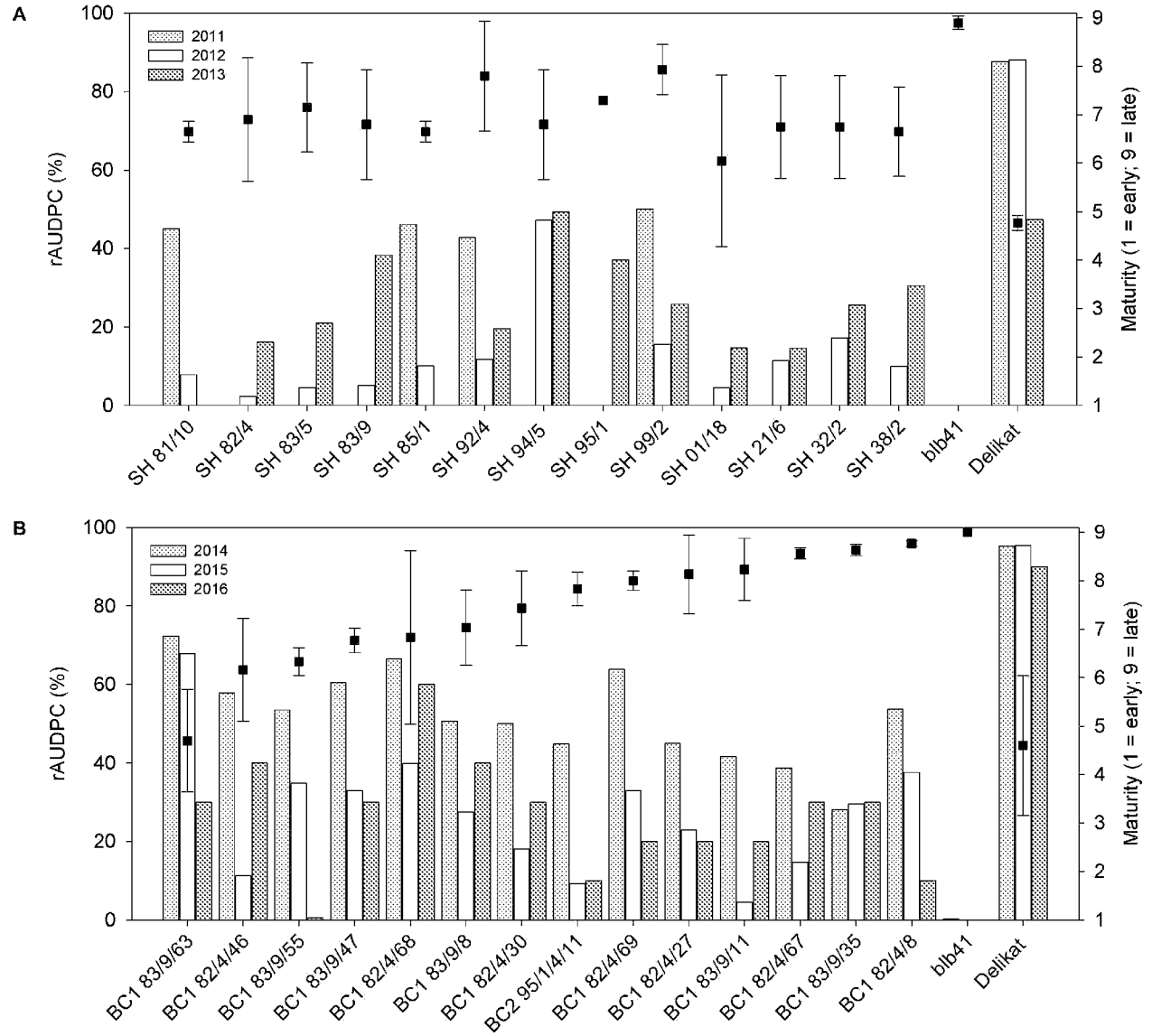

FIGURE 4 | Percentage of foliage area infected by late blight expressed as relative Area Under Disease Progress Curve (rAUDPC) and maturity (1 = very early; 9 = very late), evaluated in the field, after artificial inoculation of parents, **(A)** Somatic hybrids and **(B)** Their back-crossed progenies, 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 1095

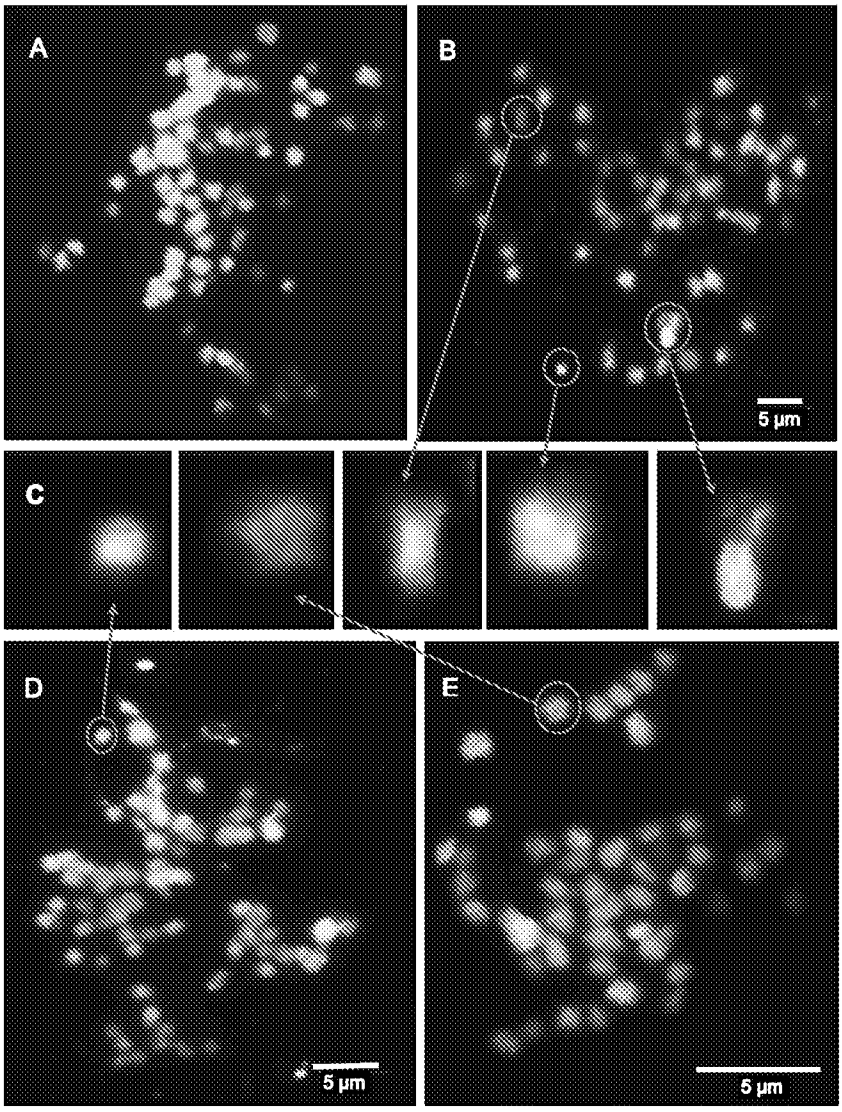

FIGURE 5 | The results of a mcGISH analysis of the genetic composition of somatic hybrids (SH), back-cross progenies (BC_1 and BC_2) – examples; green labels *S. tuberosum*, red labels *S. bulbocastanum* GLKS 33741 (*blb*41) chromosomes. **(A)** SH 83/9 - total 72 chromosomes, in red *S. bulbocastanum* = 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 1096

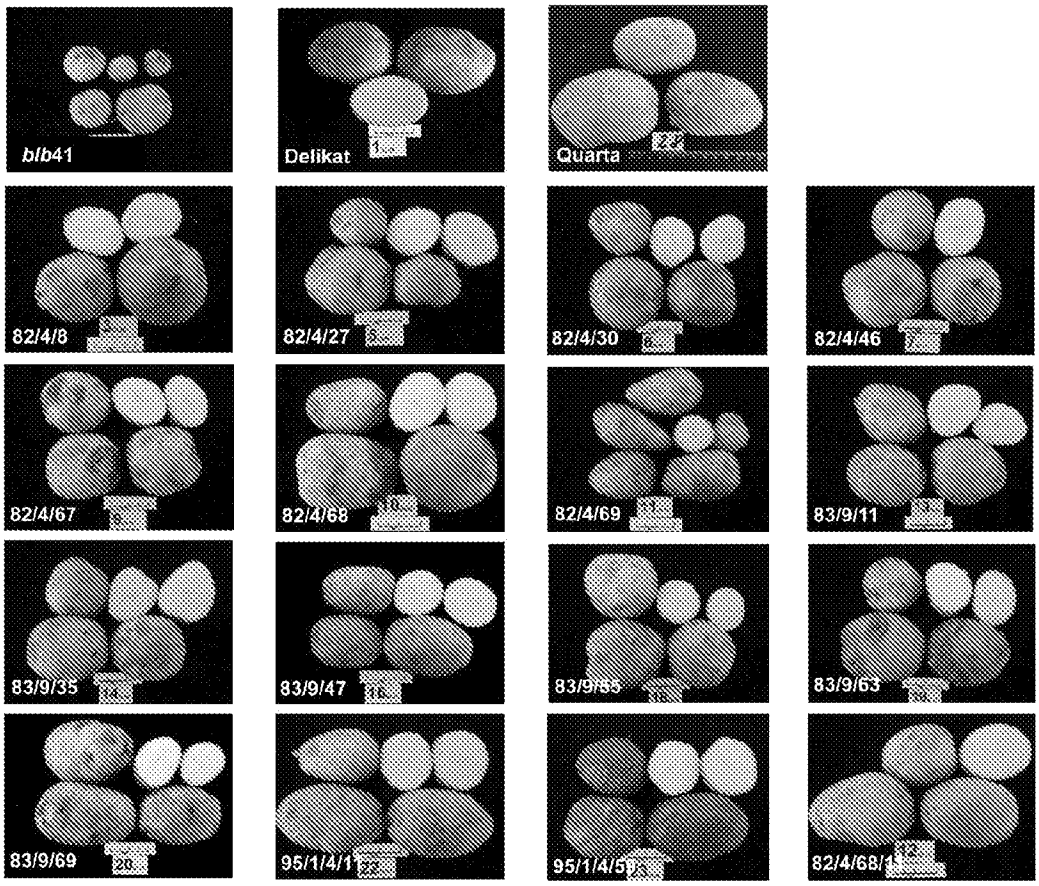

FIGURE 6 | Size and appearance of tubers of parental lines and selected BC_1 and BC_2 (the last three below) progenies of *S. bulbocastanum* GLKS 31741 (*blb*41) (+) cv. 'Delikat' somatic hybrids after cultivation in a field.

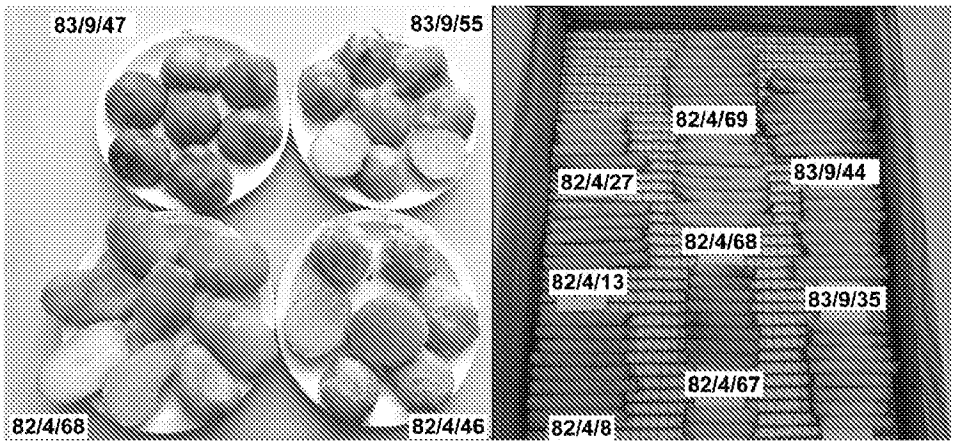

FIGURE 7 | Assessment of quality traits: Discolouration of cooked tuber flesh (left) and raw tuber tissue of different BC progenies derived from *S. bulbocastanum* GLKS 31741 (*blb*41) (+) cv. 'Delikat' somatic hybrids 82/4 and 83/9 (back-crossed with cv. 'Quarta').

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 1097

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.

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Aardevo Exhibit 1010
Page 1098

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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).

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Aardevo Exhibit 1010
Page 1100

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ORIGINAL PAPER

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Characterization and mapping of $R_{Pi\text{-}ber}$, a novel potato late blight resistance gene from *Solanum berthaultii*

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Abstract *Phytophthora infestans*, the causal agent of late blight, threatens potato production worldwide. An important tool in the management of the disease is the use of resistant varieties. Eleven major resistance genes have been identified and introgressed from *Solanum demissum*. However, new sources of resistance are continually sought. Here, we report the characterization and refined genetic localization of a resistance gene previously identified as *Rber* in a backcross progeny of *Solanum tuberosum* and *Solanum berthaultii*. In order to further characterize *Rber*, we developed a set of *P. infestans* isolates capable of identifying each of the 11 R-genes known to confer resistance to late blight in potato. Our results indicate that *Rber* is a new resistance gene, different from those recognized in *S. demissum*, and therefore, it has been named $R_{Pi\text{-}ber}$ according to the current system of nomenclature. In order to add new molecular markers around $R_{Pi\text{-}ber}$, we used a PCR-based mapping technique, named MASP-map, which located $R_{Pi\text{-}ber}$ in a 3.9 cM interval between markers CT240 and TG63 on potato chromosome X. The location of $R_{Pi\text{-}ber}$ coincides with an area involved in resistance to different pathogens of potato and tomato.

Abbreviations AUDPC: Area under the disease progress curve · BCT: Backcross to *S. tuberosum* progeny · BSA: Bulk segregant analysis · CAPS: Cleaved amplified polymorphic sequences · MASP-map: Multiplex allele-specific PCR mapping · R-gene: Resistance gene · chr: Chromosome · RFLP: Restriction fragment length polymorphisms

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Introduction

Potato is the fourth most important crop in the world. In 2004, more than 328 million metric tons of potatoes were produced worldwide (FAOSTAT 2005). Potato production is threatened by diverse pathogens, the worst of which is the oomycete *Phytophthora infestans*, the causal agent of late blight. Late blight epidemics can be devastating, sometimes causing total crop losses (Fry and Goodwin 1997). It has been estimated that the management of the disease costs $3.5 billion annually in developing countries alone (GILB 2004).

Management strategies for late blight include the application of fungicides, the use of healthy seed tubers for planting and host resistance. However, the occurrence of isolates resistant to some modern fungicides emphasizes the need for host resistance (Deahl et al. 1993; Goodwin et al. 1996; Grunwald et al. 2001). Additionally, the high cost of fungicide applications, increasing awareness of health and environmental risks and world-wide pressures to minimize the use of chemical sprays (Fry and Goodwin 1997) also make the use of host resistance a priority.

Two types of resistance to late blight have been described in potato. First, general resistance slows the spread of the disease and it is understood to be often polygenic (Leonards-Schippers et al. 1994; Umaerus and Umaerus 1994), and strongly correlated with maturity type (Bormann et al. 2004; Simko 2002) which makes it difficult to breed into new varieties (Wastie 1991). Second, specific resistance confers immunity or near immunity to the plant through a hypersensitive response and is thought to be monogenic. Genes governing such resistance have been termed R-genes, and are thought to produce proteins involved in pathogen recognition and the initiation of defense responses. Eleven resistance genes have been introgressed into the cultivated potato (*Solanum tuberosum*) from its wild relative *Solanum demissum* (Van der Plank 1963; Wastie 1991), and they are "named" *R1*, *R2*,..., *R11*. Of these, only five have been located on the genetic map of potato: *R1* on chromosome V (Leonards-Schippers et al. 1992); *R2* on chromosome IV (Li et al. 1998); and *R3a, R3b* (Huang et al. 2004), *R6*, and *R7* on chromosome XI (El-Kharbotly et al. 1996). However, *R5–R11* have recently been identified as alleles of *R3* (Huang 2005), which would also locate them in chromosome XI. Of the R-genes from *S. demissum*, only *R1*(Ballvora et al. 2002) *and R3a* (Huang et al. 2005) have been cloned and sequenced.

R-genes from other hosts of *P. infestans* have been reported. Three resistance genes against *P. infestans* have been mapped in tomato—a close relative to potato: *Ph-1* on chromosome 7, *Ph-2* on chromosome 10 (Moreau et al. 1998), and *Ph-3* on chromosome 9 (Chunwongse et al. 2002). An *R*-gene from *Solanum bulbocastanum* on chromosome VIII (Helgeson et al. 1998; Naess et al. 2000), named *RB*, has now been cloned (Song et al. 2003). *Rpi* has been found in *S. pinnatisectum* and mapped to chromosome VII (Kuhl et al. 2001). We previously reported an R-gene located chromosome X of *Solanum berthaultii* (Ewing et al. 2000), named *Rber*, since it segregates from the *S. berthaultii* parent in a backcross with *S. tuberosum*.

Unfortunately, *P. infestans* has been shown to rapidly overcome the classic R-genes from *S. demissum* when they were deployed in potato cultivars. The result is that such specific resistance has had short durability (Wastie 1991), and the immunity hoped for in such R-genes has contributed little to practical late blight management. However recent reports (Bormann et al. 2004; Stewart et al. 2003) suggest that there may be some residual disease suppression effect of R-genes even when interacting with a "compatible" isolate. Knowledge of signaling pathways and downstream events may open new approaches to disease suppression, so it is important to learn as much as possible about such genes.

Rber was mapped to a 13 cM interval between the restriction fragment length polymorphism (RFLP) markers CT214b and TG63 in a diploid backcross progeny. However, it was uncertain if *Rber* was a new gene or an allele of a resistance gene previously introgressed from *S. demissum*. The isolate of *P. infestans* used for the identification of *Rber* was a complex race, compatible with (i.e., not recognized by) R-genes: *R1, R2, R3, R4, R5, R6, R7*, and *R10*. Therefore, we inferred that *Rber* might be a new R-gene (Ewing et al. 2000).

The goal of the research reported here was to determine if *Rber* was different from *R8, R9* or *R11*, and to provide a finer map location of this R-gene. We determined the identity of *Rber* using a set of *P. infestans* isolates known as a "tester set", since they allowed potato plants to be screened for the 11 known R-genes and therefore the identification of new R-genes. We also investigated a series of markers from diverse sources to provide a finer map location of this R-gene.

Materials and methods

Plant material

Host differential set

We used a differential set of *S. tuberosum* genotypes, each one containing one known resistance gene (Table 1). This differential set was obtained from the US Department of Agriculture—Potato Germplasm Introduction Station in Sturgeon Bay, WI, USA and had been used previously (Abu-El Samen et al. 2003; Spielman et al. 1989). Historical and collection data are available for each accession (USDA 1999). The cultivars Norchip or Katahdin were used as R-gene-free controls with no known R-genes, and served to indicate the success of inoculations (Mastenbroek 1952). For all experiments, plants were grown and maintained under greenhouse conditions at the Cornell University facilities (Ithaca, NY).

Backcross mapping population

The backcross population was developed by Bonierbale et al. (1994). Briefly, an interspecific progeny was developed by crossing a dihaploid *S. tuberosum* clone (USW-2230 Saco, GS 193 in the GRIN database) (USDA 1999) as female with an individual of the accession PI473331 of *S. berthaultii* from Cochabamba, Bolivia (USDA 1999). One individual from the F1 progeny (M200-30) was then backcrossed with the 2x *S. tuberosum* parent clone HH1-9 to generate the backcross to *S. tuberosum* (BCT) progeny (Bonierbale et al. 1994).

Originally the progeny consisted of 158 diploid individuals, of which 133 were still available for this study. A total of 665 genotypes of the BCT progeny were produced at Centro Internacional de la Papa (CIP–Lima, Peru) and shipped to Cornell University as a true seed to use in this study. They were tested for, and found to be free of, major potato viruses and other pathogens. For both the original and extended progenies, in vitro plants were obtained and maintained at 15°C until planting them in the greenhouse and field in July 1999.

Table 1 Differential set of *Solanum tubersoum*

R-gene	Accession no.	Origin	Reference
R1	Kennebec	Wisconsin, USA	
R2	PI 203905	Limburg, Netherlands	Mastenbroek (1952)
R3	PI 203902		Spielman et al. (1989)
R4	PI 203900	Netherlands	Mastenbroek (1952)
R5	PI 303146	Scotland, UK	Malcomson and Black (1966)
R6	PI 587059	Netherlands	
R7	PI 303148	Scotland, UK	Malcomson and Black (1966)
R8	PI 303149	Scotland, UK	Malcomson and Black (1966)
R9	Hogdson 2573	Wisconsin, USA	Malcomson and Black (1966)
R10	PI 423656	Netherlands	
R11	PI 587060	Netherlands	

The accession numbers correspond to the plant introduction numbers assigned by USDA. Origin stands for the country collecting the material, which subsequently donated it to the Potato Germplasm introduction station (Sturgeon Bay, WI)

Plants were then stored as tubers for future use. For the characterization of *Rber* we used the pedigree clones of the BCT progeny.

P. infestans isolates

A set of 30 isolates of *P. infestans* was characterized for compatibility/incompatibility with each of the 11 known R-genes. The set of *P. infestans* isolates was obtained from the Cornell University *P. infestans* culture collection, and included strains from Ecuador, Kenya, Mexico, Uganda, United States, Peru, South Africa and Poland (Table 2). The strains were chosen on the expectation that phenotypic differences would occur among isolates from the geographically diverse regions. Specific information regarding the isolates is available upon request. For the assays, isolates were first grown on Rye B medium (Caten and Jinks 1968) in a growth chamber at 15°C to encourage sporulation and then transferred to new media every other week.

The isolates were tested on each host differential plant using a detached leaflet assay (Black et al. 1953; Dorrance and Inglis 1997). Each inoculation involved recently expanded detached leaflets from 5-, 6-week-old plants of each host differential. Fifty microliters of a suspension bearing at least 15,000 zoospores/ml were deposited on the abaxial side of the leaflets using Petri dishes with water agar (15 g/l) on the bottom as moist chambers (Tooley et al. 1986). Each inoculated leaflet was incubated for 7 days at 15°C and scored for presence/absence of infection (detected as sporulation from the leaflet) on the seventh day after inoculation (Dorrance and Inglis 1997; Rivera-Peña 1990).

Wastie (1991) and Dorrance et al. (1997) have suggested that small-scale tests, such as detached leaflets, should be interpreted with caution, because the conditions may not reliably reflect the real resistance or susceptibility interaction other than in extreme cases. Therefore, we scored as "compatible" only those interactions in which sporulation was evident. All cases without sporulation, and where hypersensitive response was present, were scored as incompatible. Each test was repeated at least five times and only those strains that showed consistent reactions were chosen to comprise a "tester set" (Table 2). A dichotomous key (Fig. 1) was then built to facilitate the identification of each of the 11 known R-genes from *S. demissum*.

Once the tester set of *P. infestans* isolates was developed, it was used to characterize the pedigree clones parents of the BCT progeny (USW22-30, HH1-9, and M200-30; the B11B clone was used as a representative of *S. berthaultii*), two of which were known to carry the *Rber* gene (M200-30 and B11B). Detached leaflet assays were again used to identify specific reactions against each isolate. The cultivar Norchip was used as a positive control in every inoculation due to its high susceptibility and absence of known R genes to *P. infestans*.

Phenotypic characterization of the BCT progeny

The presence or absence of *Rber* was determined in the extended progeny and reconfirmed in the original BCT progeny. We used isolate US940480 (ATCC# 208834, a member of the US-8 clonal lineage) obtained from the Cornell University *P. infestans* culture collection. We had previously shown that this isolate can distinguish *Rber* from 8 out of the 11 known R-genes (Ewing et al. 2000), and so it was used in both field inoculations and detached leaflet assays. Field inoculations are desirable because they reflect better resistance under agricultural conditions. The original BCT progeny had been previously exposed twice to the US8 isolate (US940480) (Ewing et al. 2000). The US8-compatible individuals from the original progeny and all individuals from the extended progeny were inoculated in the field at Freeville, NY, during the summer of 1999.

For field studies in 1999, plantlets from in vitro cultures were transplanted into Jiffy7-Peat Pellets (Jiffy Products) supports and grown under greenhouse conditions between May 15 and June 15; from June 15 to June 18 they were conditioned to the field environment in cold frames and transplanted to the field in single plant plots during the week of June 18. The spacing between plots was 0.86 m, and the space between rows was 0.9 m. The cultivar Atlantic was included as a positive control because of its high general susceptibility to late blight.

Table 2 Set of *Phytophthora infestans* isolates tested and the correspondent reaction with the R-genes carried by the respective members of the differential host set

Isolate/plant	Country of origin	R0	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11
EC1	Ecuador	C	C	I	C	I	I	I	C	I	I	I	I
KE980002(KE2)	**Kenya**	**C**	**C**	**C**	**I**	**I**	**C**	**C**	**C**	**I**	**I**	**C**	**C**
KE980004	Kenya	C	C	C	C	C	C	C	C	C	I	C	C
KE980008 (KE8)	**Kenya**	**C**	**C**	**C**	**C**	**I**	**C**	**C**	**C**	**I**	**I**	**I**	**I**
KE980012	Kenya	C	C	C	I	I	C	C	C	I	I	C	I
KE980014 (KE14)	**Kenya**	**C**	**C**	**I**	**C**	**I**	**C**	**I**	**C**	**I**	**I**	**C**	**I**
KE980016	Kenya	C	C	I	C	I	C	I	C	I	I	C	C
MX980002	Mexico	C	C	C	I	I	C	C	C	I	I	C	C
MX980005	Mexico	C	C	C	I	I	C	C	C	I	I	C	C
MX980006	Mexico	C	C	C	I	I	C	C	C	I	I	C	C
MX980082	Mexico	C	C	C	I	I	C	C	C	I	I	C	C
MX980083	Mexico	C	C	C	I	C	C	I	I	I	I	C	C
MX980084	Mexico	C	C	C	I	I	C	I	I	I	I	C	C
MX980085 (MX85)	**Mexico**	**C**	**C**	**C**	**I**	**I**	**C**	**I**	**I**	**I**	**I**	**C**	**C**
MX980099	Mexico	C	C	C	I	I	C	C	I	I	I	C	C
MX980100 (MX100)	**Mexico**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**I**	**I**	**C**	**C**
PE970002	Peru	C	C	C	C	C	C	C	C	I	I	C	C
PO149	Poland	C	C	C	I	C	C	C	C	I	I	C	C
SA960001	South Africa	C	C	C	C	I	C	I	I	I	I	I	C
UG980001	Uganda	C	C	C	C	C	C	C	C	V	V	C	I
UG980005 (UG5)	**Uganda**	**C**	**C**	**C**	**I**	**I**	**C**	**C**	**C**	**I**	**I**	**I**	**I**
UG980006	Uganda	C	C	C	I	I	C	C	C	I	I	I	I
UG980008	Uganda	C	C	I	C	I	C	I	C	I	I	C	C
UG980010	Uganda	C	C	C	I	I	C	C	C	I	I	I	C
US980066 - *US11*	USA	C	C	C	C	C	C	C	C	I	I	C	C
US970001 (*US17*)	**USA**	**C**	**I**	**I**	**C**	**C**	**C**	**I**	**C**	**I**	**I**	**I**	**I**
US940480 (*US8*)	**USA**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**I**	**C**	**C**	**C**
US940501 – *US1*	USA	C	I	I	I	I	I	V	I	I	I	I	I
IPO428.2	Netherlands	C	C	I	C	I	C	C	C	I	I	C	C
VK98014	Netherlands	C	C	I	C	I	C	I	C	I	I	I	C

The accession numbers are given by the year of isolation (i.e., 1998) and the consecutive number from the FryLab collection (Cornell University), isolates IPO428.2 and VK98014 were provided by W. Flier (Plant Research International, The Netherlands). Isolates in bold were chosen for the tester set, codes in parenthesis correspond to Fig. 1, italics represent clonal lineages. Reaction to inoculation is recorded as *C* Compatible; *I* Incompatible and *V* Variable

Potato production practices typical of northeastern USA were used to encourage plant growth. Fertilization was at the rate of 167 kg/ha of a 13-13-13 blend of nitrogen, phosphorous and potassium. Weeds were controlled as needed by applications of Lorox DF (lineron, Dupont, Wilmington, DE, USA) and Dual II (metalochlor, Syngenta, Greebsboro, NC, USA) at recommended label rates. Insects were controlled with the

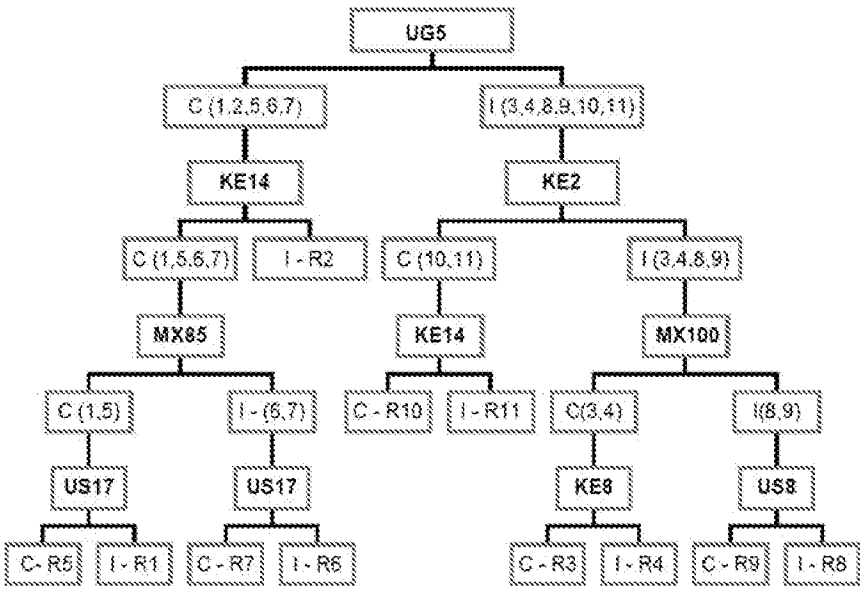

Fig. 1 Flow chart for identification of resistance genes to *Phytophthora infestans* in potato germplasm. The names in *bold* correspond to Table 2. C/I stand for compatible/incompatible reactions, respectively, and the numbers in *parentheses* are the resistance genes that show the corresponding reaction

Aardevo Exhibit 1010
Page 1104

applications of Provado 3.75 oz/A (imidacloprid, Bayer CropScience, Durham, NC, USA), Sevin XLR 1 qt/A (carbaryl, Bayer CropScience), or Ambush 6.4 oz/A (permethrin, Amvac chemical corp, Newport Beach, CA, USA) when necessary.

Inoculation with *P. infestans* took place during the evening of August 13 by applying a suspension (10 ml) containing 150 sporangia/ml of isolate US940480 to each plant. Immediately prior to inoculation, the entire area had been sprinkler-irrigated to an equivalent of about 0.2 in. of rainfall. The inoculum was applied with a hand-held sprayer. Sporangia had been obtained from sporulating lesions on leaflets of potato cv. Atlantic.

The epidemic developed rapidly and rate of disease development on each progeny genotype was recorded. The amount of foliar disease (as a percentage of the total tissue affected) was evaluated every 3 days between September 5 and October 2 using the methods and guidelines previously described (Ewing et al. 2000). These data were used to calculate the area under the disease progress curve (AUDPC) as described by Shaner et al. (1977). Those plants that showed inconsistent results or that died in the field for reasons other than late blight were subsequently tested using detached leaflets from plants grown in the greenhouse as described before.

DNA extraction

DNA was obtained from each of the individuals in the original and extended BCT progeny. Expanding leaflets were collected from 826 plants growing under greenhouse conditions and were placed in liquid nitrogen. DNA was extracted using a CTAB extraction protocol (Doyle and Doyle 1990). The precipitated DNA was treated with 5 µl of a solution (10 mg/ml) of RNAse A for 30 min at 37°C. It was then visualized in a 0.8% agarose gel in TAE buffer to verify its integrity, and concentration was measured using a Hoefer DyNA QUANT 200 fluorometer (Amersham Biosciences, San Francisco, CA, USA).

Restriction fragment length polymorphism

Restriction fragment length polymorphism probes for chromosome 10 were chosen based on the tomato genetic map and obtained from Dr. Steve Tanksley's lab (Cornell University). To obtain sufficient probe DNA for RFLP hybridization, each cloned probe was amplified by PCR using M13 universal primers in a 25 µl final volume. For the template, we used 1 µl of a liquid culture of *Escherichia coli* transformed with the probe which had been diluted to half concentration in distilled water and incubated in boiling water for 5 min. PCR conditions were: 2.5 mM $MgCl_2$, 0.2 µM of each primer, 200 mM dNTPs, and 1 U of *Taq* Polymerase (Invitrogen Corporation, Carlsbad, CA, USA). Each probe was then amplified as follows: initial denaturation 5 min at 94°C, followed by 30 cycles of 94°C for 60 s, 55°C for 30 s, and 72°C for 90 s, and a final extension at 72°C for 5 min. The amplification products were visualized on 1% agarose gels and cleaned using the Wizard PCR preps DNA purification system (Promega, Madison, WI, USA).

To find polymorphisms in the progeny we performed Southern hybridizations with each tomato probe. DNA from each of the BCT pedigree clones was digested in separate reactions with: *Dra*I, *Eco*RI, *Eco*RV, *Hae*III (all from Invitrogen Corporation), and *Bst*NI (New England Biolabs, Beverly, MA, USA). Digestion and Southern blot procedures were done as described by Sambrook et al. (1989) using Hybond N+ membrane (Amersham Pharmacia Biotech) for capillary transfer. For hybridization buffer we used Hyb-9 Hybridization solution (Gentra Systems, Plymouth, MN, USA).

Probes were labeled with ^{32}Pα-dATP with the Random Primers DNA Labeling system (Invitrogen Corporation) and cleaned with Bio-Spin 30 Tris columns (Bio Rad, Hercules, CA, USA). Hybridization took place for at least 4 h at 65°C. Washes were performed with 2× SSC—0.1% SDS for 15 min, 1× SSC—0.1% SDS for 15 min, and 0.5× SSC—0.1% SDS for 15 min at 65°C. The membranes were then exposed to BioMax MS film (Eastman Kodak Company, Rochester, NY, USA) overnight at −80°C and developed with a Kodak X-Omat automatic developer.

Multiplex allele-specific polymorphism mapping

We used a mapping technique that we named Multiplex Allele-Specific Polymorphism (MASP-map), since it allows the simultaneous amplification of one allele and a control band. It is designed to follow a single allele through a mapping progeny, via a modification of the similar methods such as PASA (Okimoto and Dogson 1996) and tetra-primer ARMS-PCR (Ye et al. 2001). MASP-map allowed us to change RFLP probes into PCR markers. The first step is to design primers from known RFLP probe sequences and use them to amplify and sequence the parents of the cross (Fig. 2). These sequences are then used to find single nucleotide polymorphisms that allow the unique identification of one of the parental alleles. A new allele-specific primer is designed so that the 3′ end anneals to the polymorphic site. In addition a mismatch is added in the penultimate nucleotide on the 3′end of the specific primer to help improve allele specificity.

To screen individuals of the progeny for the parental polymorphism of interest, PCR is carried out with three primers simultaneously; two that amplify both alleles and a third that is specific for the allele with the targeted polymorphism. Thus, individuals lacking the allele of interest amplify only the control band, while in individuals containing the allele of interest, both the specific allele and the control band are amplified.

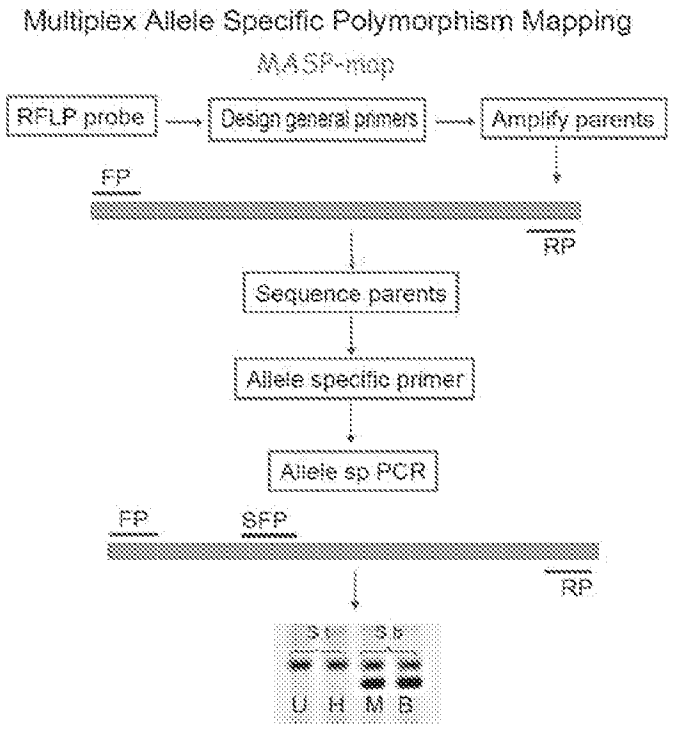

Fig. 2 Diagram of the MASP-map method. See text for explanation of the methodology. FP and RP stand for forward and reverse primer, respectively. SFP stands for specific forward primer. The *last box* shows results of MASP-map: *S.t.* and *S.b.* stand for *Solanum tuberosum* and *Solanum berthaultii,* respectively. UHMB are U: *S. t* parent USW 22-30; H: *S. t.* Recurrent parent HH1-9; M: hybrid parent M200-30; B: *S.b.* representative B11B, respectively

The amplification products are then separated and visualized in agarose gels.

In order to add markers to the interval between CT214 and TG63, the sequences of six tomato RFLP probes were obtained from the Solanaceae Genome Network (http://www.sgn.cornell.edu). From these, forward and reverse primers were designed using Primer Select from the DNASTAR DNA analysis software package (DNASTAR, Inc.), trying to localize them within the 100 initial or terminal base pairs on each probe.

These primers were used to amplify alleles in each individual parent from 50 ng of template DNA in a reaction containing 0.8 U of *Taq* DNA Polymerase (Invitrogen Corporation), 1× PCR buffer (10× stock: 200 mM Tris–HCl pH 8.4, 500 mM KCl), 2.5–3.0 mM $MgCl_2$, 200 nM of forward and reverse primers, 200 nM of dNTPs, and double distilled sterile water. The reactions were carried out in a final volume of 50 µl set on ice to minimize nonspecific amplification. Prior to sequencing, 1 µl of the PCR product was visualized to verify amplification and size of the products on a 1% agarose gel, and the remaining 49 µl were purified using the Wizard PCR PrepDNA purification system (Promega). Amplification products were sequenced by the Cornell Biotechnology Sequencing Facility using only the forward primer for short products (around 500 bp) or both forward and reverse primers for longer products.

The two parental sequences obtained for each probe were compared with Sequencher (version 4.0.5, Gene Codes Corp. MI, USA) to look for single nucleotide polymorphisms that were specific to the *S. berthaultii* parent. Specific primers for *S. berthaultii* polymorphisms were designed from the parental sequences with a T_m about 5°C lower than those of the initial primers (Table 3) and included a mismatch at the penultimate base at the 3′ end of the primer to improve specificity as described by Cha et al. (1992).

When the polymorphism was close to the 5′ end of the fragment a forward primer was designed (5/6 probes, Table 3); when polymorphisms were closer to the 3′ end, a reverse primer was designed (1/6 probes, Table 3). For the sake of clarity, the three primers used will henceforth be referred to as allele-specific (usually forward), general-forward and general-reverse.

To facilitate mapping the region of interest, we screened only individuals with a recombination at any point along chromosome X as first determined by Bonierbale et al. (1988a). Using this method, markers mCT11, mCD5, mCT238, mCT240, and mT1682 were added to the genetic map of chromosome X, the "m" refers to RFLP makers detected by MASP-map.

Tomato marker sequences have been published by Ganal et al. (1998). In order to assess percent similarity between potato and tomato sequences, they were aligned, visually inspected to identify sites with signal from more than one nucleotide (i.e., double peaks), and coded according to standard IUPAC degenerate code. Sequences were then trimmed to the same size using Sequencher version.4.2 (GeneCodes Corp.). Since tomato sequences were obtained from ESTs, for accurate comparison, introns were edited out of potato sequences. However, alignments among potato accessions were done using the whole length of the sequences. To find sequence similarity, the ClustalW utility of the software package MegAlign (Lasergene Corp, Clewley and Arnold 1997) was used. Multiple alignments were performed using the slow/accurate option weighted to account for diploidy.

Finally, sequences were compared to previously reported sequences at GenBank using the BLAST algorithm. ESTs were compared to the dbEST database, which contains GenBank, EMBL, and DDBJ sequences from ESTs. Sequences for the genomic marker TG63 were compared both with the nonredundant and the EST databases.

Cleaved amplified polymorphic sequences (CAPS)

The sequence of tomato probe TG63 was used to design primers to amplify homologous sequences in potato. However, due to multiple insertions and deletions in the sequence, it was not possible to design MASP-map primers. Therefore, we decided to use CAPS to transform this RFLP probe into a PCR marker. After screening the PCR products of all the pedigree

Table 3 Primers used for MASP-map

Name	Sequence 5′ → 3′	T_m (°C)	°C/S	Prim. size (bp)	Diff. (bp)
CT214F	**GAA CGC GAA AGA GTG CTG ATA G**	**57.1**			
CT214BF1	***GAT TCC AAC ATT CAC AAG GGT***	**54.5**	**63/45**	**618/537**	**81**
CT214R2	**CCC GCT GCC TAT GGA GAG T**	**59.7**			
CT238F2	**GGA TAA GGC GGT TCT GTC**	**50.9**			
CT238BF1	***TTC GAT GCC AAT CTC CTA***	**51.0**	**53/30**	**300/225**	**75**
CT238R1	**AAT TTC TCC ATG TTT TTC AG**	**47.6**			
CD5F	**TTG AGG CTA TTG TAC GAG TGT GCG**	**60.6**			
CD5BF1	***TGA GCA ACG TAA TGT GGA AAA***	**54.3**	**57/30**	**464/270**	**194**
CD5R	**AAA GCC TCT TAG GTA CAT TAT GTC G**	**56.2**			
CT11F1	**AGA TTG CTT GTT TGG TGG TT**	**54.3**			
CT11BR1	***TCT CAA AAG GAA TCT TGA CAC AG***	**54.4**	**61/20**	**316/250**	**66**
CT11R1	**TGG AGC AGT CAA CAG AGG**	**54.7**			
CT240F1	**CCA AAG CCC AGG CTG TCA AG**	**59.7**			
CT240BF1	***GGT TCT AAA ATG TCC TCT TAA A***	**49.5**	**55/20**	**890/718**	**172**
CT240R1	**AGT CGG GTG TCA CAA TAA**	**59.9**			
T1682F2	**CGG AAG AAC ATG GAT TTG AAG C**	**55.8**			
T1682BF4	***CAT CTC CCA GCT CAT CAT***	**51.9**	**56/10**	**523/325**	**198**
T1682R2	**CGT CAT TTT CCG ACG AGG ATT T**	**56.9**			

Sequences of primers used to develop and validate the MASP-map technique as well as their melting temperatures and specific amplification conditions. The specific primers are shown in italics; the *Solanum berthaultii* specific nucleotide is underlined and mismatches are noted in bold. The third column shows each one of the primer melting temperatures (T_m). The next column shows the anneal temperature (°C) and the extension time (s) for each PCR reaction. The product sizes (Prim. Size) difference in size (Diff.) between the products of general and allele specific products are illustrated in base pairs (bp)

accessions (USW22-30, HH1-9, M200-30, and B11B) with multiple restriction enzymes, a polymorphism unique to the *S. berthaultii* allele was discovered using *Eco*RV. This polymorphism could then be used as a cleaved amplified polymorphic sequence (CAPS, Konieczny and Ausubel 1993) marker for mapping. For the final screening, 50 ng of genomic DNA were amplified using primers TG63F1 and TG63R (Table 3), and a master mix identical to that described for amplification of RFLP probes, except that the $MgCl_2$ concentration was 3 mM. The PCR conditions for this marker were: initial denaturation 95°C for 2 min; 35 cycles of 95°C for 30 s; 50°C for 30 s; 72°C for 90 s; and final extension at 72°C for 7 min.

The PCR product was digested with 2.5 U of *Eco*RV (Invitrogen Corporation) at 37°C for 3 h and visualized with ethidium bromide on 1.5% agarose gels in 1× TAE buffer. The gels were examined with a FotoAnalyst Investigation Column Mount System (Fotodyne Inc. Hartland, WI, USA).

Simple sequence repeats

Previous studies have shown that SSR marker STM1056 is located close to TG63 in potato (Milbourne et al. 1998), and hence was also screened for progeny that are recombinant on chromosome X. For amplification we followed the procedure and primers described by Milbourne et al. (1998). PCR products were run on a 5.5% Long Ranger Gel (Cambrex Corporation, East Rutherford, NJ, USA) in a SequiGen GT Nucleic Acid Electrophoresis Cell (Bio Rad Inc.). The gels were run in 0.6× TBE buffer at 2,000 V for about 2 h, dried and exposed to Kodak BioMaxMR film (Eastman Kodak Company) overnight at room temperature.

Search for new markers with bulk segregant analysis and resistance gene analogs (RGA)

To increase the chances of finding bands linked to *Rber* using RGA mapping (see subsequently), we used bulk segregant analysis (Michelmore et al. 1991). DNA of selected BCT progeny was bulked in two groups, one with 10 incompatible and the other with 10 compatible individuals. The respective bulks were made based on the genetic data (Bonierbale et al. 1994) and phenotypic (Ewing et al. 2000) information confirmed for the original BCT progeny in chromosome X. The resistant and the susceptible bulks each comprised individuals with recombination events similar in the area of interest between CT240 and TG63, but differing for the rest of the chromosome to minimize the area of interest. The bulks contained the same amount of DNA from each of 10 individuals in such a way that the incompatible group had the *S. berthaultii* allele (segregating from M200-30 and in common with B11B) for the area of interest, regardless of the rest of the chromosome; and the compatible bulk contained alleles from the *S. tuberosum* progenitors in the area between CT240 and TG63 but could differ in the rest of the chromosome.

We used resistance gene analog (RGA) amplification to search for markers linked to *Rber* because previously cloned R-genes to late blight are known to code for nucleotide binding sites (NBS) and leucine-rich repeats (LRR) (Ballvora et al. 2002; Song et al. 2003). Primers designed to amplify resistance gene analogs, including

regions containing NBS, LRR, and protein kinase (Table 4) were obtained. PCR was carried out using 50 ng of DNA and 0.8 U of *Taq* DNA Polymerase (Invitrogen Corporation), 1x PCR buffer (10× stock, 200 mM Tris–HCl pH 8.4, 500 mM KCl), 2.5 mM $MgCl_2$, 100 nm of 33P-γATP labeled forward primer and 200 nM of reverse primer, 200 nM of dNTPs, and bidistilled sterile water in a final volume of 15 µl. The thermocycler (Hybaid Touchdown TD7200, Thermo Electron Corporation, Waltham, MA, USA) was programmed as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s; annealing of 51–56°C depending on the primer pair for 30 s, extension at 72°C for 20 s, and a final extension step of 72°C for 2 min. Each of the reactions was mixed with 5 µl of formamide loading buffer (80% formamide, 10 mM EDTA, 1 mg/ml bromophenol blue, 1 mg/ml xylene cyanol), denatured for 3 min at 94°C and quickly chilled. RGA products were separated and visualized using the same procedure described for SSRs.

Data analysis

The BCT mapping data set was obtained directly from the Solgenes database (currently available at http://www.grain.jouy.inra.fr/gendatabasemirror.html). The RFLPs, MASP-map markers, RGA, and SSR gels were scored visually for presence or absence of the bands originating in the *S. berthaultii* parent. The genotype for each individual was coded as A (homozygous) or H (heterozygous) for each marker, and these data were combined with the previously published framework markers. The chromosome map was assembled with MapManager QTX, version 0.27 (Manly et al. 2001) using a backcross linkage evaluation with $P = 1\times10^{-5}$ and the Kosambi mapping function. We added the information for the new markers to a different (new) chromosome and used the distribute command. Once the markers were located on chromosome X, we found the best localization for them using the 'ripple' command.

Results

Characterization of *Rber*

The 30 isolates of *P. infestans* we tested were diverse in their interaction (compatible/incompatible) with the differential plants (Table 2). Of these isolates, about half had unique phenotypes; 20% were compatible with R-genes 1, 2, 5, 6, 7, 10, and 11; 10% were compatible with 1, 2, 3, 4, 5, 6, 7, 10, and 11; 6.6% were compatible with 1, 3, 5, 7, 10, and 11; 6.6% with 1, 2, 5, 10, and 11; and 6.6% with 1, 2, 5, 6, and 7. In general, isolates with the same compatibility phenotype came from diverse geographical locations, except for some isolates from Mexico that showed the same compatibility pattern.

We designed a flow chart using the "tester set" of nine isolates (Table 2, Fig. 1) to identify known R-genes. The chart is used as a dichotomous key as follows. An unknown potato genotype is inoculated with each member of the tester set independently and the reactions recorded. The results are then read sequentially following the chart. For example, if a plant is incompatible with the strain UG980005, the next step is to follow the right branch. If it is compatible with the strain KE980002, the left branch should be followed, and so on, until a terminal point is reached, which will show the

Table 4 Resistance gene analogs (RGA) and cleaved amplified polymorphic sequences (CAPS) primers used

Name	Sequence 5′ → 3′	Target	Reference
S1	GGT GGC GTT GGG AAG ACA ACG	P-loop motif and NBS	Leister et al. (1996)
AS1	CAA CGC TAG TGG CAA TCC		
AS2	IAA IGC IAG IGG IAA ICC		
AS3	IAG IGC IAG IGG IAG ICC		
NBS F1	GAA ATG GGN GTN GGN AAR AC	NBS	Yu et al. (1996)
NBS R1	YCT AGT TGT RAY DAT DAY YYT RC		
LM638	GGI GGI GTI GGI AAI ACI AC	P-loop and a transmembrane region	Kanazin et al. (1996)
LM637	ARI GCT ARI GGI ARI CC		
LRR F1	CGC AAC CAC TAG GAG TAA C	Leucine-rich repeats of *RPS2* (F1/R1), *Xa21* (F2/R2) and *Cf9* (F3/R3)	Chen et al. (1998)
LRR R1	ACA CTG GTC CAT GAG GTT		
LRR F2	CCG TTG GAC AGG AAG GAG		
LRR R2	CCC ATA GAC CGG ACT GTT		
LRR F3	TTT TCG TGT TCA ACG ACG		
LRR R3	TAA CGT CTA TCG ACT TCT		
PtoKIN 1	GCA TTG GAA CAA GGT GAA	Protein kinase from tomato *Pto*	Chen et al. (1998)
PtoKIN2	AGG TGG ACC ACC ACG TA		
PtoKIN3	TAG TTC GGA CGT TTA CAT		Zhang et al.(2002)
PtoKIN4	AGT GTC TTG TAG GGT ATC		
TG63F1	CCC AGA GTC CCC CTT CCT ATT	RFLP probe TG63	This study
TG63R	CGA GAT GTT GAA TTT GCG TAA GA		

Resistance gene analog primers used for PCR amplification and search of candidate sequences linked to $R_{Pi\text{-}ber}$. Codes are Y=C/T; R=A/G; D=A/G/T, N=A/C/G/T. See text for details

R-gene that is present. All interactions are recorded and analyzed to determine the number and identification of R-genes present in the potato genotype.

The tester set was applied to three of the pedigree genotypes of the BCT progeny and a representative individual of *S. berthaultii* PI 473331 (Table 5). The *S. tuberosum* 'grand'parent USW-2230 was compatible with all isolates but US17, indicating the presence of *R1*. We also found that the recurrent *S. tuberosum* parent HH1-9 was compatible with all isolates, including US970001, which is only compatible with plants lacking all resistance genes; therefore, no resistance genes were detected in HH1-9. We do not have the original *S. berthaultii* donor (a genotype of PI473331), and therefore could not assess its phenotype. However, the hybrid parent M200-30 (product of a cross between PI473331 and USW22-30) carries *Rber* and is resistant to all isolates in the tester set, suggesting that *Rber* is a new resistance gene. Field tests performed in the Toluca Valley, Mexico allowed the isolation of several strains from plants carring $\boldsymbol{R_{Pi\text{-}ber}}$ allele showing that $\boldsymbol{R_{Pi\text{-}ber}}$ can be overcome by isolates MX990005, MX010003, MX010004, and Mx010008. Testing of these isolates with the differential set also demonstrated their compatibility with *R1–R11* (unpublished results).

Genetic mapping of *Rber*

Phenotypic characterization

We confirmed the phenotype of all individuals in the original BCT progeny evaluated by Ewing et al. (2000) and determined the phenotype of the extended BCT progeny using either field or detached leaflet assays. For plants from the original progeny inoculated in the field with the US8 isolate US940480 during the summer of 1999, the AUDPC score ranged from 0 to 3,652 U. Only those individuals with AUDPC less than 1 were scored as incompatible and thus containing $\boldsymbol{R_{Pi\text{-}ber}}$. The average AUDPC for compatible individuals was 1,580. For the extended population, we tested 336 individuals, of which 180 were incompatible (53.57%). For the combined progeny (original and extended) 52% were resistant (428). For mapping purposes we coded incompatible individuals as carrying the *S. berthaultii* allele ($\boldsymbol{R_{Pi\text{-}ber}}$) in heterozygous condition and the compatible ones as carrying only alleles from the tuberosum pedigree (for purposes of the mapping program these are considered being "homozygous" for the *S. tuberosum* allele, although the pedigree did permit heterozygosity).

MASP-map sequences

Given that MASP-map marker development required the characterization of nucleotide sequences in the pedigree clones of BCT (GenBank acc. AY874391 to AY874411), we thought it would be useful to find the level of similarity among them and with tomato. On average, potato and tomato sequences were over 90% similar. The biggest differences were found in noncoding sequences, e.g., for TG386, where tomato was only 70.1 and 69.2% similar to *S. tuberosum* and *S. berthaultii*, respectively. Regarding *S. tuberosum* and *S. berthaultii*, sequences were over 97% similar on an average. Interestingly the same average similarity was found between the *S. tuberosum* accessions (USW22-30 and HH1-9), most differences were caused by heterozygosity in one of the accessions, especially HH1-9.

Blast searches confirmed previous reports by Ganal et al. (1998) except for CT217, where we found the best homology with an *Avr9/Cf9* elicited protein. Also, mCT11, mCT214, mCT238, and mCT240, each showed homology with sequences from EST libraries made from potato leaves challenged with *P. infestans* (*P* value $> e^{-111}$). These results, the presence of an R-gene, and QTLs for resistance to other pathogens (Gebhardt and Valkonen 2001) may suggest that this area of chromosome X could be involved in resistance responses common to other pedigrees including tomato.

For the marker TG63, forward and reverse sequences from tomato, obtained from the Solanaceae Genomics Network, were compared to sequences in GenBank. No matches were found when the search used the nonredundant database, but the sequences share homology with one EST from tomato roots (EST303128, $1e^{-85}$) and one from the tomato callus (EST542946, $1e^{-55}$). However, since TG63 is a marker from a total genome library, these findings are inconclusive. We also sequenced the amplification products of all pedigree genotypes from primers TG63F1 and TG63R; however, heterozygosity for insertion/deletions precluded clean direct sequences that could be tested for homologies.

Table 5 Results of testing parents of backcross to *S. tuberosum* (BCT) with tester set. C/I refers to compatible and incompatible reactions, respectively. (Norchip was a positive control)

Isolate	Norchip	USW22-30	HH1-9	M200-30	B11B
KE980002	C	C	C	I	I
KE980008	C	C	C	I	I
KE980014	C	C	C	I	I
MX980085	C	C	C	I	I
MX980100	C	C	C	I	I
UG980005	C	C	C	I	I
US17 (US 970001)	C	I	C	I	I
US8 (US940480)	C	C	C	I	I

Genetic markers

Several new genetic markers were added to chromosome X in order to assess the precise location of $R_{Pi\text{-}ber}$. First, we confirmed the identity of each individual from the original BCT progeny by testing with the flanking markers mCT214 and TG63. Doing this, we were able to assess previously missing genotypes for five individuals for CT214 and six individuals for TG63. There was disagreement between the genotyping results in this study and a previous one (Bonierbale et al. 1994) for three individuals that were originally scored for TG63 as double recombinants. In our study, these individuals were recorded as heterozygous (presence of *berthaultii* alleles), whereas in the previous study they were recorded as homozygous, which caused them to show as double recombinants. For a progeny of $N = 158$ and given the original map distance between the markers TG63–*Rber*–CT214b of 4.8 and 8.2 cM, respectively (Ewing et al. 2000), the number of double recombinants should be about 1(0.62). The probability of the three double recombinants reported is 0.0012 suggesting that the original designation is highly unlikely. Using the revised designations determined in this study we found a new distance between TG63 and $R_{Pi\text{-}ber}$ of 0.6 cM, instead of the 4.8 cM originally reported (Ewing et al. 2000); the corrected distance between CT214 and $R_{Pi\text{-}ber}$ is 5.8 cM.

We added the RFLP markers CT217, CD32, CD72, and CT20, from the tomato genetic map to potato chromosome X. Because of the inversion between tomato and potato chromosomes, we found that only CD32 was located in the area of interest at 0.6 cM from CT214. Marker CT217 is located closer to TG52; CD72 and CT20 are located in a terminal position relative to TG63 (Fig. 3).

Using MASP-map, three new markers (mCD5, mCT238, and mCT240) were placed within the interval between mCT214 and TG63. Markers mCT240 and TG63 are the closest to $R_{Pi\text{-}ber}$ at 3.9 and 0.63 cM, respectively (Fig. 3). Two other markers, mCT11 and mT1682, were added outside the interval which confirmed the area where an inversion took place in potato chromosome X with respect to tomato chromosome 10.

When the entire progeny (original and extended; 826 individuals) was tested with the flanking markers mCT214 and TG63 to identify recombinant individuals in this interval, we found 54 recombinants, very close to the 52 expected for a 6.4 cM distance. There were six additional individuals for which results were inconsistent and from which we did not use data. By adding marker mCT240 to the 54 recombinants, 29 recombination events were found between CT240 and $R_{Pi\text{-}ber}$ (a genetic distance of 3.51 cM) and seven recombinants between $R_{Pi\text{-}ber}$ and TG63 (0.84 cM).

The microsatellite STM1056 resulted in a total of 12 markers, most of which are located on chromosome VIII. The band STM1056-7 showed linkage to markers on chromosome X, with LOD scores between 10.9 for AS1-13 and 26.3 for $R_{Pi\text{-}ber}$. It is most likely located between $R_{Pi\text{-}ber}$ and TG63 but precise localization was not possible.

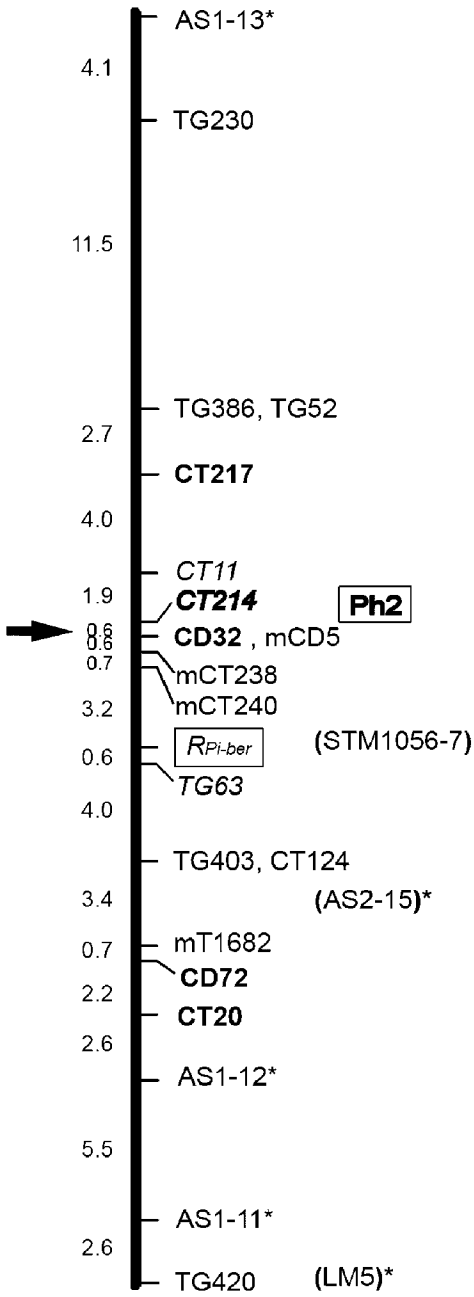

Fig. 3 Localization of $R_{Pi\text{-}ber}$ on chromosome X in the potato map. Previously published framework markers are TG230, TG386, TG52, TG403, CT124, and TG420. RFLP markers are shown in *bold*; MASP-map markers are preceded by "m"; RGA markers have *asterisks* after the name. Approximate locations of RGA and SSR markers are shown in *brackets*, names of cosegregating markers are separated by *commas*. Resistance genes $R_{Pi\text{-}ber}$ and *Ph2* (position inferred from tomato map) are surrounded by *boxes*. Inversion point between tomato and potato is indicated by an *arrow*

From all the RGA primers tested we found three bands linked to chromosome X. These were detected with primers S1 × AS1, which were designed to amplify the P-loop motif and the nucleotide binding site

common to resistance genes (Leister et al. 1996). They are located at 4.1 cM distal to TG230 (AS1-13), and between markers CT20 and TG420 (AS1-11 and AS1-12). There were two other bands, AS2-15 and LM5, with linkage to the chromosome (LOD 18.3 and 18.6, respectively), but we were not able to locate them confidently between other markers on the chromosome. It is possible that the *S. tuberosum* and *S. berthaultii* alleles do not differ enough to show differential amplification with the RGA primers used; therefore, we were unable to find markers closely linked to $R_{Pi\text{-}ber}$. Approximate locations are shown in Fig. 3.

Discussion

The objective of this study was to determine the novelty and precise location of the *Rber* resistance gene from *S. berthaultii*. The first step was to design a method that could rule out known R-genes in potato which are responsible for the resistance reaction conferred by the gene. Using a detached leaflet assay, a host differential set and *P. infestans* isolates from diverse geographic regions. We were able to select nine isolates that together differentiate each of the previously characterized resistance genes through their known incompatibility reactions. Using this tester set and the flow diagram illustrated (Fig. 1), we determined that *Rber* is a new previously uncharacterized R-gene against *P. infestans*. In order to avoid confusion with the recently published RB gene and maintain the currently used nomenclature, that describes R-genes as $R_{\text{pathogen-donor of resistance}}$ (Kuhl et al. 2001; Solomon-Blackburn and Barker 2001) we renamed the gene $R_{Pi\text{-}ber}$.

The rationale for characterizing $R_{Pi\text{-}ber}$ as a locus with a novel specificity, is as follows: it is incompatible with UG980005 suggesting that it could only be *R3, R4, R8, R9, R10, R11, or new;* it is incompatible with KE980002, ruling out *R10* and *R11*; it is again incompatible with MX980100, eliminating *R3* and *R4*, besides *R3* is located on Chr. XI; incompatibility with US8 rules out *R9*. Finally, M200-30 is incompatible with the R8-compatible isolate Ke980004, allowing us to conclude that $R_{Pi\text{-}ber}$ is also not *R8*. Besides, new findings by Huang (2005) suggest that *R5–R11* are allelic to *R3*, ruling them out from chromosome X. This leaves only the proposition that $R_{Pi\text{-}ber}$ is different from previously identified and "named" R-genes. Finally, as previously stated, $R_{Pi\text{-}ber}$ is overcome by several Mexican isolates including Mx990005 and Mx010003. In order to keep the current nomenclature of resistance genes to *P. infestans* in potato, we will refer to *Rber* (Ewing et al. 2000) as $R_{Pi\text{-}ber}$.

While the development of the R-gene tester set proved to be a useful tool, its widespread application is unfortunately limited. Some avirulent characteristics in a few of the isolates have changed over time. Others have also observed this phenomenon (Abu-El Samen et al. 2003). Thus, we found it necessary to include the appropriate host differential plant as positive controls in all inoculation experiments.

The use of a new genotyping method for this study allowed us to add several specific RFLP markers to the map with relative ease. The MASP-map method provides an efficient alternative to RFLPs for mapping in large populations. It is based on the use of primers that specifically amplify one parental allele and that can be used to screen segregation in a progeny. This system is inexpensive and efficient, needing only one PCR step, three primers per set, the usual PCR reagents, and visualization on agarose gels. Another significant advantage of this method is the quantity of DNA required; only 50 ng of DNA per reaction was used in this study. In contrast, the same study using RFLP methods would have required at least 10 μg of DNA per probe. Possibly its most important advantage is that it provides amplification of a control band in addition to the allele-specific band. The lack of the target allele is visualized by amplification of just the control band; the absence of all amplification products in a sample indicates a failure of the PCR.

The original map placed $R_{Pi\text{-}ber}$ between markers CT214b and TG63, an area spanning 13 cM. Through confirmation of phenotypes and genotypes at these markers, we have demonstrated this area to be smaller than originally thought (6.4 cM). While the precise map position of $R_{Pi\text{-}ber}$ is still not known, we were able to add four new markers within the interval and locate $R_{Pi\text{-}ber}$ between CT240 and TG63, to an area spanning 3.8 cM. Map distances calculated for the original progeny increased slightly when the extended BCT progenies were added, from 3.2 between CT240 and $R_{Pi\text{-}ber}$ and 0.63 cM between $R_{Pi\text{-}ber}$ and TG63 to 3.51 and 0.84 cM in the same interval. A possible explanation for this difference is the dissimilar environments in which the crosses were made, as suggested by Paterson (1996). Since the size of the potato genome is close to 1,000 cM (Meksem et al. 1995), which corresponds to 1,000 Mb (haploid genome equivalent, Leister et al. 1997), 1 cM is roughly equivalent to 1 Mb. This means that (on average) there is predicted to be about 3.8 Mb of DNA in the area of interest, and the closest marker TG63 may be only 630 Kb away from $R_{Pi\text{-}ber}$.

The molecular maps of potato and tomato show a strikingly high level of colinearity (Bonierbale et al. 1988a; Gebhardt et al. 1991; Tanksley et al. 1992). The tomato map, consisting of more than 1,030 markers (Tanksley et al. 1992), has been saturated to a higher degree than the potato map. For this reason, our effort to saturate the area with new markers began by using orthologous probes from tomato. Despite the similar genetic structure and colinearity between potato and tomato, their chromosome X homologues differ by a paracentric inversion (Bonierbale. et al. 1988b), close to $R_{Pi\text{-}ber}$. We found the break point to be in a 2.7 cM area between markers TG280 and TG408, in agreement with Tanksley et al. (1992). In contrast to previous expectation, however, our results indicate that CT217 in potato

is located closer to TG386 and is not within the inversion (Tanksley et al. 1992).

One question of interest is whether resistance genes found in one species are orthologous to those found in other species. This is particularly interesting because a recent study by Grube et al. (2000) showed that there are clusters of resistance genes that are conserved across the Solanaceae. Not all proposed homologies, however, turn out to be accurate. For example, *R6* was originally thought to be present in both *S. demissum* and *S. stoloniferum* (Schick et al. 1958). Later work showed these to be two different loci (Malcomson and Black 1966). The same seems to be the case with $R_{Pi\text{-}ber}$. A previous study (Ewing et al. 2000) considered the possibility that $R_{Pi\text{-}ber}$ was orthologous to *Ph2* on tomato chromosome X (Moreau et al. 1998) due to the map position of both genes. Based on the position of the inversion point, we show here that they are not located at the same map position. *Ph2* is located between markers CT240 and TG233 in tomato, which are upstream of $R_{Pi\text{-}ber}$ in the potato map, and which therefore occupy different loci in the long arm of Chromosome X (Moreau et al. 1998).

In our study, resistance gene analogs (RGAs) were amplified with primers designed for nucleotide-binding sites, leucine-rich repeats and *Pto*-kinase. We identified five bands located in chromosome X, three of which could be added to the map (Fig. 2). The other two had high LOD scores for linkage to markers in chromosome X, but we were unable to map them confidently due to too many apparent double crossovers. Another RGA sequence was reported by Leister et al. (1996) on the long arm of chromosome X (St 1.2.4), which could be allelic to AS1-11 or AS1-12. These results may be suggestive of the presence of a resistance gene cluster on chromosome X.

Our results provide a more detailed characterization and improved understanding of $R_{Pi\text{-}ber}$, including a more precise map position on potato chromosome X. Because we converted RFLP markers into MASP-map and CAPS markers, selection in the future for individuals containing $R_{Pi\text{-}ber}$ will be easier and faster, particularly if the gene is used in breeding programs. In addition, the improved localization of the inversion on chromosome X will make it easier to identify new markers from the always-growing tomato map to saturate the area between mCT240 and TG63. Further steps are needed to clone and characterize $R_{Pi\text{-}ber}$. These future analyses will provide insights on resistance interactions between potato and *P. infestans*, and perhaps contribute to the management of the disease.

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The starch-related R1 protein is an α-glucan, water dikinase

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To determine the enzymatic function of the starch-related R1 protein it was heterologously expressed in *Escherichia coli* and purified to apparent homogeneity. Incubation of the purified protein with various phosphate donor and acceptor molecules showed that R1 is capable of phosphorylating glucosyl residues of α-glucans at both the C-6 and the C-3 positions in a ratio similar to that occurring naturally in starch. Phosphorylation occurs in a dikinase-type reaction in which three substrates, an α-polyglucan, ATP, and H_2O, are converted into three products, an α-polyglucan-P, AMP, and orthophosphate. The use of ATP radioactively labeled at either the γ or β positions showed that solely the β phosphate is transferred to the α-glucan. The apparent K_m of the R1 protein for ATP was calculated to be 0.23 μM and for amylopectin 1.7 mg·ml^{-1}. The velocity of *in vitro* phosphorylation strongly depends on the type of the glucan. Glycogen was an extremely poor substrate; however, the efficiency of phosphorylation strongly increased if the glucan chains of glycogen were elongated by phosphorylase. Mg^{2+} ions proved to be essential for activity. Incubation of R1 with radioactively labeled ATP in the absence of an α-glucan showed that the protein phosphorylates itself with the β, but not with the γ phosphate. Autophosphorylation precedes the phosphate transfer to the glucan indicating a ping-pong reaction mechanism.

Starch is the storage carbohydrate most widely distributed in the plant kingdom. In storage organs it serves as a long-term carbon reserve, whereas in photosynthetically competent tissues it is transiently accumulated to provide both reduced carbon and energy during periods unfavorable for photosynthesis. Starch consists of essentially linear (amylose) and highly branched (amylopectin) glucose polymers that are arranged as semicrystalline particles, the starch granules. Amylopectin from many sources contains phosphate-monoesters that are covalently bound at the C6 and C3 positions of the glucosyl residues (1). In potato tuber starch ≈0.1–0.5% of the glucose moieties are phosphorylated. The amount of phosphate monoesters in starch strongly influences its physicochemical properties (2) and, therefore, affects the ability of different starches to be used by industry.

In potato tubers the starch-bound phosphate comprises a significant proportion of the total tuber phosphate content, but its function in starch metabolism is not clear. The same holds true for the biochemical reaction(s) leading to the formation of the starch phosphate monoesters. A protein (designated as R1) has been recently identified using a proteomic approach, and circumstantial evidence suggests that it is involved in the phosphorylation of starch (2, 3). The C-terminal sequence of the R1 protein shows some homology to bacterial PEP synthases (pyruvate, water dikinase EC 2.7.9.2), which transfer phosphate from ATP in a dikinase reaction to pyruvate and water. Antisense repression of R1 in potato leads to a strong reduction in the amount of starch-bound phosphate, whereas the expression of R1 in *Escherichia coli* resulted in an increase in the phosphorylation of the bacterial glycogen (2). In addition, a mutation in a homologous gene in *Arabidopsis* leads also to a reduction in the phosphate content of leaf starch (3).

Interestingly, the degradability of starch was strongly impaired in these transgenic and mutant plants leading to a starch-excess phenotype in leaves and a repression of cold-sweetening in potato tubers. It cannot be ruled out, therefore, that the R1 protein is involved in starch degradation and that the decrease in starch phosphorylation seen in plants with reduced R1 protein amounts is the result of some pleiotropic effect. This view is supported as the R1 protein binds to leaf starch granules during periods of net starch degradation, but not during synthesis (4). Furthermore, in various plant species R1 levels did not correlate with the degree of starch phosphorylation (5).

In the present communication we describe the purification of the heterologously expressed potato R1 protein to apparent homogeneity and its functional analysis *in vitro*. The results clearly show that R1 catalyses the phosphorylation of starch-like glucans.

Materials and Methods

Materials. The following commercial biochemicals were used: amylopectin from potato and corn (Sigma), glycogen from bovine liver (Fluka), and amylose from potato (Merck), myokinase (EC 2.7.4.3) from rabbit muscle (Sigma), pyruvate kinase (EC 2.7.1.40) from rabbit muscle (Roche), and phosphorylase a (EC 2.4.1.1) from rabbit muscle (Sigma). [β-^{33}P]ATP [1 mCi/ml; 3000 Ci/mmol (1 Ci = 37 GBq)] and [γ-^{33}P]ATP (10 mCi/ml, 3000 Ci/mmol) were purchased from NEN.

Glucose-3-phosphate was synthesized in 45% overall yield from β-D-(1,2)(4,6)-diacetone glucose (Aldrich) following a procedure described by Perich and Johns (6, 7), which includes 3-phosphorylation using dibenzyl diisopropyl-phosphoramidite and subsequent deprotection. The product was identified to be (α, β)-D-glucose-3-phosphate by NMR and mass spectrometry. The following characteristics were obtained. R_f (MeOH): 0.52; matrix-assisted laser desorption ionization (MALDI)-MS (positive ion mode, matrix: THAP): 299.1 (M+K), 283.1 (M+Na), 261.1 (M+H); ^{1}H NMR (300 MHz, D_2O) δ-: 5.19 - 5.20 (d, J = 3.2, 1 H, H-1, α), 4.62–4.65 (d, J = 7.9, 1 H, H-1), 4.22–4.26 (q, J = 8.6, 1 H, H-3), 4.02–4.06 (q, J = 8.5, 1 H, H-3), 3.31–3.85 (m, 10 H, H-2, 4, 5, 6); ^{13}C NMR (75 MHz, D_2O) δ-: 100.0 C-1 (α), 96.5 C-1 (β), 86.2, 86.1 C-3, 84.0, 83.9, 79.9, 77.8, 75.5, 73.2, C-2,4,5, 65.12, 64.9 C-6). ^{31}P-NMR [121 MHz, citrate buffer (pH 4.5), D_2O, 85% H_3PO_4 as external standard] resulted in a chemical shift (δ-: 2.05 ppm) as described by Blennow *et al.* (8).

SDS/PAGE and Immunoblotting. SDS/PAGE and immunoblotting (using the polyclonal anti R1 holoprotein antibody) were performed as described (4).

Abbreviations: G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; G3P, glucose-3-phosphate; HPAEC-PAD, high-performance anion exchange chromatography with pulsed amperometric detection.

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Page 1115

Analytical Techniques. Proteins were quantified according to Bradford (9), using the Bio-Rad protein assay and BSA as a standard. Glucose, ADP, and AMP were measured as described (10). Pi was determined according to Parvin and Smith (11). glucose-6-phosphate (G6P) and glucose-3-phosphate (G3P) were analyzed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as described (5). However, a CarboPac PA-100 column (Dionex) was used. Alternatively, G6P was analyzed enzymatically (12).

Purification of Recombinant R1 Protein. Potato R1 was heterologously expressed in *E. coli* BL21(DE 3) carrying the plasmid pET21d R1-tp as described (4). However, R1 expression was induced by addition of isopropyl β-D-thiogalactoside (IPTG) to give a final concentration of 1 mM. The cells were lysed and extracted in 50 mM Tris·HCl (pH 7.5), 2.5 mM EDTA, 2.5 mM dithioerythritol (DTE), 0.5 mM PMSF, and 2 mM benzamidine. Following centrifugation, the soluble protein fraction was subjected to anion-exchange chromatography (2). The R1-containing fractions were pooled and concentrated using ultrafiltration (Diaflo PM30, Amicon). Subsequently, the concentrate was passed through a PD10 column (Pharmacia) equilibrated with buffer A [100 mM 4-morpholinepropanesulfonic acid (Mops)-KOH (pH 7.6)/1 mM EDTA/2 mM DTE/0.5 mM PMSF]. The eluate (3.5 ml containing 3–4 mg protein) was then loaded onto a column (1.6 × 8.5 cm) filled with native starch granules from potato tubers (Merck). The granules had been washed twice in deionized water and once in buffer A before they were filled into the column. Following sample application, the starch column was washed with 20 ml of buffer A and, subsequently, with 30 ml buffer A supplemented with 50 mg/ml of malto-oligosaccharides (Glc_2–Glc_6; Merck) at a flow rate of 0.5 ml/min. Those fractions apparently containing only R1 were combined and concentrated to a volume of 1.5 ml by ultrafiltration. The buffer in the R1 preparation was changed by passage through a HiTrap desalting column (Pharmacia) equilibrated with buffer B [50 mM Hepes-KOH (pH 7.5)/1 mM EDTA/1 mM DTE/0.5 mM PMSF/10% glycerol]. Aliquots of the R1 preparation were stored at −80°C until use.

Synthesis of a Glucan Substrate for R1 by Chain Elongation of Glycogen ("Elongated Glycogen"). Twenty milligrams of glycogen dissolved in 18 ml of 50 mM Hepes-KOH (pH 7.5), 1 mM EDTA, 40 mM glucose-1-phosphate (G1P), and 10% glycerol were incubated with 22 units of phosphorylase for 20 min at 25°C. Phosphorylase was then inactivated by heating for 10 min at 100°C. The polyglucan was precipitated by adding EtOH to a final concentration of 60%. Following centrifugation, the polyglucan was washed twice by resuspension in 24 ml of water and precipitation as above. This glucan was used in the nonradioactive "postelongation assay" (see below).

The maximum chain lengths (detected using MALDI-MS) in debranched samples of the untreated glycogen and the elongated glycogen were 33 and 55, respectively.

R1 Activity Assay. If not otherwise stated activity assays (final volume 0.5 ml) were performed in buffer C [50 mM Hepes-KOH (pH 7.5)/1 mM EDTA/6 mM $MgCl_2$/10% glycerol] that was supplemented with polyglucans and different potential phosphate donors as indicated. Reactions were started by adding purified R1 (20–40 μl in buffer B). In control samples R1 was replaced by an equal volume of buffer B.

Nonradioactive Assays. Two different nonradioactive assays were used. (*i*) In the postelongation assay R1 was reacted with 1.4 mg "elongated glycogen" (see above). (*ii*) In the coelongation assay R1 was incubated with 0.5 mg of glycogen, 40 mM G1P, and 0.6 units of phosphorylase.

Following incubation at 25°C (as indicated; see *Results*), the reaction was terminated by heating for 5 min at 95°C. Aliquots (0.3–0.5 ml) were withdrawn and the polyglucans precipitated by adding cold EtOH to give a final concentration of 70%. After incubation for 1–2 h on ice and centrifugation (20,000 × *g*, 10 min at 4°C) the pellet was resuspended in 0.5 ml of water and precipitation was repeated once (as above). The pellet was dried in a speed vac for 5 min and subsequently resuspended in 150–250 μl of 0.7 M HCl. Following incubation at 80°C for 15 min, an aliquot (50–70 μl) was removed and neutralized with 2.8 N KOH, and G6P was determined enzymatically. The remaining solution was then hydrolyzed at 95°C for 2.5 h. Following neutralization, G6P and glucose were determined enzymatically. Because the phosphorylase preparation may contain small amounts of contaminating phosphoglucomutase, we checked for the presence of free (not glucan bound) G6P in the solution. To calculate for glucan bound G6P residues the amount of G6P determined before hydrolysis at 95°C was subtracted from the value determined following hydrolysis (free G6P was observed only in the coelongation assay).

Radioactive Assays. Two elucidate whether the γ-P or the β-P of ATP is transferred to the glucan we used two different labeled ATP preparations in the initial radioactive experiment (results in Table 3). (*i*) [γ-^{33}P]ATP and (*ii*) a mixture of [γ-^{33}P]ATP and [β-^{33}P]ATP. The latter was obtained by mixing 1 μl of [γ-^{33}P]ATP with 350 μl of a buffer that contained 50 mM Hepes-KOH (pH 7.5), 1 mM EDTA, 10% glycerol, 5 mM $MgCl_2$, 5 mM KCl, 0.1 mM ATP, and 0.3 mM AMP. The formation of [β-^{33}P]ADP was initiated by the addition of 2.4 units of myokinase. Following the incubation for 25 min at 37°C, the reaction was terminated by heating for 10 min at 95°C and filtration through a 10-kDa filter (Microcon YM-10, Amicon). [β-^{33}P]ADP was then converted to [β-^{33}P]ATP by adding 0.86 mM PEP and 2 units of pyruvate kinase. After 20 min incubation at 37°C the reaction was heat terminated.

In all other radioactive assays pure [β-^{33}P]ATP was used; 0.2–1 μCi were applied in each assay (0.5 ml sample). The mixture was incubated at 25°C as indicated and was then terminated by heating for 10 min at 95°C. The polyglucan was separated from the soluble components (including the labeled ATP) by use of centrifugal filter units (Microcon YM-10; see above). 230 μl of the suspension were mixed with 270 μl of 2 mM cold ATP and passed through the 10-kDa filter by centrifugation (30 min, 14,000 × *g*). The polyglucan that remained on top of the filter was further washed four times by resuspension in 450 μl of cold 2 mM ATP solution and subsequent centrifugation. Finally the filter unit was placed in a scintillation vial, scintillation fluid was added (Ready Safe, Beckman), and the vial was vortexed vigorously for 10 s before the incorporated radioactivity was determined in a scintillation counter.

***In Vitro* Phosphorylation of the Purified R1 Protein.** R1 was incubated with labeled ATP in buffer C (further details are given in the legend to Fig. 5). Following denaturation, samples were subjected to SDS/PAGE. Gels were either dried or proteins were blotted to nitrocellulose before autoradiography. Treatments with heat, alkali, or acid were adapted from Rosenberg (13). However, the final concentrations of NaOH and HCl were decreased to 0.5 M because 1 M NaOH caused significant hydrolysis of the R1 protein, as revealed by protein staining on the membrane.

Results

Purification of R1. R1 from potato was heterologously expressed in *E. coli* and then purified to apparent homogeneity. As a first step, anion exchange chromatography proved to be efficient (Fig. 1, lane 2). For further purification we took advantage of the interaction of R1 with starch granules (2, 4). The concentrated protein fraction obtained by anion-exchange chromatography was passed through a column filled with native starch granules from potato tubers. When

Aardevo Exhibit 1010
Page 1116

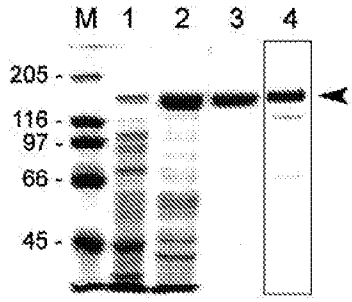

Fig. 1. Purification of recombinant R1. Proteins were separated by SDS/PAGE and stained with Coomassie (lanes M and 1–3) or subsequently transferred to nitrocellulose and probed with an antibody raised against R1 (lane 4). M, molecular weight marker; 1, crude *E. coli* extract following induction (10 μg); 2, protein fraction derived from anion-exchange chromatography (10 μg); 3, purified R1 following passage through the starch column (2.5 μg); 4, Western blot of the purified R1-fraction (0.7 μg).

the starch column was eluted with buffer A (without maltooligosaccharides), contaminating proteins were eluted first, whereas release of most of the R1, because of the interaction with the granules, required a larger buffer volume (data not shown). However, under these conditions elution of R1 results in a considerable dilution. To prevent the dilution, a two-step procedure was established that consists of elution with 20 ml of buffer A to remove contaminating proteins and, subsequently, with a malto-dextrin mixture dissolved in buffer A. Those R1 molecules that had remained on the column until then were eluted as a sharp peak (data not shown). The resulting R1 preparation apparently lacks contaminating proteins and the identity of R1 was confirmed by Western blotting (Fig. 1, lanes 3 and 4).

R1 Catalyzes an ATP-Dependent Phosphorylation of Polyglucans. No starch-related phosphotransferase activity has ever been demonstrated and neither the phosphate donor nor the actual phosphate acceptor molecule for this type of reaction has been identified. In potato tubers starch phosphorylation appears to occur during biosynthesis of the polysaccharide (12). To mimic the process of starch biosynthesis we used a system composed of glycogen, G1P, and phosphorylase from rabbit muscle (coelongation assay; see *Materials and Methods*). Glycogen served as primer for the glucosyl transfer from G1P catalyzed by phosphorylase. If purified R1 and different possible phosphate donors are added to this mixture, elongating α-glucan chains of the glycogen can act as acceptors for a putative phosphotransferase reaction. Following overnight incubation, the polyglucans were precipitated and hydrolyzed to release glucose and, if phosphorylation has occurred, glucose-phosphates as well. G6P and glucose were determined enzymatically. As shown in Table 1, R1 catalyzes glucan phosphorylation in the presence of ATP. No phosphorylation was observed in the presence of PEP, PPi, UTP, or GTP (or in the absence of ATP). Thus, among all compounds tested only ATP functions as a phosphate donor. As a control experiment, we transformed *E. coli* with an empty vector and subjected the extracted proteins to the purification procedure described for R1. No phosphorylating activity was detectable in the resulting protein preparation. This finding demonstrates that the enzyme activity described here was neither due to any contaminating bacterial protein that copurifies with R1 nor derived from the native starch granules applied for affinity chromatography.

During incubation the amount of polyglucan per reaction mixture increased from 0.5 mg (amount of glycogen added) to about 2 mg (as calculated from the glucose quantification following acid hydrolysis). It should be noted that no phosphate incorporation was detected by using the nonradioactive assay when R1 was incubated with 2 mg of glycogen and ATP but phosphorylase was omitted (data not shown).

Table 1. The phosphorylation of glucans catalyzed by R1 depends on ATP

Substrates	Phosphate incorporation, nmol G6P/μmol Glc
ATP, PEP	9.31 (9.29, 9.32)
ATP, PPi	8.60 (9.52, 7.67)
ATP	8.55 (7.81, 9.28)
PEP, PPi	ND
PEP	ND
PPi	ND
UTP	ND
GTP	ND
None	ND

Activity of R1 was determined using the coelongation assay. R1 (7.2 μg each) was incubated for 14 h with 0.5 mg glycogen, 40 mM G1P, 0.6 units of phosphorylase, and different possible phosphate donors (5 mM ATP, PEP, UTP, and GTP; 2.5 mM PPi). Following precipitation and hydrolysis of the polyglucan, G6P and glucose (Glc) were determined enzymatically. Values are means of two independent experiments. Values in parentheses are the individual measurements for experiments 1 and 2 (each run in duplicate). In controls (lacking R1) no phosphate incorporation was observed (not shown). ND, not detectable.

R1 Phosphorylates both the C-6 and C-3 Positions. *In vivo* starch phosphorylation results in esters linked to both the C-6 and C-3 of glucosyl moieties (1). To study whether the same phosphorylation pattern is achieved by the *in vitro* action of R1, the polyglucan fraction (obtained in the coelongation assay) was subjected to acid hydrolysis and the resulting monomers were analyzed by HPAEC-PAD. As shown in Fig. 2, both G6P and G3P were clearly detectable in the R1-containing reaction mixture, but absent in the control lacking R1. The identity of the two glucose-phosphates was confirmed by comparison with authentic G6P and G3P (Fig. 2) and by disappearance of the respective peaks if the hydrolyzed samples were treated with alkaline phosphatase (not shown). This shows that under *in vitro* conditions R1 catalyzes the phosphorylation of both C-6 and C-3 position of the glucose residues. The ratio of G6P to G3P residues is comparable to that obtained in native potato amylopectin (not shown).

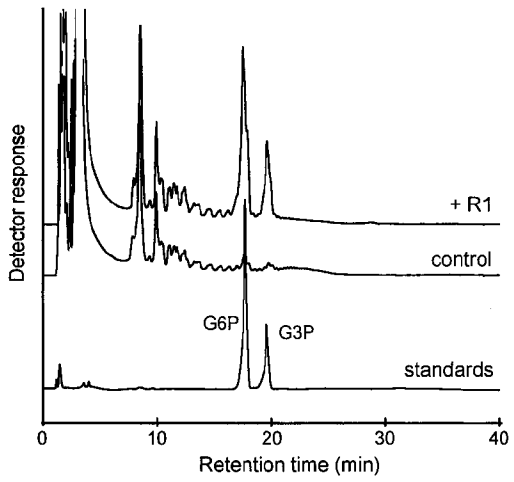

Fig. 2. G6P and G3P residues are products of *in vitro* phosphorylation by R1. Following the coelongation assay (7.2 μg R1,5 mM ATP, 14 h incubation) the synthesized polyglucan was precipitated and hydrolyzed. Samples containing equal amounts of glucose were then analyzed by HPAEC-PAD. The elution profile of authentic G6P and G3P (4 nmol each) is also shown.

Aardevo Exhibit 1010
Page 1117

Table 2. R1 is a dikinase

	A (without glucan)	B (+ glucan)	B − A
P-incorporation (G6P)	—	8.8 (9.3, 8.2)	8.8
P-incorporation (G6P + G3P)	—	12–16	**12–16**
ADP	2.4 (2.8, 2.0)	4.8 (4.6, 4.9)	2.4
AMP	3.0 (2.4, 3.6)	17.6 (18.5, 16.7)	**14.6**
Pi	7.6 (7.3, 7.8)	23.1 (22.6, 23.6)	**15.6**

In a total volume of 0.5 ml with or without 1.4 mg of glucan substrate ("elongated glycogen," see *Materials and Methods*), 2.6 μg of R1 were incubated with 250 nmol of ATP for 15 h. Phosphate incorporation and the increase in ADP, AMP, and Pi were determined and given as nmol. Values are means of two independent experiments. Values in parentheses are the individual measurements for experiments 1 and 2 (each run in duplicate). The glucan substrate was free of the analyzed metabolites (not shown).

R1 Is a Dikinase. In principle, either a kinase or a dikinase reaction could transfer a phosphate group from ATP to the α-glucan. If R1 acts as a kinase, the incorporation of phosphate and the formation of ADP are expected to occur in a 1 to 1 stoichiometry. However, because a sequence stretch of R1 displays some homology to bacterial PEP synthases (2, 3) it has already been speculated that R1 might act as a starch, water dikinase (3). In this case, phosphate incorporation, AMP production, and the release of orthophosphate should be equimolar. To identify the reaction catalyzed by R1, we determined the stoichiometry of glucan phosphorylation, and the production of ADP, AMP, and orthophosphate (Pi). The coelongation assay is not appropriate for these measurements because the phosphorylase also releases Pi. However, by preincubation of glycogen with G1P and phosphorylase and the subsequent removal of the enzyme, G1P and the Pi formed we were able to generate a polyglucan (postelongation assay; see *Materials and Methods*) that functioned effectively as a phosphate acceptor for R1. This polyglucan (designated as elongated glycogen) was then incubated with R1 and ATP. Following *in vitro* phosphorylation, the polyglucan was hydrolyzed and the amount of glucosyl-6 phosphate residues was determined enzymatically. The formation of G3P was confirmed by HPAEC-PAD (not shown; cf. Fig. 2). Because small amounts of ADP, AMP, and Pi were also formed in the absence of the elongated glycogen, we subtracted these values from those measured in the complete reaction mixture (Table 2). The amount of ADP produced was about six times lower than the observed phosphate incorporation (a minimum and maximum estimation based on enzymatic G6P determination and HPAEC-PAD-analysis is given in Table 2). However, both AMP and Pi increased by nearly the same amounts compared with the total phosphate incorporation. These results strongly suggest that R1 is a dikinase that transfers phosphate from ATP to starch (or starch like glucans) and water as paired acceptors and, thereby, generates starch-P, AMP, and Pi.

R1 Transfers the β-P of ATP to the Glucan. The assumption that R1 catalyzes a dikinase-type reaction was further confirmed using radioactive assays. Two differently labeled [^{33}P]ATP preparations were used: one was [γ-^{33}P]ATP, and the other was a mixture of [β-^{33}P]ATP and [γ-^{33}P]ATP (see *Materials and Methods*). Various glucan substrates (potato amylopectin, post- and co-elongated glycogen) were incubated in the presence or absence of R1 with either [γ-^{33}P]ATP or the mixture of [β-^{33}P]ATP and [γ-^{33}P]ATP. The glucans were then separated from the labeled adenine nucleotides and the radioactivity incorporated into the α-glucan was quantified (Table 3). Two observations can be made: First, labeling of all of the glucans was detectable only if R1 and [β-^{33}P]ATP were added. Thus, R1 phosphorylates α-glucans according to the following equation:

Table 3. The β-P of ATP is transferred to the glucan

	Phosphate donor			
	[γ-^{33}P]ATP		[β-^{33}P]ATP (+ [γ-^{33}P]ATP)	
Substrate	− R1, cpm	+ R1, cpm	− R1, cpm	+ R1, cpm
Amylopectin	43	49	31	1,512
Elongated glycogen	60	42	161	10,423
Glycogen + G1P + Pho	59	233	45	110,749

Buffer C was used supplemented with either 2 mg of potato amylopectin, 1.4 mg of postelongated glycogen (see *Materials and Methods*), or 0.5 mg of glycogen, G1P, and phosphorylase (coelongation assay) and 0.5 mM ATP. Either 1.1 μCi [γ-^{33}P]ATP or 1.1 μCi of a preparation in which [γ-^{33}P]ATP had been partly converted to [β-^{33}P]ATP (see *Materials and Methods*) were added. Reactions were started by addition of R1 (2.6 μg each) and terminated after 15 h. The radioactivity incorporated into the glucan was counted.

$$\text{glucan} + \text{ATP} + \text{H}_2\text{O} \rightarrow \text{glucan-P}_\beta + \text{AMP} + \text{P}_\gamma\text{i}$$

Secondly, the efficiency of phosphorylation strongly depends on the type of the α-glucan. Phosphate incorporation using the coelongation assay (glycogen + G1P + phosphorylase) proved to be most effective.

To test whether the phosphorylation reaction is reversible (as is the phosphorylation of pyruvate by PEP synthase) we incubated R1 (4 μg) with 5 mM Pi, 2 mM AMP, and potato amylopectin that had been prelabeled with ^{33}P (either by *in vitro* phosphorylation or by feeding potato plants with ^{33}P). However, there was no release of radioactivity during a 7-h incubation. Thus, the phosphorylation reaction is irreversible, at least under our experimental conditions.

Kinetics of Amylopectin Phosphorylation. The coelongation assay does not allow for a detailed kinetic analysis of the glucan phosphorylation because during the R1 action the glucan substrate is not constant. Therefore, we used potato amylopectin for a more detailed analysis of *in vitro* phosphorylation. In these experiments pure [β-^{33}P]ATP served as phosphate donor to allow for quantification of phosphate incorporation. As shown in Fig. 3, the phosphorylating reaction continued over the entire incubation period of 18 h; however, the rate decreased with time. This may be caused by a limited stability of the enzyme activity or by decreasing accessibility of the phosphate acceptor sites.

Under the assay conditions used extremely low concentrations of ATP were required to saturate the velocity of potato amylopectin phosphorylation (Fig. 4*A*). The apparent K_m was 0.23 μM

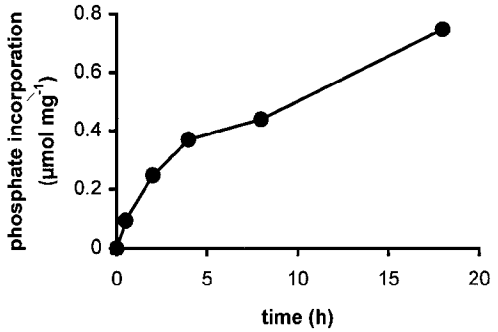

Fig. 3. Time dependence of potato amylopectin phosphorylation. Potato amylopectin (4 mg·ml^{-1}) was incubated with 0.5 mM ATP containing 1 μCi [β-^{33}P]ATP and R1 (2.6 μg) at 25°C for the indicated times. Two experiments were performed with very similar results. Data from one experiment are shown.

Aardevo Exhibit 1010
Page 1118

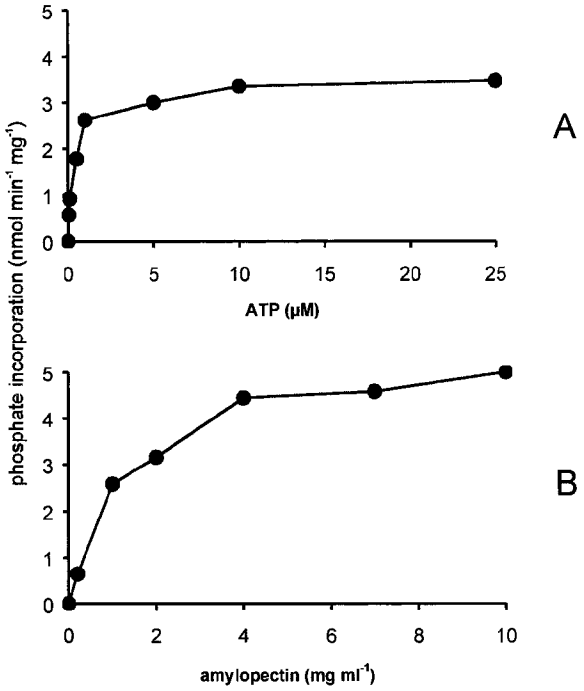

Fig. 4. Analysis of potato amylopectin phosphorylation. Experiments were performed using [β-^{33}P]ATP. Incubation time, 5 min. (*A*) Fixed amylopectin concentration (10 mg·ml^{-1}) and different ATP concentrations. In the range of 1 μM to 25 μM ATP, 2.6 μg of R1 were used. 0.5 μg R1 were used in the samples containing 0.05, 0.1, 0.5, 1, and 10 μM ATP. (*B*) R1 (1 μg) was incubated with 25 μM ATP and different amylopectin concentrations. The phosphorylation rate remained essentially unchanged in the range of 10 mg·ml^{-1} to 50 mg·ml^{-1} (data not shown).

(as calculated from a Lineweaver–Burk plot). The rate was unchanged in the range of 0.01 mM to 5 mM ATP (data not shown). At a fixed ATP concentration (25 μM), the rate of glucan phosphorylation was determined using varying amylopectin concentrations (Fig. 4*B*). The half maximum rate of phosphorylation was observed at an amylopectin concentration of 1.7 mg·ml^{-1}. The apparent maximum velocities were 3.2 and $\approx$5 nmol·min^{-1}·mg^{-1} in Fig 4 *A* and *B*, respectively. Slightly different V_{max} values were obtained because two separate R1 preparations were used in the two sets of experiments.

Incubation of R1 with saturating concentrations of ATP and potato amylopectin at different pH values revealed a pH optimum for R1 activity of 7.0 (data not shown).

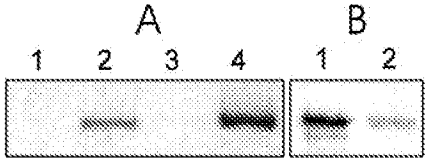

Fig. 5. Autocatalytic phosphorylation of R1. (*A*) The β phosphate of ATP is transferred to R1, forming a heat-labile linkage. R1 was incubated with either 1 μCi [β-^{33}P]ATP or [γ-^{33}P]ATP for 25 min. Incubation was terminated by adding SDS sample buffer and denaturation either for 5 min at 95°C or for 20 min 30°C. Equal protein amounts were subjected to SDS/PAGE and autoradiography. 1, [γ-^{33}P]ATP, 95°C; 2, [β-^{33}P]ATP, 95°C; 3, [γ-^{33}P]ATP, 30°C; 4, [β-^{33}P]ATP, 30°C. (*B*) The phosphorylated R1 is alkali-stable and acid-labile. R1 (35 μg) was incubated with 35 μCi of a preparation in which [γ-^{33}P]ATP had been partly converted to [β-^{33}P]ATP (see *Materials and Methods*) for 1 h at room temperature. The sample was then divided and either NaOH (1) or HCl (2) were added to give final concentrations of 0.5 M. Following incubation for a further 30 min, the samples were neutralized, mixed with SDS-sample buffer, and denatured for 20 min at 30°C. Equal amounts of protein were analyzed by SDS/PAGE and autoradiography.

Mg^{2+} proved to be essential for the phosphorylation reaction. If $MgCl_2$ was omitted from the assay mixture activity decreased by 90–95% (data not shown). No significant inhibition (or stimulation) of the phosphorylation reaction was observed in the presence of Pi (5 mM) or AMP (1 mM). Likewise, G1P (present in the coelongation assay), pyruvate, and dithioerythritol (DTE) did not significantly influence the *in vitro* phosphorylation of potato amylopectin (data not shown).

Autophosphorylation of R1 Precedes the Phosphotransfer to the Glucan. A phosphohistidine (containing the β-P) is an intermediate in the dikinase-type reaction catalyzed by PEP-synthase (14). To analyze whether R1 follows a similar mechanism, the purified protein was incubated with either [γ-^{33}P]ATP or [β-^{33}P]ATP in the absence of glucan. The reaction was terminated by adding SDS-sample buffer and denaturation at either 30°C or 95°C. Following SDS/PAGE, radioactivity was visualized by autoradiography. As shown in Fig. 5, R1 is capable of autocatalytic phosphorylation. The β-P is transferred to the protein and the linkage proved to be heat-labile (Fig. 5*A*). This finding is consistent with a phosphohistidine being formed because phosphohistidine is heat-sensitive in contrast to phosphoserine, phosphotyrosine, and phosphothreonine (13). Moreover, the phosphorylated R1 was stable if treated with strong alkali but labile if treated with acid (Fig. 5*B*), thereby fulfilling another criterion that indicates a phosphoramidate linkage suggestive of histidine phosphorylation (13, 14). In any case, the autophosphorylation of R1 precedes the phosphotransfer to glucosyl residues (Table 4). R1 was preincubated with [β-^{33}P]ATP in

Table 4. Autophosphorylation of R1 precedes the phosphotransfer to amylopectin

	Addition			43 min radioactivity,
Sample	0 min	30 min	33 min	cpm
A	R1 + [β-^{33}P]ATP	+ Nonlabeled ATP	+ Amylopectin	58, 63
			+ Buffer	28, 34
B	R1	+ [β-^{33}P]ATP, + nonlabeled ATP	+ Amylopectin	4, 12
			+ Buffer	7, 0

R1 (5 μg) was preincubated with 0.15 μCi [β-^{33}P]ATP (nonlabeled ATP was absent) in buffer C for 30 min in a total volume of 110 μl. Subsequently 10 μl of 0.1 M nonlabeled ATP was added and the suspension was incubated for a further 3 min. Fifty microliters each were then withdrawn and mixed with 5 mg of amylopectin dissolved in 450 μl of buffer C or in buffer C alone (A). Following incubation for 10 min the reaction was terminated by heating at 95°C for 15 min. The sample was divided and 230 μl each were filtered through microcon filter units (see *Materials and Methods*) and the radioactivity retained on the filter was counted. Another sample was treated identically; however, [β-^{33}P]ATP was omitted during the preincubation and was instead simultaneously applied together with nonlabeled ATP (B). The background counts (scintillation fluid alone, 43 $\pm$ 7) were subtracted.

Aardevo Exhibit 1010
Page 1119

the absence of any α-glucan. Nonlabeled ATP (in large excess) and amylopectin were then sequentially added and the suspension was incubated for a further 10 min. As a control, amylopectin was omitted. Under these conditions labeling of amylopectin was detectable (Table 4*A*), whereas no incorporation was detectable on simultaneous addition of labeled and unlabeled ATP (Table 4*B*). A comparison of the samples containing or lacking amylopectin revealed that under conditions of preincubation with [β-^{33}P]ATP (Table 4*A*) labeled protein also contributes to the total radioactivity retained on the filter units used to separate free and bound label (see *Materials and Methods*). However, this contribution was insignificant if labeled and nonlabeled ATP were added simultaneously as usually done in the activity assays (data not shown).

Discussion

In higher plants, R1 deficiency results in two significant changes in phenotype: a strongly reduced starch phosphorylation (below the limit of detection in plants lacking R1) and an enhanced starch accumulation resulting in a starch-excess phenotype. Both effects have been observed in transgenic potato plants expressing an R1 antisense construct (2) and in the *sex*1 mutant of *Arabidopsis* (3). By complementation of the *Arabidopsis* mutant with the wild-type R1 gene, phosphorylation of starch was restored and starch accumulation was adjusted to that of the wild type. On a biochemical basis, these two effects were difficult to understand because the enzymology of glucan phosphorylation was not known.

The data presented in this study show that R1 is an α-glucan, water dikinase and, therefore, provide a biochemical explanation for the effect on starch phosphorylation, but not yet for the starch excess phenotype. The dikinase-type reaction is in accordance with the reported homology of the C-terminal region of R1 to sequence regions of both PEP synthase (2) and pyruvate, phosphate dikinase (PPDK, EC 2.7.9.1) containing the ATP-binding site (3). PEP synthase and PPDK catalyze the transfer of the β-P of ATP to pyruvate and the transfer of the γ-P to water or orthophosphate, respectively. Likewise R1 transfers the β-P to the glucan and the γ-P to water. Furthermore, the autocatalytic phosphorylation of R1 (Fig. 5) supports the occurrence of a phosphohistidine intermediate (containing the β-P) as reported for both PEP-synthase (13) and PPDK (15). Recently, a conserved stretch of amino acids has been identified in the potato R1 protein and in the R1 homologue of *Arabidopsis* (SEX1) that is similar to the phosphohistidine domains of PEP synthase and PPDK (3). In any case, autophosphorylation of R1 precedes the phosphate transfer to the α-glucan, indicating a ping pong mechanism.

R1 strongly discriminates between various types of polyglucans. Glycogen from bovine liver was an extremely poor substrate (6% ^{33}P incorporation compared with potato amylopectin, data not shown). Phosphate incorporation dramatically increased if the glucan chains of glycogen were elongated and was most effective if phosphorylase and R1 were added simultaneously (coelongation assay). However, it should be noted that the glycogen-derived polysaccharides obtained in the co- or postelongation assay differ. During the coelongation assay, a glucan of low water solubility was formed (presumably because of the generation of very long chains) that to some extent precipitated during incubation. In contrast, the postelongated glycogen (under the conditions chosen) remained water soluble. Thus, currently we do not know whether the activity of R1 increases when the glucan chains are simultaneously elongated. In any case, ongoing chain elongation is no prerequisite for R1 activity because the postelongated glycogen and potato amylopectin were also phosphorylated.

Increased activity of R1 during, or following, glucan chain elongation concurs with the fact that *in vivo* phosphate groups are mainly located in relatively long chains of the amylopectin fraction (chain length 30–100 glucose units) (16, 17). However, it is possible that following *in vivo* phosphorylation the chains are further elongated by starch synthases or modified by branching enzyme activity (18). Amylopectin from wild-type potato plants contains a relatively large amount of phosphate esters, but it can be further phosphorylated by the recombinant R1 protein and, therefore, possesses additional phosphorylation sites. In contrast, amylopectin from R1 antisense potato plants (in which the starch phosphate is decreased by about 85%) and also corn amylopectin (that does not contain detectable amounts of covalently bound phosphate) proved to be less effective as phosphate acceptors. *In vitro* phosphate incorporation in corn amylopectin was less than 15% compared with wild-type potato amylopectin (data not shown). The same holds true for potato amylose, concurring with amylose being essentially free of phosphate esters *in vivo* (19).

Taken together, the data indicate that R1 phosphorylates distinct glucan targets. Accordingly, the modification of the starch structure by antisense repression of starch synthases or branching enzymes significantly affect the starch phosphate content (20–23). The actual target structure(s) that are recognized by R1 as a phosphorylation site(s) remain(s) to be identified.

The identification of R1 as an α-glucan, water dikinase clearly shows that the strongly impaired starch degradability in the R1 antisense potato plants and in the *sex*1 mutant of *Arabidopsis* is the consequence of reduced starch phosphorylation. Because of an increased hydrophilicity, the phosphorylated starch may be more easily accessible for degrading enzymes such as amylases. Alternatively, the activity and/or binding of starch degrading enzymes, yet to be identified, may directly depend on phosphate residues within starch. The regulatory link between phosphorylation and degradability of starch is the subject of our current work.

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Aardevo Exhibit 1010
Page 1120

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RFLP mapping on potato chromosomes of two genes controlling extreme resistance to potato virus X (PVX)

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Summary. Two different chromosomal locations of major genes controlling extreme resistance to potato virus X (PVX) were found by restriction fragment length polymorphism (RFLP) analysis of two populations segregating for the resistance. The resistance gene *Rx1* mapped to the distal end of chromosome XII, whereas *Rx2* was located at an intermediate position on linkage group V in a region where reduced recombination and segregation distortion have also been observed. These linkage anomalies were due to abnormal behaviour of the chromosome contributed by the resistant parent P34. The results presented were obtained using two different strategies for mapping genes of unknown location. One approach was the use of probes revealing polymorphic loci spread throughout the genome and resulted in the mapping of *Rx1*. The second approach was based on the assumption of possible linkage between the resistance gene and clone-specific DNA fragments introduced from a wild potato species. *Rx2* was mapped by adopting this strategy.

Key words: Potato virus X – Resistance genes – RFLP – *Solanum tuberosum* – Genetic introgression

Introduction

Among potato viruses, virus X (PVX) may induce heavy yield losses. Types of resistance to infection by this virus have been described as (1) field resistance based on minor genes and (2) localized hypersensitivity and extreme resistance due to single dominant major genes (reviewed in Ross 1986). Genes conferring a localized hypersensitivity reaction can be overcome by several virus strains, while extreme resistance protects against all important virus isolates from North America and Europe. Resistance genes, mainly derived from wild potato species, are present in several modern cultivars.

Until recently the practical use by plant breeders of major genes active against virus infection has been, to some extent, hindered by the poor state of potato genetics. This is due to the tetraploidy of the cultivated varieties of *Solanum tuberosum* but also to the low vitality and fertility of their diploid derivatives. Recently a new molecular marker class, termed restriction fragment length polymorphisms (RFLPs), has become available and provides a useful tool for constructing dense genetic chromosomal maps for the species (Bonierbale et al. 1988; Gebhardt et al. 1989). The availability of chromosome-specific RFLP probes has rendered attractive the mapping of major genes affecting morpho-physiological or resistance traits. Different strategies have been developed in various plants of agricultural significance for the mapping of resistance genes via RFLP markers. Nearly isogenic lines (NILs) have facilitated the mapping of gene *I2* conferring resistance to *Fusarium oxysporum* race 2 in tomato (Sarfatti et al. 1989), a resistance to maize dwarf mosaic virus strain A (McMullen and Louie 1989) and gene *Tm-2a* conferring resistance to tobacco mosaic virus (Young et al. 1988). Addition lines have permitted the detection of RFLP markers linked to a resistance to *Heterodera schachtii* in sugar beet (Jung et al. 1990). Recently Barone et al. (1990) have mapped the gene *Gro1* controlling resistance to *Globodera rostochiensis* in an F_1 population after crossing diploid potato lines.

The population used by Barone et al. (1990) for mapping the *Gro1* gene also segregated according to Mendelian ratios for extreme resistance to PVX. For the same trait a second segregating F_1 population was available that originated from a resistant parent different in origin from the one used in the previous cross. This paper describes the experiments that revealed that in the two crosses the genes conferring resistance to PVX were different and mapped at two different positions on the RFLP map of potato.

Materials and methods

Plant material. A set of 38 diploid potato clones (Gebhardt et al. 1989) from the collection of the Max-Planck-Institut für Züchtungsforschung was screened for ex-

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treme resistance to PVX. The resistant line H82.337/49 (P18) was crossed with the susceptible line H80.696/4 (P40) to produce an F_1 population. The offspring segregated for PVX resistance, indicating that P18 was heterozygous for the resistance allele. The test population consisted of 123 F_1 seedlings which were tested for resistance to PVX. Leaves and shoots from 100 genotypes were harvested and freeze-dried to be used for DNA extraction and RFLP analysis (see Barone et al. 1990). A second diploid line, H77.409/13 (P34), also characterized by extreme resistance to PVX in the heterozygous state, was crossed with the susceptible line H82.309/5 (P16) to produce a second segregating F_1 population of 110 seedlings which were grown in pots and tested for resistance. From this population DNA was extracted from 21 resistant and 23 susceptible plants for RFLP analysis. The two F_1 populations considered will be designated as F1840 and F3416 respectively.

Tests for resistance to PVX. Four different PVX isolates, originating from different potato varieties, and the strain PVX BS from the tobacco variety Xanthi were used to screen the original set of 38 potato clones. Only the strain PVX BS was used to classify the two segregating populations. All virus isolates were provided by H.-L. Weidemann, Biologische Bundesanstalt, Braunschweig. The isolates were propagated on tobacco Samsun NN. Resistance to PVX was tested essentially as described by Cockerham (1970). Sap as the source of inoculum was prepared from two ground leaves of systemically infected tobacco plants. The sap was diluted with 50 ml 0.05 M phosphate buffer, pH 7. The solution was smoothly rubbed with a small sponge on potato leaves previously dusted with Carborundum (400 mesh). After 1 min the leaves were rinsed with water and the procedure was repeated 1 and 2 weeks later.

Potato plants were considered resistant when they showed localized micro-necrosis or no response at all to the infection. Susceptible plants showed a pronounced mottling typical of virus reproduction in the plant. The classification of plants as resistant was confirmed by a serological test (ELISA, Biochemica Test Combination, Potato virus X; Boehringer/Mannheim). The mechanical inoculations were performed directly on F_1 seedlings and on their tuber descendants. The infection of the original set of clones and of the two segregating populations was carried out twice by grafting potato scions on plants of tomato cv. Estrella EZ previously infected by mechanical inoculation with PVX. Potato genotypes were classified as susceptible if viruses were detected by the ELISA test in at least one of all assays.

RFLP analysis. DNA extraction, restriction digests, electrophoresis, blotting and hybridization procedures were as described by Gebhardt et al. (1989).

Probes. To screen the population F1840 the inserts of 32 genomic and 25 cDNA clones of potato were used as probes. In addition, the genomic tomato clone TG68 provided by S.D. Tanksley (Cornell University, Ithaca, N.Y., USA) was also utilised. The 58 markers map to the 12 linkage groups of potato (Gebhardt et al. 1989; C. Gebhardt et al., in preparation). Population F3416 was tested with only seven potato probes.

Data and linkage analysis. Data analysis, linkage tests, estimation of recombination frequencies and determination of the linear order of linked loci, including multipoint linkage and the EM algorithm for handling missing data, were performed as described in Gebhardt et al. (1989), Ritter et al. (1990) and Barone et al. (1990). Resistance was treated in our linkage analysis as an extra RFLP fragment, being present in resistant and absent in susceptible plants.

Results

Segregation of PVX resistance

On screening the set of 38 diploid potato clones for PVX resistance, only lines 18 and 34 showed extreme resistance. F_1 seeds of crosses of these resistant genotypes with susceptible genotypes were available. In the F_1 from cross F1840, 53 resistant and 64 susceptible plants were found (6 out of 123 could not be classified). This fitted the segregation ratio of 1:1 ($\chi^2_{1:1}=1.03$; n.s.) expected when a genotype heterozygous for a single dominant gene is crossed to a homozygous recessive. From the 100 plants selected for RFLP analysis, 6 did not produce tubers and could not be exposed to all virus tests. They have been omitted from our analysis. The second F_1, F3416, segregated 31 resistant and 79 susceptible plants. This segregation ratio ($\chi^2_{1:1}=20.95$; significant, $P<0.001$) did not fit the type of inheritance found for the previous cross (see Discussion for interpretations).

RFLP analysis

Marker selection and characteristics of the polymorphisms found for the cross F1840 have been described elsewhere (Barone et al. 1990). Probes distributed throughout the genome were screened first. After having detected linkage with a specific marker, neighbouring markers identified from the RFLP map were tested for tighter or looser linkage. To align our linkage data with those of other groups, tomato probes were mapped to potato linkage groups allowing an alignment of potato chromosomes to the homeologous chromosomes of tomato (C. Gebhardt et al., in preparation). In the cross F1840 linkage was established between the resistance gene and seven markers mapping to linkage group XII (Fig. 1A, new nomenclature, previously X in Gebhardt et al. 1989). These included the loci *GP91(c)*, *CP106*, *CP114*, *GP34*, *CP103(b)* and *CP60*. One linked locus was revealed by the genomic tomato probe TG68 from tomato chromosome XII. All loci located in the cross F1840 by a multipoint linkage test occupied positions with the same order as established in the potato map of C. Gebhardt et al. (in preparation). The resistance gene *Rx1* was mapped to the most distal region of chromosome XII, 3.3 cM from the marker CP60 (Fig. 1A).

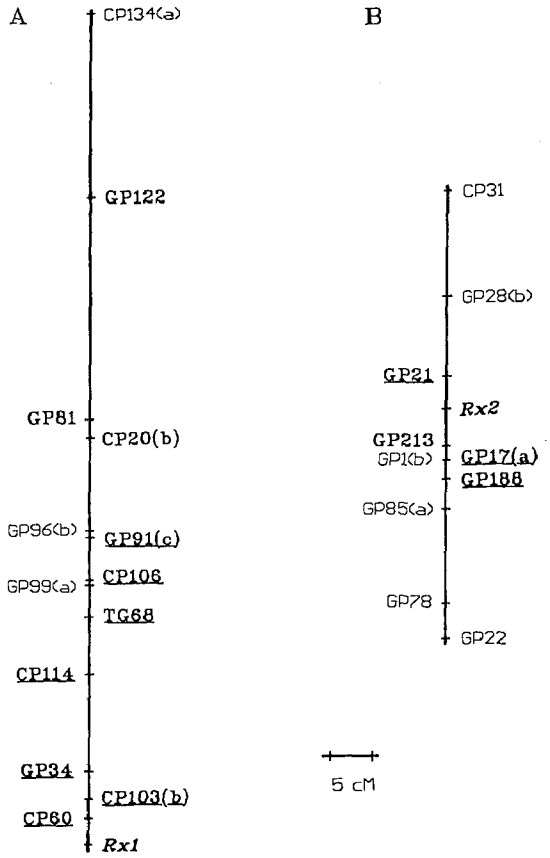

Fig. 1 A and B. The positions of the loci *Rx1* and *Rx2* controlling extreme resistance to potato virus X (PVX) on chromosome XII (**A**) and V (**B**) of potato. Chromosome numbers are according to the tomato nomenclature (Bonierbale et al. 1988). Chromosome XII is equivalent to linkage group X, and chromosome V to linkage group V in a previous potato restriction fragment length polymorphism (RFLP) map (Gebhardt et al. 1989). The chromosome map in **A** was obtained from cross F1840, that in **B** from cross F3416. Linkage orders were inferred from multipoint estimates. Distances are given in centimorgans (Kosambi units, Kosambi 1944) based on the recombination frequencies between RFLP fragments of the resistant parents. Potato markers are of genomic (GP) and of cDNA origin (CP). *Letters in parentheses* indicate that more than one locus was detected with the same probe. TG68 is a reference marker of tomato. Probes revealing linkage with the resistance genes in the crosses F1840 and F3416 are *underlined.* A few additional markers (given in *lighter letters*) of the potato RFLP map are included at their approximate positions in order to outline the total structure of the chromosomes

For the cross F3416 a different strategy was adopted: probes having a higher probability of being linked to the resistance gene were initially selected. The RFLP analysis of the set of 38 clones of *S. tuberosum* (described in Gebhardt et al. 1989) had indicated 12 fragments, corresponding to loci at several positions in the genome, that were specifically present only in clone 34, the resistant parent of the cross F3416. The loci associated with these fragments were considered to be candidates for linkage with the resistance trait because they would indicate chromosomal segments introduced into the *S. tuberosum* genome from wild *Solanum* species that carried a PVX resistance gene (Debener et al. 1991). Following this strategy, we first considered the 12 probes mentioned above for the linkage studies. The first probe tested, GP21 on chromosome V (homeologous to tomato chromosome V, C. Gebhardt et al., in preparation), provided evidence of linkage, showing a recombination frequency of 4.5% with the resistance gene *Rx2*. As shown in Fig. 2, only two recombinants were found among the susceptible plants analysed with GP21. Loci *GP17(a)* and *GP188*, also mapping on chromosome V (Fig. 1 B), showed linkage with *Rx2* with recombination frequencies of 6.8% and 9.1% respectively. Two additional loci mapping to the same region, *GP213* and *GP28(b)*, were not suitable for this cross: Probe GP213 indeed revealed a polymorphism but only the susceptible parent was heterozygous for the fragment, and the restriction fragment determining the locus *GP28(b)* was missing in F3416. Two markers from the chromosomal region encompassing *Rx1* (on linkage group XII) as well as GP21 were informative for the resistant parent in both populations and could, therefore, confirm the independence of *Rx1* and *Rx2*: neither CP60 nor GP34 showed linkage with *Rx2* (recombination frequencies of 48% and 50% respectively). Conversely, GP21 was not linked to *Rx1*.

The linear order of the RFLP loci tested was identical in F3416 and in the map of chromosome V developed from a different cross (C. Gebhardt et al. 1989, in preparation). The linkage with three markers therefore allocated *Rx2* to chromosome V between *GP21* and *GP17(a)* (Fig. 1 B). The interval between the loci *GP21* and *GP188* revealed considerable differences in the extent of recombination between genotypes: the interval length was 29.6 cM when measured between alleles of parent P16 and 13.6 cM between alleles of parent P34. In the cross used for constructing the RFLP map of potato, the distance between the same loci was 33.0 cM, a value similar to that measured in line 16. The recombination frequency obtained for the same chromosomal region in the cross F1840 also indicated a distance of ca. 30 cM. The reduced recombination observed in the interval *GP21-GP188* therefore seems to be specific for the chromosome V carried by line 34. We should point out that an influence of size of the sample of plants selected for RFLP analysis on the extent of recombination can be excluded because the recombinant to non-recombinant ratio was not statistically significantly different in resistant and susceptible plants.

Discussion

According to Cockerham (1970) two independent genes for extreme resistance to PVX exist: one can be traced back to the potato seedling USDA 41956, the first resistant genotype described (Schultz and Raleigh 1933) and to the *S. andigena* clone CPC 1673. The second was derived from *S. acaule* and is present in the clone MPI 44.1016/10 (Ross 1986). The potato clones MPI 44.1016/10 and CPC 1673 have frequently been used in potato breeding at the Max-Planck-Institut für Züchtungsforschung (H. Ross, personal communication). This explains our observation that in the segregating popula-

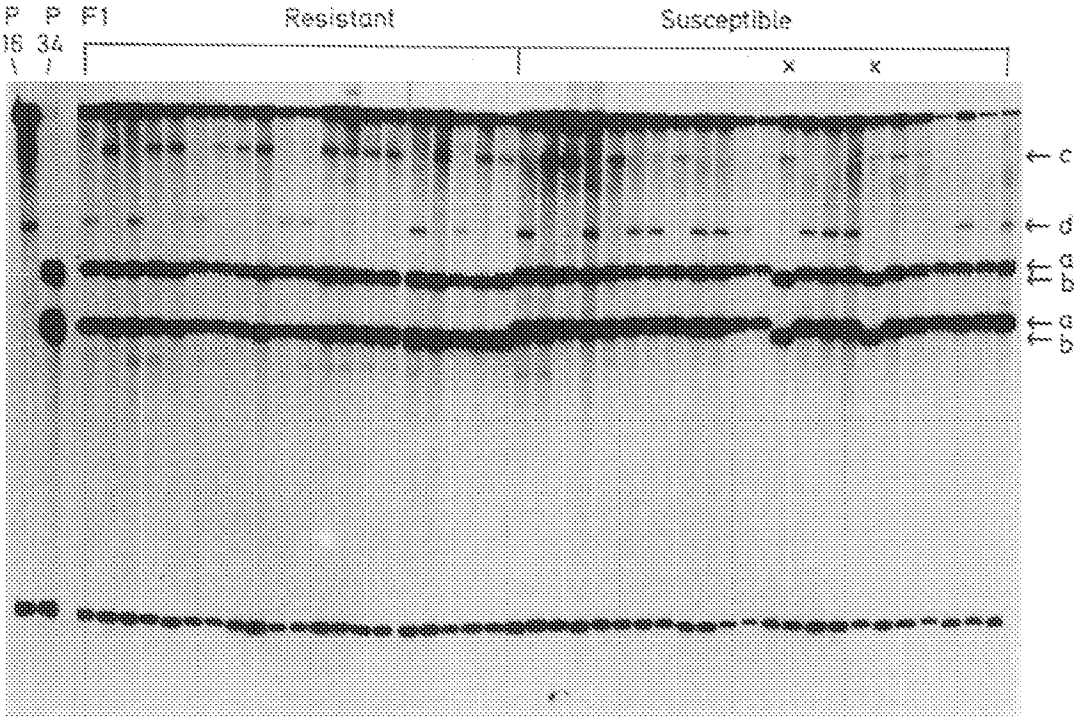

Fig. 2. Southern blot of potato DNA probed with the RFLP marker GP21 showing cosegregation with the PVX resistance locus *Rx2*. The first two lanes on the left correspond to the susceptible (P16) and the resistant parent (P34), followed by resistant and susceptible genotypes of the segregating F_1 progeny. Of the 21 resistant genotypes analysed, only 20 are shown. The four segregating RFLP alleles are given on the right. Alleles a and b each consist of two cosegregating restriction fragments and are derived from P34. Alleles c and d are derived from P16. Allele a is specific for P34 and is linked in repulsion with the resistance locus *Rx2*. The two recombinants are indicated by (x)

tions two different locations for genes controlling extreme resistance to PVX were found: one on the distal end of chromosome XII and the other in an intermediate position on chromosome V. Due to the complex crossing programme followed at the institute it was, however, impossible to assess unambiguously the source of resistance from the pedigrees of the resistant lines P18 and P34. The pedigree of line 34 included the resistant clone MPI 44.1016/10 (H. Uhrig, unpublished results): it is then likely that the resistance gene of line 34 (*Rx2*) is descended from *S. acaule*. The second resistance gene *Rx1*, present in P18, could have been inherited from CPC 1673 and would correspond to the resistance gene from *S. andigena*.

Two different approaches for gene mapping are presented in this paper. With population F1840 we adopted the strategy of systematically screening the genome with probes choosen according to their map position. As soon as linkage was detected with a particular locus, neighbouring markers were tested until the gene was located in an interval between two consecutive markers, or, as in the case of *Rx1*, at the distal end of one chromosome. The following considerations help to define the necessary number of probes: a total genomic length of 1000 cM would require 25 markers to detect linkage within a range of 20 cM. One further marker is necessary for orienting the interval on the chromosome and, depending on the marker density of the map and on the variation of marker interval lengths in different populations, a few further probes are needed to determine the position of the gene with the best possible accuracy.

The second approach was successful with population F3416. It was based on the assumption that the resistance gene had been introduced from a evolutionarily distant *Solanum* species together with several 'exotic' RFLP alleles (Debener et al. 1991). Whereas a large part of the genome of this species must have been eliminated during further breeding, one or more DNA fragments descending from the wild species and linked to the resistance gene should have been conserved in the genome of line P34. Although exotic RFLP alleles are common to many wild species widely used in potato breeding (Debener et al. 1991) and several such DNA fragments were present outside chromosome V in the genome of line P34, linkage to *Rx2* was detected with GP21, the first such probe tested. It must be mentioned however that, contrary to our expectations, *Rx2* was found to be linked in repulsion with the exotic fragment revealed by probe GP21 (allele a in Fig. 2). The simplest explanation for this finding is that an *Rx2*-bearing ancestor was used intensively during potato breeding at the Max-Planck-Institut. During the generations of breeding, the linkage in coupling between the resistance gene *Rx2* and the wild species allele revealed by GP21 was broken by crossing over but both recombinant segments of chromosome V were retained in the germplasm pool. Both parents of P34 therefore carried the complementary recombinant segments.

While segregation of the resistance gene in F1840 agrees with the ratio of 1:1 expected for a genotype heterozygous for a single dominant gene crossed to a homozygous recessive, the segregation found in F3416 shows significant deviations from this ratio and resembles more a 1:3 ratio (resistant versus susceptible). This could occur as a result of the existence of a complementary effect of two independent genes both contributing to the expression of extreme resistance. Assuming the two gene hypothesis, however, in the fraction of susceptible genotypes, independent segregation of the allelic fragments revealed by probe GP21 would be expected. This was not the case because only two recombinants were found among the susceptible plants (Fig. 2): within the set of 21 resistant and 23 susceptible plants the RFLP allele linked to *Rx2* was present 23 times and absent 21 times. This distribution among susceptible and resistant plants can be obtained by chance only with a probability lower than 5×10^{-13}. It can be concluded that the extreme resistance of P34 is due to a single dominant gene located in a chromosomal region with distorted segregation. This finding is not uncommon. While mapping RFLPs in different progenies of potato in the chromosomal region surrounding *Rx2*, aberrant segregation ratios were also observed (C. Gebhardt et al., in preparation).

Other abnormalities, like the reduced recombination found between the loci *GP21* and *GP188* for alleles of P34 as compared with alleles of P16, could result from the presence of chromosomal regions descended from wild species (C. Gebhardt et al., in preparation; Debener et al. 1991). The occurrence of segregation distortion would also explain the segregation ratios found in tetraploid potato crosses by Stevenson et al. (1939), who suggested a complementary effect of two genes controlling extreme resistance to PVX. This hypothesis was rejected later by Mills (1965) and Cockerham (1970).

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Improving Potato Storage and Processing Characteristics through All-Native DNA Transformation

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The dominant potato (*Solanum tuberosum*) variety for French fry production in the United States is the 131-year-old Russet Burbank. Market penetration of the higher yielding and more uniform Ranger Russet variety is limited to about one-fifth of that of the Russet Burbank because of two storage deficits: black spot bruise sensitivity and high levels of cold-induced sweetening. Here, these trait weaknesses are turned into strengths by simultaneously lowering the expression of Ranger Russet's tuber-expressed polyphenol oxidase (*Ppo*), starch-associated *R1*, and phosphorylase-L (*PhL*) genes. This genetic modification was accomplished without inserting any foreign DNA into the plant genome. French fries from the intragenic potatoes also contained reduced amounts of the antinutritional compound acrylamide while, unexpectedly, displaying enhanced sensory characteristics.

KEYWORDS: Genetic engineering; acrylamide; intragenic crops; potato

INTRODUCTION

Highly successful breeding programs have limited the average varietal age for many agronomically important crops to 4−6 years only (*1*). In contrast, the dominant potato variety Russet Burbank was developed 131 years ago. Although suffering from multiple deficits, this potato's excellent storage characteristics solidified its position as the preferred "white-fleshed" variety for French fry processing in the United States. Efforts to develop improved germplasm were hampered by complex tetrasomic genetics and inbreeding depression (*2*). One alternative variety that is considered to be the grower's favorite but has not been able to challenge Russet Burbank is Ranger Russet. Market penetration of this 15-year-old variety has been limited to only ~20% of that of Russet Burbank. Although Ranger Russet combines superior yield with disease resistance, adaptability, tuber uniformity, and high levels of starch, it is particularly sensitive to tuber discolorations that are linked to impact-induced bruise. This phenomenon is caused by leakage of polyphenol oxidase (Ppo) from damaged plastids into the cytoplasm. The subsequent oxidation of polyphenols triggers a precipitation of black melanin that greatly affects tuber quality during prolonged storage (*3*). Furthermore, Ranger Russet accumulates high levels of glucose and fructose during cold storage. These reducing sugars react with free amino acids during high-temperature processing of the potato. Accumulation of the resulting Maillard reaction products lowers consumer appeal by darkening French fries. Therefore, the quality of Ranger Russet is generally compromised if tubers are stored for longer than about 8 weeks.

One Maillard reaction product that was recently identified in processed potato products is acrylamide. This compound reacts with the amino-terminal valine of hemoglobin to form adducts. The extent of adduct formation is a good marker for exposure levels and implies daily intakes approximating 100 μg, about 36% of which is derived from fried and baked starchy foods (*4*, *5*). These intake levels are much lower than those that are known to cause toxicological effects including neurological symptoms, decreased fertility, and cancer in rodents (>2 mg/kg of body weight) (*6*). Indeed, a recent population-based study in Sweden found that long-term dietary exposure to acrylamide in amounts typically ingested had no measurable impact on cancer (*7*, *8*). Nonetheless, public and scientific concerns may justify attempts to limit the content of acrylamide in processed food.

Both the storage and nutritional characteristics of potato can be improved by employing methods in genetic engineering. Silencing of the *Ppo* gene was shown to lower the extent of black spot bruise sensitivity (*9*, *10*), and the down-regulated expression of the starch-associated *R1* or phosphorylase-L (*PhL*) genes lowered the accumulation of reducing sugars in cold-stored potato tubers (*11*, *12*). Here, the simultaneous silencing of the *Ppo*, *R1*, and *PhL* genes is shown to provide black spot bruise tolerance and greatly reduced levels of cold-induced sweetening. Interestingly, French fries derived from the modified tubers displayed a strongly enhanced visual appearance and improved aroma while accumulating much lower levels of acrylamide.

EXPERIMENTAL PROCEDURES

Plasmid Construction. The DNA segment used to produce a multigene silencing construct contained the 154-base pair 5′-untranslated trailer of the POT32 *Ppo* gene (coordinates 1−154 of Genbank accession no. AY566556), the 179-bp 5′-untranslated leader of the *R1* gene (homologous to accession no. Y09533), and the 267-bp 5′-untranslated leader of the *PhL* gene (coordinates 1989−2256 of accession no. AF143202). Basic vectors are as described previously (*9*). The 573-bp spacer element used to separate the inverted repeats of the various silencing constructs represents the intron of the potato ubiquitin-7 gene (coordinates 1215−1788 of accession no. U26831).

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Aardevo Exhibit 1010
Page 1126

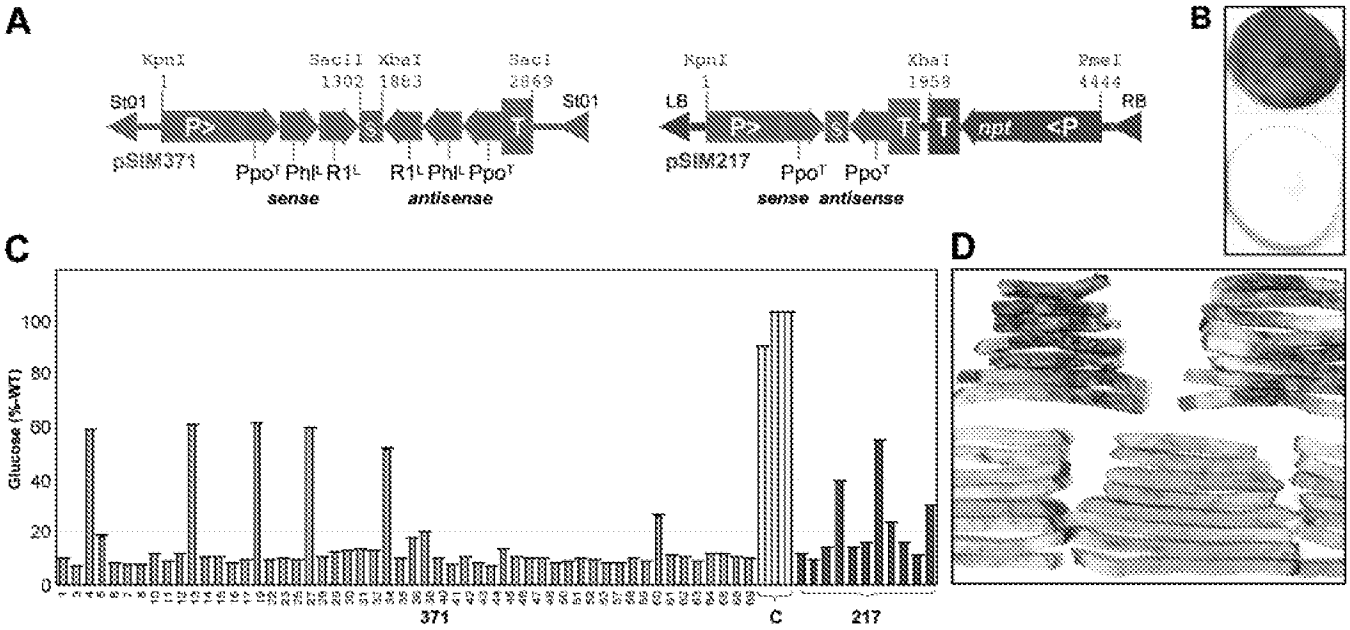

Figure 1. Mutigene silencing and bruise tolerance. (**A**) Diagram of constructs for multigene (pSIM371) and single-gene (pSIM217) silencing. P>, promoter of the Gbss gene; Ppo^T, trailer of the *Ppo* gene; PhL^L, leader of phosphorylase-L gene; R1^L, leader of *R1* gene; S, spacer from the ubiquitin-7 gene intron; T, terminator of the ubiquitin-3 gene. (**B**) Levels of Ppo tuber activity as assayed by pipetting a catechol solution onto cut tuber surfaces. Phenotypes are indicative for tubers of pSIM371 lines that displayed (top) and lacked (bottom) activity. (**C**) Ppo enzyme activities for 48 pSIM371 lines (green bars) and 11 of 50 pSIM217 benchmarks (red bars) that displayed reduced catechol-induced browning, as well as for untransformed Ranger Russet controls (yellow bars). Data are shown as percentage of average wild-type levels (ΔOD_{520} g^{-1} = 22.48 ± 0.98) and represent the mean ± SE of three independent experiments. (**D**) French fries from impacted untransformed (top) and pSIM217 (bottom) tubers. Note that the dark areas at the ends of intragenic fries represent the Russet skin and are not indicative of local discolorations.

Plant Transformation and Genotyping. Production of transgenic and intragenic plants was carried out as described (*9*). Transformed plants were genotyped for the presence or absence of P-DNA, T-DNA, and backbone DNA by employing Polymerase Chain Reactions (PCR) with appropriate primer sets (*9*).

Tuber Assays. Phosphate levels in potato starch were determined by using AOAC method 995.11, Phosphorus (Total) in Foods (45.1.33 *Official Methods of Analysis of AOAC International*, 17th ed.). Samples were prepared by dry ashing in a muffle furnace followed with an acid digestion. The dissolved samples were then neutralized and treated with a molybdate−ascorbic acid solution and compared to a series of phosphorus standards (treated similarly). A dual-beam spectrophotometer was used for the colorimetric analysis at 823 nm. A glucose oxidase/peroxidase reagent (Megazyme, Dublin, Ireland) was used to determine the glucose levels of cold-stored tubers. Tubers were screened for Ppo activity by pipetting 0.5 mL of 50 mM catechol in 50 mM MOPS (pH 6.5) onto freshly cut tuber surfaces and assaying for color development. The levels of Ppo enzyme activity were compared with wild-type levels by mixing pulverized tubers (1 g) for 1 h in 20 mM catechol and 50 mM 3-(*N*-morpholino)propanesulfonic acid buffer at pH 6.5 (5 mL). After precipitation of the solid fraction, the change of OD_{520} was determined over time.

RNA Analysis. Gene expression levels were assessed by performing quantitative real-time RT-PCR as described (*11*).

Processing. Field-grown tubers were either processed immediately or first stored in humidity-controlled chambers set at 4 °C. Tubers were washed, blanched for 8 min at 74 °C, cut into shoestring strips, dipped in a 1% sodium acid pyrophosphate solution at 71 °C, dried at the same temperature until 14 ± 2% weight loss was achieved, fried at 200 °C for 40 s to attain 64 ± 2% first-fry moisture, and frozen for 20 min at −26 °C, shaking the tray two to three times in the first 6 min. Processed fries were then finish-fried at 168 °C for 3 min and 15 s.

French Fry Assays. Color was assessed by using an E30-FP Agtron Process Analyzer (Agtron, Reno, NE) whereby a lighter color is reflected by a higher number and values above 40 are generally considered to be acceptable. Samples were biochemically analyzed for acrylamide levels by employing liquid chromatography−mass spectrometry/mass spectrometry (LC-MS/MS). Total polyphenol levels were determined as described (*13*). Sensory evaluations of French fries were performed by a panel of eight professionally trained experts at the optimum time of 3 min out of the fryer.

RESULTS AND DISCUSSION

In an attempt to turn the storage deficits of Ranger Russet into strengths, we employed a multigene silencing approach. For this purpose, a DNA segment was produced that contains fragments of three "undesirable" genes. One of the fragments was derived from the 5′-untranslated trailer of the *Ppo* allele POT32 that is predominantly expressed in mature tubers (*3*). The other two fragments represent the 3′-untranslated leaders of the *R1* and *PhL* genes, the down-regulated expression of which is known to slightly lower the extent of cold-induced sweetening (*11*, *12*).

Two copies of the "triple-gene fragment" DNA segment were inserted as inverted repeat between the tuber-specific promoter of the granule-bound starch synthase (*Gbss*) gene (*14*) and the terminator of the potato ubiquitin-3 (*Ubi3*) gene (**Figure 1A**). Positioning of the resulting silencing construct between two tandemly repeated potato St01 elements that function as T-DNA border alternatives created an all-native potato transfer (P-) DNA (*15*). A plasmid carrying this P-DNA, designated pSIM371, was introduced into an *Agrobacterium* LBA4404 strain (*16*) also harboring a conventional binary vector. This second "LifeSupport" vector, pSIM368, carried both the bacterial neomycin phosphotransferase (*nptII*) positive selectable marker gene and the cytosine deaminase (*codA*) gene for negative selection inserted between conventional T-DNA borders (*3*).

The resulting double-vector strain was used to infect 21900 Ranger Russet stem explants. Upon cotransfer of the P-DNA and T-DNA, the explants were subjected to kanamycin for 5

Aardevo Exhibit 1010
Page 1127

days to select for transient *nptII* gene expression. Proliferation of cells containing stably integrated *nptII* T-DNAs was subsequently prevented by transferring explants to media containing 5-fluorocytosine (5FC), a chemical that is converted into toxic 5-fluorouracil (5FU) by the *codA* gene product (*17*). A total of 3822 shoots that survived the double selection were genotyped by PCR for the presence of the desired P-DNA and the absence of foreign DNA. This analysis found ~85% of plants to contain plasmid backbone DNA. After elimination of both this large group of backbone-DNA-containing plants and an additional group that still carried the T-DNA, 256 marker-free and all-native DNA (intragenic) plants were selected for propagation and planting in the greenhouse.

Tubers from 1-month-old intragenic plants were prescreened for Ppo activity by pipetting a catechol "indicator" solution onto cut surfaces (**Figure 1B**). Ppo activity levels were then biochemically quantified in tubers that displayed reduced catechol-induced browning. These analyses demonstrated that 48 intragenic lines (19%) were effectively silenced for Ppo, often at levels below 15% of untransformed controls (**Figure 1C**). Both the frequency and extent of silencing were comparable to those of plants that had been transformed with pSIM217, a construct designed to only down-regulate the expression of the POT32 *Ppo* gene (**Figure 1A,C** and data not shown). To confirm that reduced Ppo activity levels would provide black spot bruise tolerance, tubers were physically impacted and, after 2 weeks, used for processing. French fries obtained from both pSIM371 and pSIM217 tubers were found to be free of discolorations, including visible white spot impact damage. In contrast, control fries developed extensive black spot bruise symptoms that covered about half of the fry surface (**Figure 1D** and data not shown).

Phenotypes of the 48 intragenic and bruise-tolerant lines were further analyzed by growing them in the field. Untransformed controls and three groups of transgenic "benchmark" lines were included in this trial. The first group of benchmarks consisted of five of the above-described *Ppo*-silenced pSIM217 lines. A second group of five lines was successfully silenced for the *PhL* gene through expression of the silencing construct of pSIM216 (*11*), whereas the *R1*-targeted silencing construct of pSIM332 had been used to produce a third group of gene-suppressed lines (**Figure 2A**).

The extent of cold-induced sweetening and its impact on tuber quality was assessed by storing field-grown tubers for 3 months at 4 °C. Tuber analyses demonstrated that 43 of the bruise-tolerant pSIM371 lines accumulated 30−60% of wild-type glucose levels. These levels were generally lower than those obtained with the benchmark lines that had been silenced for only one of the starch-associated genes (60−90%) (**Figure 2B**). Thus, our results confirm that a single multigene silencing construct can be used effectively to down-regulate the expression of multiple genes. Furthermore, they indicate an either additive or synergistic effect of the combined *R1*/*PhL* gene-silencing approaches.

Reduced glucose levels are known to lower processing-induced fry darkening (*18*). However, it was not clear to what extent the overall appearance of French fries from the 43 intragenic "bruise-tolerant and low-glucose" lines would be different from that of untransformed controls. Spectrophotometrical analysis demonstrated that the fries from Ranger Russet and Russet Burbank tubers were relatively dark, with Agtron levels between 42 and 46 (**Figure 2C** and data not shown). These levels barely met the minimum requirement for commercial applications, which is generally set at values >40. In contrast, the much lighter fries from five selected intragenic lines, 371-23, -28, -30, -38, and -68, surpassed the target that was set by us for the commercial application of a modified potato (>55), reaching levels between 58 and 60 (**Figure 2C** and data not shown). Intermediary Agtron values of about 50 were obtained with the pSIM216 and pSIM332 benchmark lines.

One physiological disorder that was found in about 15% of untransformed Russet Burbank and Ranger Russet tubers, as well as in those from the benchmark lines, was sugar-end formation. Triggered by drought-stimulated sugar migration to the lower end of potato tubers, this stress response is manifested as darkly colored French fry "ends" (*18*). Unexpectedly, the intragenic French fries lacked such local discolorations (data not shown). Thus, this stress-induced defect, which can result in extensive product loss and reduced quality, is effectively addressed by simultaneously lowering *R1* and *PhL* gene expression.

The enhanced appeal of French fries from pSIM371 lines was confirmed by a panel of eight professionally trained individuals. By rating for color, color variation, and appearance defects, fries from tubers of five intragenic Ranger Russet lines were found to outperform those of both Ranger Russet and Russet Burbank (**Figure 2D**). In contrast, the visual appearance of the benchmark lines was not significantly different from that of untransformed controls (data not shown).

In addition to improved visual appearance and consistency, the intragenic fries from freshly harvested tubers also displayed a significantly enhanced overall aroma as sensed by the olfactory epithelium, which is located in the roof of the nasal cavity (**Figure 2E**). This observed improvement was even more dramatic in tubers that had been stored for 10 weeks at 4 °C. In fact, French fries from the cold-stored intragenic lines 371-28, -30, -38, and -68 still met or exceeded the sensory attributes of those from freshly harvested untransformed varieties. Although the biochemical basis of enhanced aroma was not studied as part of this work, it is possible that the combined silencing of the starch mobilization genes *R1* or *PhL* prevented the accumulation of certain Maillard reaction products that negatively contributed to flavor aroma. The aroma improvements were not detected in the single-gene-silenced pSIM216 and pSIM332 lines that lowered glucose accumulation only partially (data not shown). Apart from aroma, the fries from intragenic lines were not different from control and benchmark fries in terms of texture, toughness, or mealiness. Collectively, our data indicate that the simultaneous suppression of the starch mobilization gene *R1* or *PhL* enhances key quality and sensory attributes to deliver a more consistent and flavorful processed product.

Given that both *R1* and *PhL* genes impinge on the starch phosphorylation pathway, we performed a detailed chemical analysis of the tuber starch. From this study, we found that silencing of the *R1* and *PhL* genes triggered opposite effects on phosphate levels. Single-gene silencing of *R1* (pSIM332) resulted in lower levels of starch phosphate ($P = 0.0001$), whereas levels were increased in tubers silenced only for *PhL* (pSIM216; $P = 0.05$) (**Figure 2F**). Interestingly, tuber starch phosphate levels of three intragenic lines tested resembled those of the pSIM332 benchmarks, indicating that the action of *R1* appears, at least at this level, to be epistatic over *PhL*. The replacement of conventional potato tubers by tubers from the intragenic lines could potentially reduce phosphate levels in the wastewaters of French fry plants. Currently, these levels are at 25−40 mg of phosphate L^{-1}, about half of which is derived from potato starch. Prior to environmental release, wastewater

Figure 2. Tuber and French fry quality improvements. All data represent the mean ± SE of at least three independent experiments. Symbols and abbreviations are the same as in **Figure 1**. (**A**) Diagram of the single-gene silencing constructs pSIM216 and pSIM332. (**B**) Glucose levels in field-grown tubers after 3 months of cold storage for pSIM371 lines (green bars), untransformed Ranger Russet controls (yellow bars), and pSIM216 and pSIM332 benchmarks (red bars) as expressed as percentage of average wild-type levels (3.89 ± 0.15 mg of glucose/g of FW). (**C**) Color of French fries derived from cold-stored tubers of untransformed Russet Burbank (RB), Ranger Russet (RR), and intragenic lines 371-30 and -38. (**D**) Overall fry appearance of RB (gray bars), RR (yellow bars), and intragenic pSIM371 lines (green bars) based on visual assessments of color, color variation, and appearance defects by a professionally trained sensory panel. Rating was performed by using a scale from 0 to 100%. French fries were obtained from both freshly harvested tubers (left bars) and tubers that had been stored at 4 °C for 10 weeks (right bars). An arrow at 60% indicates the quality threshold for most French fry applications. (**E**) Overall aroma of fries from fresh (left bars) and cold-stored (right bars) tubers as assessed by a professional sensory panel. (**F**) Tuber starch phosphate levels. (**G**) Acrylamide levels in French fries of cold-stored tubers in parts per billion (PPB).

Aardevo Exhibit 1010
Page 1129

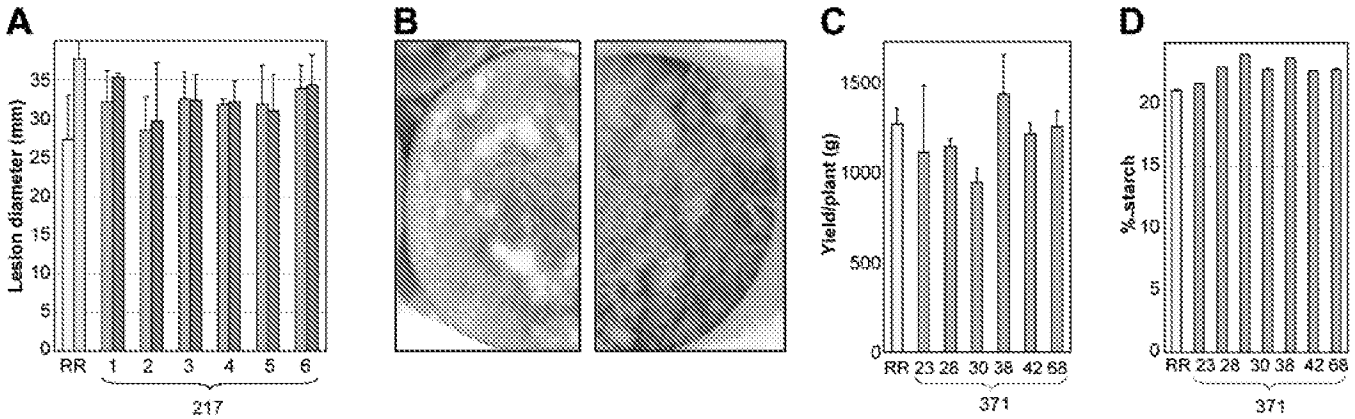

Figure 3. Agronomic characteristics. (**A**) Tuber response to infection with *Phytophthora infestans*. Tubers of both Ranger Russet (RR) control and PPO-silenced pSIM217 plants were subjected to 10^5 (left bars) and 10^6 (right bars) fungal spores and incubated at 18 °C. Disease progression was assessed by determining lesion size (millimeters) 5 days postinfection. All data represent the mean ± SE of at least three experiments. (**B**) Representative symptoms of infected pSIM217 (left) and control (right) tuber slices, 10 days after infection. (**C**) Yield of mid-sized to large (>100 g) tubers per plant. Data represent the mean ± SE of four replicated plots of 25 plants each. (**D**) Starch content. RR, Ranger Russet.

phosphates must be reduced, either by biological or physical postfactory treatment, at significant production expense. An alternative in planta method to reduce phosphate contamination would provide significant environmental and production advantages.

The low levels of reducing sugars in intragenic tubers prompted us to determine acrylamide levels in French fries obtained from these tubers. Acrylamide is a toxic Maillard compound that is largely derived from heat-induced reactions between the carbonyl group of reducing sugars and asparagine (*19*, *20*). Field-grown tubers from a second-year trial in Aberdeen, ID, were cold stored, processed, and biochemically analyzed. **Figure 2G** shows that French fries generated from intragenic tubers contained only about a third of the acrylamide that accumulates in control fries. Because consumption of French fries was recently estimated to contribute to ~16% of the total dietary intake of acrylamide (0.07 μg/kg of body weight/day) (http://www.cfsan.fda.gov), application of the "low acrylamide" fries would reduce the daily acrylamide intake by more than 10%. Further reductions may be obtainable by applying analogous acrylamide reduction strategies to oven-baked fries, potato chips, bread, and other starchy products, the consumption of which contributes to an estimated 59% of average intake levels (http://www.cfsan.fda.gov).

As a cultivar, Ranger Russet has a strong disease resistance profile, which presumably would be maintained after the intragenic improvement. In contrast to Russet Burbank, it contains natural tolerance against a range of viral diseases, including potato viruses X and Y and potato leaf roll virus (*21*). Furthermore, it displays resistance against the fungal "early die" pathogen *Verticillium dahliae*, allowing growth of Ranger Russet without need for expensive methyl bromide-based fumigation and the inadvertent loss of nontarget soil microbes (*22*). Because a constitutive reduction in *Ppo* expression levels might negatively influence plant defense responses (*23*), we sought to silence the *Ppo* gene only in the cortex and pith of the tuber, retaining enzyme activity in the epidermis (*9*). Here, we further tested pSIM217 lines for their susceptibility to the fungal pathogen *Phytophthora infestans*, causal agent of potato late blight. Tubers from both the transgenic lines and untransformed controls were infected with 10^5 and 10^6 spores of the aggressive US-8 strain (*24*). This infection resulted in a similar progression of disease as observed in control tubers (**Figure 3A**), demonstrating that *Ppo* gene silencing did not enhance the susceptibility against this important potato pathogen. Interestingly, severe tuber blackening was observed in only control tubers (**Figure 3B**). This result indicates that some of the early *P. infestans* disease symptoms are associated with *Ppo*-mediated coloration. The possibility of less obvious infection suggests that broad utilization of intragenic Ranger Russet may require monitoring for late blight, perhaps by including border rows of untransformed potato as "sentinels".

Overall data from the two field trial seasons suggested that the incorporated traits had no negative effect on agronomic performance. Analyses of tubers from the second-year trial did not reveal any differences between untransformed and intragenic lines in terms of plant and tuber typeness (data not shown). Furthermore, yields of regularly sized (>100 g) tubers were compromised only in line 371-30 (**Figure 3C**). This line displayed some morphological abnormalities and is likely to represent a somaclonal variant. The only measurable agronomic distinction between all intragenic lines and untransformed Ranger Russet related to starch levels. Tubers of the pSIM371 lines contained increased levels of starch, from 217 mg/g wild-type levels to an average of 232 mg/g in the modified lines (**Figure 3D**). This 7% increase in starch concentration is generally considered to be beneficial for the production of French fries.

Our results demonstrate that a multigene silencing construct enhanced the performance of Ranger Russet in seven different ways: black spot bruise resistance, reduced cold-induced sweetening, reduced stress-induced sugar ends, enhanced fry aroma, reduced amounts of processing-induced acrylamide, reduced starch phosphate content, and increased starch. By replacing some of the acreage that is currently occupied by Russet Burbank, it will also be possible to increase yields and lower costs for disease control. Furthermore, Ranger Russet's greater environmental adaptability will allow an expansion of territory in regions not wholly suitable for Russet Burbank cultivation. We have shown that both the sensory and nutritional characteristics of potato can be improved by simultaneously silencing the tuber-expressed *Ppo*, *R1*, and *PhL* genes. By committing to all-native DNA transformation methods for the incorporation of output rather than input traits, we hope to address at least some consumer concerns about the genetic modification of food crops (*25*).

Aardevo Exhibit 1010
Page 1130

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Low-acrylamide French fries and potato chips

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Keywords: acrylamide, intragenic, plant biotechnology, potato.

Summary

Acrylamide is produced in starchy foods that are baked, roasted or fried at high temperatures. Concerns about the potential health issues associated with the dietary intake of this reactive compound led us to reduce the accumulation of asparagine, one of its main precursors, in the tubers of potato (*Solanum tuberosum*). This metabolic change was accomplished by silencing two asparagine synthetase genes through 'all-native DNA' transformation. Glasshouse-grown tubers of the transformed intragenic plants contained up to 20-fold reduced levels of free asparagine. This metabolic change coincided with a small increase in the formation of glutamine and did not affect tuber shape or yield. Heat-processed products derived from the low-asparagine tubers were also indistinguishable from their untransformed counterparts in terms of sensory characteristics. However, both French fries and potato chips accumulated as little as 5% of the acrylamide present in wild-type controls. Given the important role of processed potato products in the modern Western diet, a replacement of current varieties with intragenic potatoes could reduce the average daily intake of acrylamide by almost one-third.

Introduction

Asparagine plays an apparently important role in the assimilation and storage of nitrogen (Lam *et al.*, 2003), and is particularly abundant in the products of wheat (*Triticum aestivum*) (Benedito *et al.*, 1989), coffee (*Coffea arabica* and *C. canephora*) (Mazzafera, 1999) and potato (Talley *et al.*, 1970). On heat processing, the amide amino acid reacts with reducing sugars to produce acrylamide (Mottram *et al.*, 2002). Average daily intake levels of this Maillard reaction product are estimated to be 0.3–0.7 μg acrylamide/kg/day (Dybing *et al.*, 2005). Ingested acrylamide is readily absorbed and metabolized, in part, by a cytochrome P450 to produce mercapturic acid and glycidamide (Boettcher *et al.*, 2005). Although mercapturic acid is excreted via the urine, both the remaining acrylamide and its reactive metabolite bind to various proteins as well as DNA (Barber *et al.*, 2007; Martins *et al.*, 2007). High levels of adduct formation have been linked to animal health issues, including cumulative nerve terminal damage (Johnson *et al.*, 1986; Friedman *et al.*, 1995). In humans, oral intake levels believed to be without an appreciable risk of deleterious effects are currently estimated to be 3.0 μg acrylamide/day (http://www.epa.gov/iris). This level of dietary intake is exceeded in small subsets of the population, particularly in young children and adolescents (Dybing *et al.*, 2005). In their preliminary JECFA/64/SC report (http://www.who.int/ipcs/food/jecfa/summaries/summary_report_64_final.pdf), the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives and Contaminants has therefore recommended reducing the acrylamide content of processed starchy foods.

Recently developed methods to limit acrylamide formation require changes in grower or processor practices, which may limit their broad application. For instance, the beneficial effect of sulphur fertilization on lowering the acrylamide potential of potato and wheat (Elmore *et al.*, 2007) is offset by increased farmer input costs and sulphur contamination issues. Furthermore, the partial decreases in acrylamide concentration that can be achieved by modifying processing variables, such as the time and temperature of heating (Rydberg *et al.*, 2005; Amrein *et al.*, 2006), yield products that have lost some of their original colour, flavor and/or texture, and may therefore be less appealing to consumers. A third approach incubates raw materials with either asparagine-metabolizing enzymes or amino acids that compete with asparagine in the Maillard reaction (Elder *et al.*, 2006; Howie *et al.*, 2006). Such treatments have been shown to be only partially effective for

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Aardevo Exhibit 1010
Page 1132

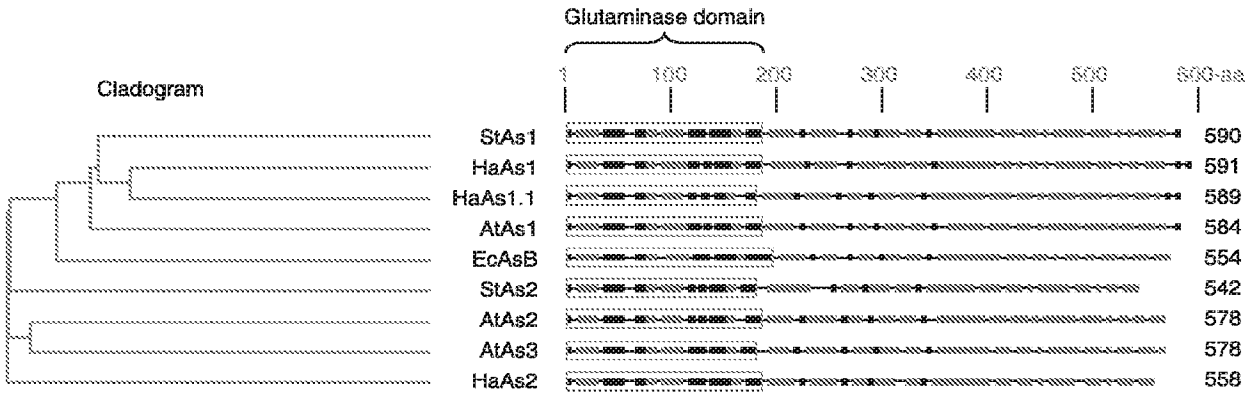

Figure 1 Phylogenetic relationship and structure of asparagine synthetase (As) proteins from a number of different species: At, *Arabidopsis thaliana*; Ec, *Escherichia coli*; Ha, *Helianthus annuus*; St, *Solanum tuberosum*. Black boxes, α helices; grey boxes, β sheets.

some raw food ingredients, require high concentrations of the additive, and are too difficult and costly to apply broadly. A preferred route to the reduction of acrylamide would be to shift to crops that are naturally poor in acrylamide precursors. However, there are currently no such varieties available that also display all the additional input, processing and quality traits demanded by the processing industry (Vivanti *et al.*, 2006). Given the complexity of wheat, coffee and potato breeding, efforts to develop such new processing varieties will require 15 years or more.

A faster route to decrease the acrylamide potential of food crops was established 2 years ago through genetic engineering. Simultaneous silencing of two tuber-expressed genes in starch degradation, which encode water dikinase R1 and amyloplast-targeted phosphorylase-L, led to a decrease in the accumulation of glucose and fructose by approximately two-fold (Rommens *et al.*, 2006). Reduced browning of processed products from these modified tubers correlated with an approximately two- to threefold decrease in acrylamide levels. In this article, an alternative approach for the production of low-acrylamide French fries and potato chips is described that does not alter their sensory characteristics. This new method is based on the tuber-specific silencing of two genes in asparagine biosynthesis, and reduces the concentration of free asparagine by up to 95%.

Results

Silencing of asparagine synthase genes limits asparagine biosynthesis in potato tubers

Asparagine synthetase (As) catalyses the ATP-dependent conversion of aspartate into asparagine. Two cDNAs for this key enzyme in asparagine biosynthesis, designated as *StAs1* and *StAs2*, were amplified from a tuber poly(A)$^+$ mRNA-derived library of the potato variety Ranger Russet. The 1.6-kb open reading frames share 71% sequence identity and encode products with a typical ~190-amino-acid N-terminal glutaminase domain (cd00712), which is involved in the hydrolysis of glutamine to produce glutamate and ammonia (Marchler-Bauer *et al.*, 2007) (Figure 1). The predicted secondary structures of both of these proteins and their functional orthologues resemble that of the crystallized AsB of *Escherichia coli* (Larsen *et al.*, 1999) (Figure 1). StAs1 shares most homology with the proteins encoded by the dark-inducible *HaAs1* from sunflower (*Helianthus annuus*) (86%) and the *AtAs1* gene from *Arabidopsis* (83%). In contrast, StAs2 is more closely related to the product of the weakly expressed and light-inducible *AtAs2* gene (86%) (Figure 1).

In an attempt to limit the accumulation of asparagine in potato tubers, a silencing construct was designed that simultaneously targets the expression of *StAs1* and *StAs2* (Figure 2a). The two genes are quite divergent at the DNA level, with short (approximately 5 bp) stretches of homology interrupted by mismatches. Therefore, 0.4-kb fragments from both *StAs1* and *StAs2* were fused to create a DNA segment, and two copies of this segment were inserted as an inverted repeat between two convergently orientated promoters. It was our primary intent to reduce the transcription of the *StAs1* and *StAs2* genes in tubers, whilst limiting the extent of gene silencing in the foliage where the gene products play important roles in both photorespiration (Ta *et al.*, 1985) and ammonium detoxification (Wong *et al.*, 2004). For this reason, promoters were employed that were at least 100-fold more active in tubers than in leaves: the

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Aardevo Exhibit 1010
Page 1133

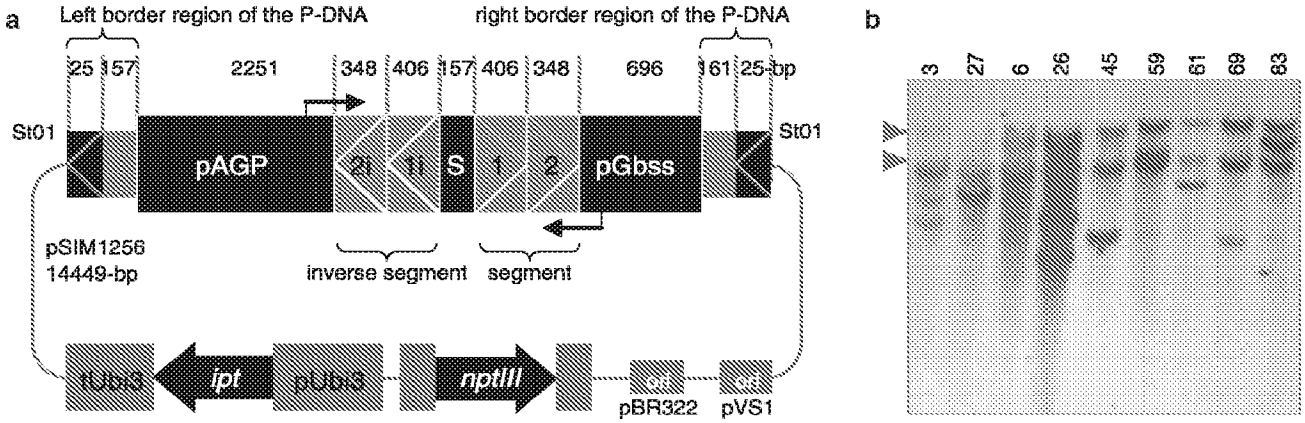

Figure 2 Plant transformation with a silencing construct targeting the potato asparagine synthetase genes (*StAs1* and *StAs2*) in tubers. (a) Diagram of vector pSIM1256. St01, potato-derived border-like element resembling the T-DNA border; pAGP, promoter of the potato *Agpase* gene; 1, *StAs1* gene fragment; 2, *StAs2* gene fragment; i, inverse complement; S, spacer; pGbss, promoter of the potato *Gbss* gene; ori, origin of replication; pUbi3, promoter of the potato *Ubi3* gene; *ipt*, Agrobacterium *ipt* gene; tUbi3, terminator of the potato *Ubi3* gene; *npt III*, kananycin resistance gene from *E. coli*. Plant-derived sequences are shown in green; vector backbone sequences are shown in grey. (b) P-DNA copy number of nine intragenic lines, as determined with an *StAs1/2*-derived probe that also visualizes two endogenous fragments (grey arrows).

granule-bound starch synthase (Gbss) and a short (2.2-kb) version of the potato ADP-glucose pyrophosphorylase (Agp) (Visser *et al.*, 1991; Muller-Rober *et al.*, 1994; C. Richael, unpubl. data). The resulting silencing construct was positioned within a potato-derived transfer (P-) DNA to create the binary vector pSIM1256.

An *Agrobacterium* LBA4404 strain containing pSIM1256 was used for marker-free and all-native DNA transformation of the 'French fry' variety Ranger Russet (Richael *et al.*, 2008). Fifteen shoots with a P-DNA$^+$/backbone$^-$ genotype were rooted, confirmed by DNA gel blot analysis to contain at least one P-DNA integration event (Figure 2b, and data not shown) and propagated *in vitro* for the production of intragenic lines. Three plants of each of the 15 transformed lines were grown in the glasshouse for 3 months. Both the yield and size of harvested tubers were similar to those of untransformed controls (Figure 3a,b). The specific gravity, which is a reliable indicator of starch content, was also unaffected by the genetic modification (data not shown). An association between the presence of the silencing construct and two- to fivefold decreased transcript levels for the *StAs2* gene in tubers of 12 of the 15 lines was subsequently demonstrated by reverse transcriptase polymerase chain reaction (RT-PCR) (Figure 3c). RNA gel blot analysis, although less sensitive, confirmed this result (Figure 3d), and also indicated that silencing of the *StAs2* gene was fully correlated with suppressed expression of the *StAs1* gene (Figure 3e). The effective establishment of *StAs1/2* gene silencing in intragenic lines was mainly attributed to the presence of fragments from the two targeted genes in the silencing construct. In addition, it is possible that the partial homology between the *StAs1* gene fragment and the resident *StAs2* gene, and vice versa, was sufficient for some cross-silencing (Jackson *et al.*, 2003).

Tubers from all the intragenic lines were analysed by high-performance liquid chromatography (HPLC) to determine the effect of *StAs1/2* gene silencing on the accumulation of asparagine. As shown in Figure 3f, the content of this amide amino acid was up to 20-fold lower in intragenic tubers than in the tubers transformed with an 'empty' vector control. Interestingly, tubers of line 1256-45 contained higher As2 transcript levels but lower concentrations of asparagine than tubers of, for instance, line 1256-26. This finding suggests that the various intragenic lines differ in their content of reducing sugars and/or other compounds that influence the potential for acrylamide formation (Rydberg *et al.*, 2005). Further studies on four randomly chosen lines, 1256-6, 1256-26, 1256-27 and 1256-45, demonstrated that reduced asparagine levels coincided with a 1.5–2.5-fold increase in the accumulation of the alternative amide amino acid glutamine, which resembles asparagine in that it contains two amino groups (Figure 3g). All other tested amino acids remained at wild-type levels (Figure 3g), and the protein content was also unaltered (Figure 3h). However, the total amount of tested free amino acids in tubers from line 1256-26, which contained the smallest amount of asparagine, was 16% less than that of controls and other intragenic lines, indicating that an almost complete impairment of asparagine formation may have a rippling negative effect on the biosynthesis of other amino acids.

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Page 1134

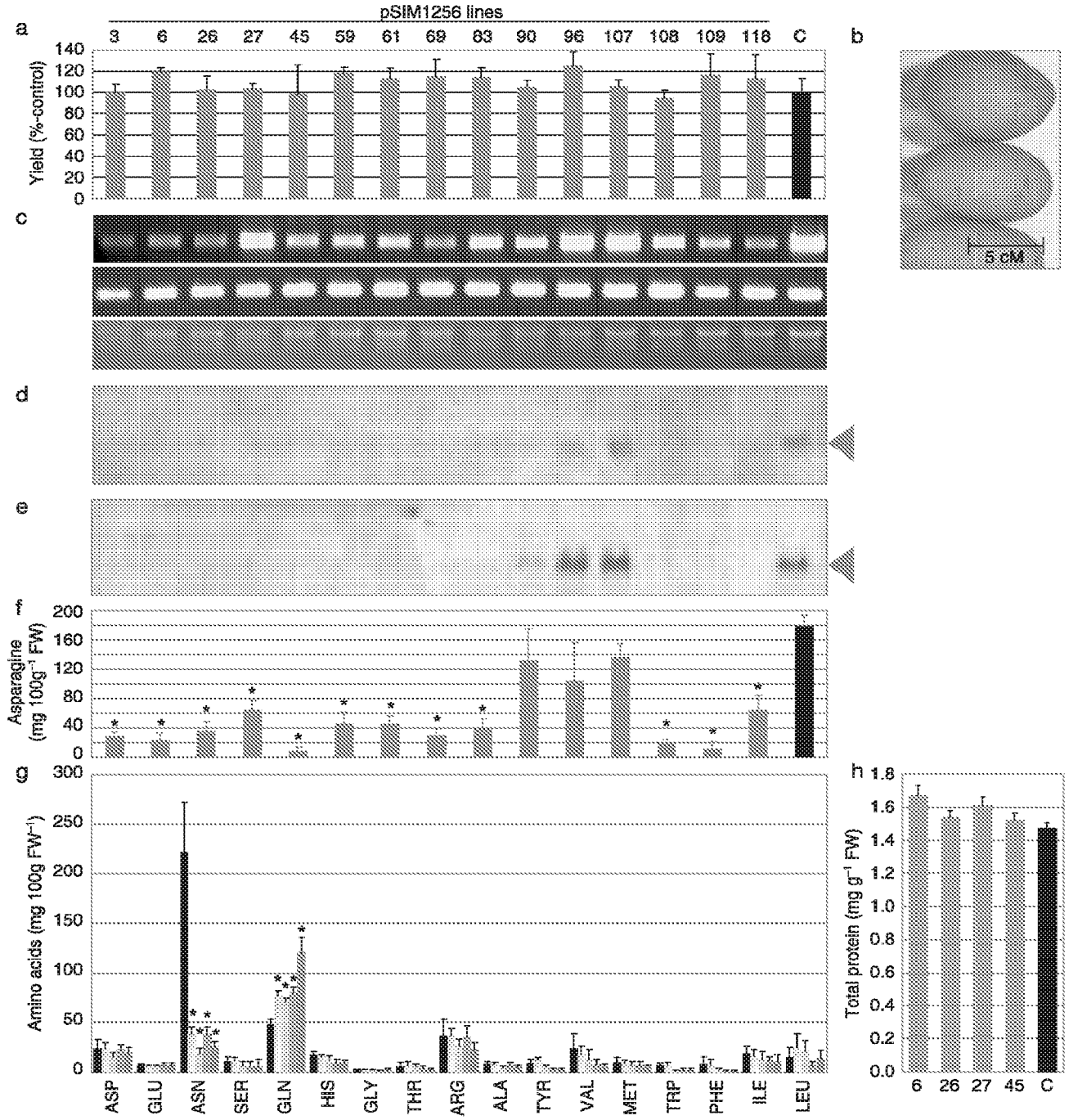

Figure 3 Silencing of the potato asparagine synthetase genes (*StAs1* and *StAs2*) decreases the accumulation of asparagine. (a) Tuber yield for 3-month-old glasshouse-grown plants of 15 intragenic lines (grey bars) and untransformed controls (C) (black bar). 100% = 361 ± 60 g/plant. (b) Phenotype of typical tubers of line 1256-26. (c) Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) for relative expression of *StAs2* (top panel) and the internal *actin* gene control (middle panel), with RNA loading shown in the bottom panel. (d) StAs2 transcript levels determined by RNA gel blot analysis. The grey arrow shows the predicted position of the RNA. Weak band intensity in the control lane indicates low *As2* gene expression levels in untransformed plants. Transcript levels were undetectable in tubers of most intragenic lines. (e) Hybridization of an RNA gel blot with an *StAs1* gene-derived probe. (f) Tuber asparagine levels. Significant differences from controls ($P < 0.05$) are indicated with an asterisk. FW, fresh weight. (g) Amino acid profiles for untransformed control tubers (black bars) and tubers from lines 1256-6 (lightest grey bar), 1256-26 (light grey bar), 1256-27 (grey bar) and 1256-45 (dark grey bar). (h) Total protein content. Data represent the mean of three experiments ± standard deviation.

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Aardevo Exhibit 1010
Page 1135

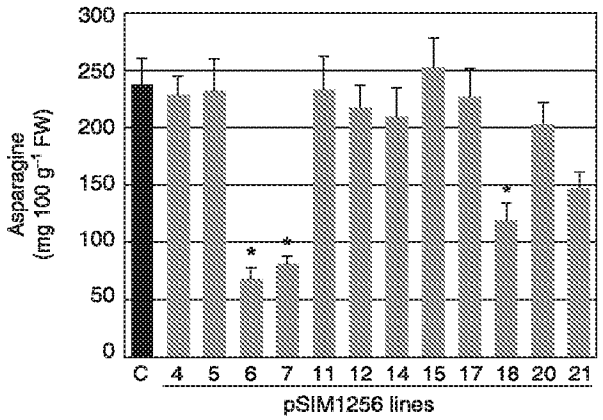

Figure 4 Asparagine levels of tubers from pSIM1151 lines. The results shown represent the average values of three measurements ± standard deviation for tubers of the untransformed control (C) (black bar) and 12 transformed lines (grey bars). Significant differences from controls ($P < 0.05$) are indicated with an asterisk. FW, fresh weight.

A second expression cassette that contained the *StAs1/2*-derived inverted repeat between two Gbss promoters was also tested for its efficacy to reduce the accumulation of asparagine in tubers. The vector carrying this alternative version of the silencing cassette (pSIM1151) was used to produce 12 transformed lines. HPLC analyses demonstrated that tubers from most of the transformed lines contained the same asparagine levels as wild-type controls (Figure 4). Only three transformed lines produced tubers with less than one-half the amount of asparagine that accumulated in controls. This result confirms earlier studies, which indicated that tuber-specific and convergent transcription-mediated gene silencing is triggered more effectively by the combination of Gbss and Agp promoters than by two copies of the Gbss promoter (Yan *et al.*, 2006). It can be concluded that silencing of the *StAs1* and *StAs2* genes caused a dramatic decrease in the accumulation of asparagine in tubers without compromising the yield of glasshouse-grown transgenic lines.

Reduced asparagine formation in tubers limits heat-induced acrylamide formation in French fries

Potatoes are generally stored at temperatures that limit cold-induced sweetening (7–10 °C). For proof-of-concept experiments designed to determine the effect of low asparagine levels on acrylamide production, the storage temperatures were decreased to 4 °C. After a 1-month incubation period, tubers contained 8.8 ± 0.37 mg/g fresh weight (FW) of glucose, which is 12-fold higher than the glucose levels in freshly harvested tubers. Initially, the cold-stored tubers of eight intragenic lines (1256-6, 1256-26, 1256-27, 1256-45, 1256-83, 1256-90, 1256-96 and 1256-109) were blanched, cut, par-fried and finish-fried for 2 min 45 s for the production of lightly coloured French fries. HPLC analyses of these materials demonstrated that the control product contained 126 ng g^{-1} of acrylamide, whereas the presence of this undesirable Maillard reaction product was often below the detection limit of 20 ng g^{-1} in intragenic fries (data not shown). The finish-frying time was therefore extended to 4 min, and produced brown French fries for both untransformed controls and intragenic tubers (Figure 5a). As expected, the longer frying time resulted in the formation of more acrylamide in controls (1097 ng g^{-1}), and low but detectable levels of this compound in intragenic fries (Figure 5b). Importantly, fries from lines 1256-26 and 1256-45 contained only approximately 5% of the acrylamide that accumulated in controls. The correlation coefficient between tuber asparagine levels and fry acrylamide levels was 0.9074, indicating that asparagine plays an important role in acrylamide formation.

The four intragenic lines 1256-27, 1256-83, 1256-90 and 1256-96 were evaluated by a team of professionally trained food scientists for various aspects of texture (crispness, mouthfeel, mealiness and texture variation) and taste (caramel taste, fresh fried taste and aroma). These assessments demonstrated that the above-described resemblance in the visual appearance of intragenic and control fries

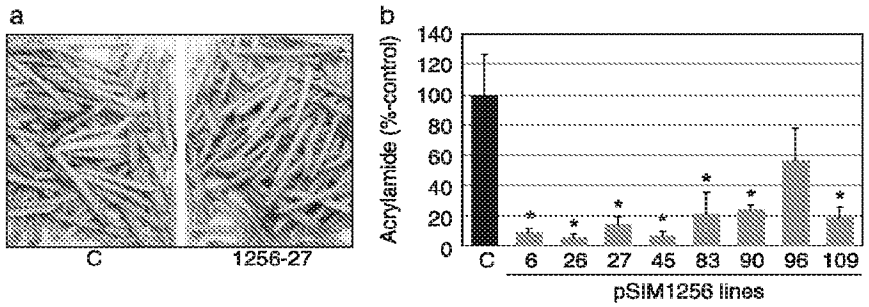

Figure 5 Acrylamide levels in French fries. (a) Visual appearance of fries from an untransformed control (C) and intragenic line 1256-27. (b) Acrylamide levels. Data are expressed as a percentage of the untransformed control value (1097 ± 258 ng g^{-1}) and represent the mean of three measurements ± standard deviation. Asterisks indicate significant differences from controls ($P < 0.05$).

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Aardevo Exhibit 1010
Page 1136

Table 1 Sensory evaluation of French fries prepared from both untransformed and intragenic Ranger Russet tubers stored for 1 month at 4 °C prior to processing. Tests were performed by a panel of eight professionally trained experts, who sampled French fries at the optimum time of 3 min. Data denote the average marking of six assessments ± standard deviation on a nine-point scale (9, excellent; 1, poor)

	Sensory characteristic	Control	1256-27	1256-83	1256-90	1256-96
Texture	Crispness	4.7 ± 0.5	4.8 ± 0.1	5.0 ± 0.1	4.5 ± 0.2	5.2 ± 0.1
	Mouthfeel	6.3 ± 0.3	5.8 ± 0.1	5.8 ± 0.3	3.8 ± 0.5	6.2 ± 0.2
	Mealiness	5.8 ± 0.3	6.2 ± 0.1	6.2 ± 0.2	5.2 ± 0.1	5.8 ± 0.2
	Variation	6.4 ± 0.3	6.2 ± 0.1	6.3 ± 0.3	6.3 ± 0.1	6.5 ± 0.3
Taste	Caramelized	6.6 ± 0.4	7.0 ± 0.0	6.3 ± 0.3	5.3 ± 0.4	5.8 ± 0.2
	Fresh-fried	2.9 ± 0.2	3.3 ± 0.4	3.0 ± 0.0	3.3 ± 0.4	3.3 ± 0.4
	Aroma	5.9 ± 0.7	6.0 ± 0.0	5.2 ± 0.2	5.3 ± 0.6	5.5 ± 0.4

coincided with a similar texture and taste (Table 1). Collectively, our results demonstrated that a 3–18-fold decrease in the asparagine content of potato tubers resulted in a similar fold decrease in the amount of acrylamide accumulated during heat processing. These changes did not affect the tuber yield or French fry quality.

Production of low-acrylamide potato chips

The high surface-to-volume ratio of sliced tubers that are used for chip production promotes the rapid formation of acrylamide during heat processing (Matthäus *et al.*, 2004). Therefore, the glucose concentration of cold-stored tubers was decreased (to 2.8 ± 0.12 mg/g FW) by incubation for 2 weeks at 15 °C. Potato chips obtained from reconditioned control tubers contained 4318 ± 1138 ng g^{-1} of acrylamide (Figure 6a,b), which is still approximately fourfold higher than that for French fries from cold-stored tubers (Figure 5b). Intragenic chips from lines 1256-27 and 1256-83 accumulated much lower levels of acrylamide (861 and 1153 ng g^{-1}, respectively). However, these levels may still be sufficiently high to pose potential health issues. Thus, the impairment of asparagine biosynthesis is not always sufficient to guarantee low levels of acrylamide formation.

In an effort to optimize the decrease in acrylamide, the potato variety Atlantic, which contains much lower levels of glucose and fructose than does Ranger Russet (Webb *et al.*, 1978), was transformed. Fifteen of 844 shoots regenerated from infected explants were shown by PCR-based genotyping to contain the *StAs1/2* gene silencing construct, whilst lacking backbone DNA, indicating a frequency for marker-free and backbone-free transformation of 1.8%. Plants of three intragenic lines that displayed reduced *StAs1* and *StAs2* gene expression levels, designated as 1256A-3, 1256A-5 and 1256A-6, were confirmed to contain at least one P-DNA insert (Figure 6c), and were grown in the glasshouse for tuber production. Harvested tubers accumulated 7.1%–18.4% of the asparagine that was produced in untransformed control tubers (Figure 6d). These fresh tubers were subsequently processed using standard procedures to produce potato

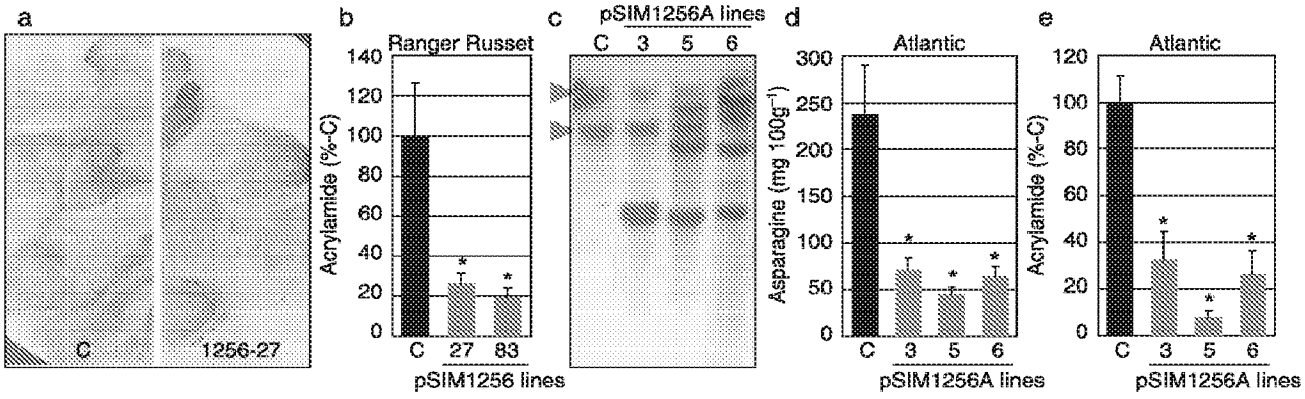

Figure 6 Potato chips from low-asparagine tubers. (a) Example of chips from tubers of untransformed Ranger Russet controls (C) and line 1256-27. (b) Acrylamide levels in chips. Data for chips from intragenic lines (grey bars) are shown as a percentage of the levels in chips from the untransformed controls (4318 ± 1137 ng g^{-1}) (black bar). (c) Copy number of intragenic Atlantic lines 1256A-3, 1256A-5 and 1256A-6, as visualized with a probe containing fragments of both the *StAs1* and *StAs2* genes. Grey arrows indicate the positions of two endogenous hybridizing DNA bands. (d) Asparagine levels in lines 1256A-3, 1256A-5 and 1256A-6 (grey bars) compared with controls (black bar). (e) Acrylamide levels in processed material from intragenic Atlantic lines (grey bars) and controls (black bar). 100% = 840 ± 90 ng g^{-1}.

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Aardevo Exhibit 1010
Page 1137

chips. Figure 6e shows that control chips from Atlantic tubers contained 840 ng g^{-1} of acrylamide, which is about fivefold lower than the levels in chips from untransformed Ranger Russet tubers. Acrylamide levels were much lower in the processed material from intragenic lines, most notably line 1256A-5. This particular intragenic line contained only 8% of the acrylamide that was present in chips from untransformed Atlantic tubers (67 ng g^{-1}), and may prove to be suitable for commercial production if none of the original agronomic characteristics of the Atlantic variety are lost as a consequence of the transformation process.

Discussion

Simultaneous silencing of the *StAs1* and *StAs2* genes decreases the accumulation of free asparagine in potato tubers by up to 95%. Because asparagine plays an important role in nitrogen assimilation and storage (Lam *et al.*, 2003; Lea *et al.*, 2007), it was somewhat surprising that the targeted metabolic change did not affect the yield or shape of tubers from glasshouse-grown plants. To some extent, the intragenic tubers adjusted to the impaired asparagine biosynthesis by both accumulating more glutamine, which is the precursor of asparagine and has only a slightly lower nitrogen to carbon ratio, and increasing protein biosynthesis. It is also possible that tubers increased the formation of γ-aminobutyric acid, which contains about 11% of soluble nitrogen (Thompson *et al.*, 1953). A full analysis of the extent to which intragenic tubers are capable of compensating for their lost ability to assimilate and store nitrogen in the form of asparagine will be carried out in future work. Regardless of the outcome of these studies, it was found that the tuber-specific silencing of the *StAs1* and *StAs2* genes did not affect the tuber yield of glasshouse-grown plants. This finding is in agreement with a previous study on transgenic potato plants down-regulated for the amino acid symporter StAap1 (Koch *et al.*, 2003). Substantially decreased levels of free amino acids in the tubers of these transgenic plants were not associated with negative side-effects on leaf metabolism, tuber yield or phenotype.

In contrast with the tuber-specific silencing strategy employed in this work, it is predicted that strong constitutive silencing of the two related *As* genes would compromise plant growth and/or development. The proteins encoded by these genes play an important role in recapturing ammonium that is lost during photorespiration, a process that exceeds primary nitrogen assimilation by 10-fold (Keys *et al.*, 1978). Although the enhanced assimilation of ammonium has been shown to increase photosynthesis (Fuentes *et al.*, 2001) and accelerate growth (Oliveira *et al.*, 2002), an impairment of this process may result in the accumulation of high and potentially toxic concentrations of ammonium (Gerendas *et al.*, 1997; Wong *et al.*, 2004). It should be mentioned that intragenic plants have not yet been exposed to the environmental stresses that are typical in the field. Such stresses include, for instance, drought, overwatering and infection, and are all known to enhance the formation of asparagine (Lea *et al.*, 2007). The physiological significance of up-regulated asparagine biosynthesis is not yet fully understood, but it is possible that an inability to increase the concentrations of asparagine in tubers would, directly or indirectly, enhance stress sensitivity in the field.

Despite the efficacy of *StAs1/2* gene silencing, intragenic tubers still contained 5%–25% of the asparagine that accumulated in untransformed potatoes. Some of this amino acid may have been synthesized through either rudimentary StAs1 and/or StAs2 activity or transamination of 2-oxosuccinamic acid (Joy, 1988). A more substantial proportion was probably produced in leaves for transport through the phloem to the tubers (Fischer *et al.*, 1998). Given the similar phenotypes of intragenic plants and their untransformed counterparts, it is unlikely that the silencing construct has a strong effect on the biosynthesis of asparagine in leaves. An additional argument for this hypothesis comes from the poor foliar activity of the promoters of the silencing construct (Visser *et al.*, 1991; C. Richael, unpubl. data).

The significance of asparagine as an important determinant of the acrylamide potential of tubers was not noted in earlier studies evaluating the effect of genotype on acrylamide levels (Williams, 2005; Viklund *et al.*, 2008). This discrepancy highlights the importance of other factors that influence the formation of acrylamide. Such factors include, for instance, glycine concentration (Bråthen *et al.*, 2005), product matrix (Claeys *et al.*, 2005), tuber moisture (Amrein *et al.*, 2006), antioxidants (Zhang *et al.*, 2007) and, most importantly, reducing sugars. The role played by each of these factors is variety dependent, which explains why asparagine concentration alone is not always a reliable predictor for the extent of acrylamide formation (Williams, 2005). Although the pSIM1256 Ranger Russet lines provided a suitable source of low-asparagine tubers for the production of French fries, they still yielded potato chips that contained relatively high levels of acrylamide. This result indicates that an impairment of asparagine biosynthesis is often necessary, but not always sufficient, to guarantee low levels of acrylamide formation during heat processing. The large amounts of reducing sugars that accumulate in cold-stored Ranger Russet tubers may accentuate reactions with compounds that represent

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Page 1138

alternative acrylamide precursors, such as glutamine (Mottram *et al.*, 2002).

Our study demonstrated that low-asparagine Ranger Russet tubers that were cold stored at 4 °C yielded potato chips that were still relatively high in acrylamide. This finding indicates that the dramatic reductions in asparagine levels accomplished through *As1/2* gene silencing are not always sufficient to guarantee a low-acrylamide product. For chip production, it will still be necessary to either store tubers at higher temperatures (7–10 °C) or to apply typical 'chip' varieties, such as Atlantic. Alternatively, it may be possible to retransform a low-asparagine Ranger Russet variety with constructs for reduced cold-induced sweetening. Such constructs could be designed to down-regulate the expression of the genes involved in starch degradation (Rommens *et al.*, 2006) or to express the maize (*Zea mays*) *Dof1* transcription factor gene (Yanagisawa *et al.*, 2004) or the potato metabolic regulator SnRk1 (McKibbin *et al.*, 2006).

The consumption of processed potato products contributes to approximately one-third of the average dietary intake of acrylamide (Boettcher *et al.*, 2005). Thus, an eventual replacement of existing potatoes by low-asparagine varieties would lower the ingestion of acrylamide by approximately 30%. Even greater reductions could be accomplished by applying the described methods to wheat and coffee. In these cases, the *As* genes would have to be silenced in the endosperm.

In conclusion, our work has demonstrated that the heat-induced formation of acrylamide can be decreased by reducing the asparagine content in potato tubers. Preliminary data have indicated that this modification does not alter the normal agronomics of the crop. If supported by consumers, all-native fry products with very low levels of acrylamide could be offered as a new market choice within the next 5 years.

Experimental procedures

Database and statistical analyses

Gene expression levels were assessed by analysing expressed sequence tag data stored at the Gene Index Database, maintained by the Dana Farber Cancer Institute, Boston, MA, USA (www.danafarber.org). Amino acid motifs associated with the functional activity of enzymes were analysed by position-specific iterated and pattern-hit initiated BLAST (Schäffer *et al.*, 2001). Databases searched using BLASTN and TBLASTS included those maintained by the National Center for Biotechnology Information, Bethesda, MD, USA (http://www.ncbi.nlm.nih.gov). The correlation coefficient between two variables was calculated according to Pearson (Gayen, 1951). Both the calculation of protein alignments and the visualization of cladograms were based on CLUSTALW (with penalties for opening and extending a gap set at 10.0 and 0.2, respectively). Protein structures were predicted using the PHYRE protein threading program version 2.0 (www.sbg.bio.ic.ac.uk/~phyre/). The *t*-test was used to study whether the means of two groups were statistically significantly different from each other.

Plasmid construction

The DNA segment used to produce a multigene silencing construct contained 348-bp fragments of the *StAs1* gene (coordinates 318–665 of GENBANK accession CK278037) and the *StAs2* gene (coordinates 314–661 of GENBANK accession CK271149). The tuber-specific Gbss promoter represents coordinates 1138–1823 of GENBANK accession X83220. The Agp promoter is identical to coordinates 2183–4407 of GENBANK accession X96771. The basic P-DNA vector used has been described previously (Rommens *et al.*, 2004).

Plant transformation, genotyping and DNA gel blot analysis

Stock plants were maintained in Magenta boxes containing 40 mL half-strength M516 medium (PhytoTechnology, Shawnee Mission, KS, USA) with 3% sucrose and 2 g/L gelrite. Plants were transformed as described previously (Richael *et al.*, 2008). Transformed plants were genotyped for the presence or absence of P-DNA, T-DNA and backbone DNA using a robust and reliable PCR method, explained elsewhere (Xin *et al.*, 2003). The P-DNA copy number of intragenic lines was determined by isolating DNA using DNAzol reagent (Invitrogen, Carlsbad, CA, USA), transferring *Eco*RI digests to positively charged membranes (Roche Applied Science, Indianapolis, IN, USA) and hybridizing the membranes with a probe derived from the 0.8-kb DNA segment, which was employed to create the silencing construct and carried fragments from both the *StAs1* and *StAs2* genes, using the non-radioactive digoxigenin system (Roche Applied Science).

RNA analysis

RNA was isolated from tubers using an RNAeasy Plant Mini Kit (No. 74904, Qiagen, Valencia, CA, USA), following the manufacturer's recommendations. cDNA was synthesized from 0.5 μg of total RNA in a 20-μL volume using an oligo dT primer and SuperscriptII RT (Invitrogen) at 42 °C in 1 h. One microlitre of resulting cDNA was used as template in the following PCR: 25-μL volume containing 20 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 0.2 μM of each primer and 1.5 U Taq DNA polymerase (Invitrogen). The cycling protocol was as follows: 3 min at 95 °C, followed by 33 cycles of 20 s at 95 °C, 20 s at 57 °C and 80 s at 72 °C, with a final step of 2 min at 72 °C. Nine microlitres of the amplification products were analysed by electrophoresis and visualized by ethidium bromide on a 1% agarose gel. Semi-quantitative PCR for the *StAs2* gene was carried out with the pair of intron-spanning primers 5′-GGGATGCCATTGGCATTACAC and 5′-TGTACTGGCTCTGATACTGGTC, using Qiagen's one-step RT-PCR kit (catalogue number 210212), according to the manufacturer's recommendations. Intron-spanning primers for the internal

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Aardevo Exhibit 1010
Page 1139

control *actin* gene were 5′-AGTGGTCGTACCACCGGTATTGTG and 5′-ATGATCAGTGAGGTCACGACCTGC. Non-radioactive digoxigenin RNA gel blot hybridization was performed according to the manufacturer's recommendations (Roche Applied Science). A labelled 1.1-kb probe for the *StAs2* gene was derived from a gene fragment amplified with the primer pair 5′-CTTGCTCATCAACGATTGGCAATAG and 5′-AGGTCGGATCATTTTCCATTCTG. The primers used to produce a 1.1-kb probe for the *StAs1* gene were 5′-GGTTGATGACTGATGTCCCCTTTG and 5′-TAGTTAGCTCCTTATTGTGAGCTC.

Tuber production, characterization and processing

Plants were grown for 3 months in 7.6 L pots in a glasshouse that was controlled for temperature (18 °C minimum/27 °C maximum) and light (16-h photoperiod with an intensity of approximately 1500 μmol/m^2/s). The specific gravities of the harvested tubers were determined by dividing the weight in air by the weight in water (Kleinkopf *et al*., 1987). For French fry production, the tubers were washed, blanched for 8 min at 74 °C, cut into shoestring strips, dipped in a 1% sodium acid pyrophosphate solution at 71 °C, dried at the same temperature, fried at 200 °C for 45 s and frozen for 20 min at –26 °C, shaking the tray two to three times in the first 6 min. The processed fries were then finish-fried at 168 °C for either 2 min 45 s (light colour) or 4 min (dark colour). Potato chips were produced by slicing fresh tubers to 1.0-mm thickness using an industrial slicer; after air drying, they were fried for 11 min at 170 °C.

Assays for glucose, amino acids and total protein content

A glucose oxidase/peroxidase reagent (Megazyme, Bray, Co. Wicklow, Ireland) was used to determine the glucose levels of cold-stored tubers. For amino acid analyses, tuber samples were extracted and analysed using an automated amino acid analyser according to the official methods of analysis of the Association of Official Analytical Chemists (AOAC) (http://www.aoac.org/omarev1/982-30.pdf). The total protein content was determined by grinding a transverse slice of approximately 0.5 cm across the middle of the tuber in liquid nitrogen with a mortar. A homogenate containing 250 mg of the ground powder in 0.5 mL of extraction buffer [25 mM Tris-acetate pH 8.5, 0.5 M NaCl, 5 mM phenylmethylsulphonylfluoride (PMSF)] was centrifuged at 12 000 ***g*** for 10 min. The protein concentration in the supernatant was then measured using the Bradford protein assay kit (Bio-Rad, Hercules, CA, USA).

Assessment of fry colour and acrylamide levels

Colour was assessed using an E30-FP Agtron Process Analyser (Agtron, Reno, NV, USA), whereby a lighter colour is reflected by a higher number and values above 40 are generally considered to be acceptable. French fries were ground to a fine powder in liquid nitrogen; the powder was shipped on dry ice to Covance Laboratories (Madison, WI, USA), where a combination of liquid chromatography and mass spectrometry was used to detect acrylamide at concentrations as low as 20 p.p.b., according to a method developed by the United States Food and Drug Administration (http://www.cfsan.fda.gov/~dms/acrylami.html), adapted from an earlier method (Schuster, 1988).

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Page 1142

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Engineered native pathways for high kaempferol and caffeoylquinate production in potato

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Summary

Flavonols and caffeoylquinates represent important groups of phenolic antioxidants with health-promoting activities. The genetic potential of potato (*Solanum tuberosum*) to produce high levels of these dietary compounds has not been realized in currently available commodity varieties. In this article, it is demonstrated that tuber-specific expression of the native and slightly modified MYB transcription factor gene *StMtf1*^{M} activates the phenylpropanoid biosynthetic pathway. Compared with untransformed controls, transgenic tubers contained fourfold increased levels of caffeoylquinates, including chlorogenic acid (CGA) (1.80 mg/g dry weight), whilst also accumulating various flavonols and anthocyanins. Subsequent impairment of anthocyanin biosynthesis through silencing of the flavonoid-3′,5′-hydroxylase (*F3′5′h*) gene resulted in the accumulation of kaempferol-rut (KAR) to levels that were approximately 100-fold higher than in controls (0.12 mg/g dry weight). The biochemical changes were associated with increased expression of both the CGA biosynthetic hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase (*Hqt*) gene and the upstream chorismate mutase (*Cm*) and prephenate dehydratase (*Pdh*) genes. Field trials indicated that transgenic lines produced similar tuber yields to the original potato variety Bintje. Processed products of these lines retained most of their phenylpropanoids and were indistinguishable from untransformed controls in texture and taste.

Keywords: chlorogenic acid, flavonoids, flavonols, metabolic engineering, potato, transgenic plants.

Introduction

Potato (*Solanum tuberosum*), a crop native to the high Andes of Bolivia, Ecuador and Peru, was first cultivated by the Inca Indians around 200 BC. Indigenous farmers grew complex mixes of primitive varieties, each of which was recognized for specific health-promoting characteristics (Glendinning, 1983). This traditional approach has gradually been replaced by a more pragmatic focus on tuber uniformity, yield and storage characteristics (Ochoa, 1975; Frolick *et al.*, 2000). The emphasis on basic input and shelf life traits is more pronounced in countries in which potatoes were introduced after the 16th century. Most modern varieties represent closely related genotypes that produce uniform white or off-white tubers with small amounts of the originally abundant health-promoting compounds. For instance, tubers of the predominant potato variety in the USA, Russet Burbank, contain 40-fold lower levels of kaempferol-rut (KAR) than traditional Andean varieties such as Morado Taruma.

It is unfortunate that commodity potatoes contain only trace amounts of kaempferol derivatives because *in vitro* studies have indicated that these compounds display important health-promoting activities. By donating hydrogen atoms from their phenolic hydroxyl groups to superoxides and Fenton-mediated hydroxyl radicals, flavonols, including kaempferol, function as potent plant-derived antioxidants (Liu, 2004; Wang *et al.*, 2006). Additional structural properties, such as the presence of both a 4′-OH group in the B ring and a 4-keto, 5-OH region, are associated with the ability of these types of compound to function as anti-inflammatory agents (Wang *et al.*, 2006). Kaempferol is unique in lowering the incidence of various oestrogen-linked diseases (Wang *et al.*, 2001; Pang *et al.*, 2006; Rice and Whitehead, 2006; da Silva *et al.*, 2007), and recent epidemiological studies have also

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indicated protective effects against coronary heart disease and pancreatic cancer (Lin *et al.*, 2006; Nothlings *et al.*, 2007).

The most important dietary sources of kaempferol, which include the cruciferous crops cabbage and broccoli, represent an almost negligible proportion of the modern diet (Hollman and Arts, 2000; Howerton *et al.*, 2007; Ransley *et al.*, 2007). The resulting low dietary intake levels, which are currently estimated at 0.6–3.9 mg/day (Hertog *et al.*, 1993; Nothlings *et al.*, 2007), could be substantially increased by metabolic engineering of the phenylpropanoid pathways of frequently consumed vegetable crops. Transgenic tomato (*Solanum esculentum*) fruits expressing the *Petunia hybrida* chalcone isomerase gene have already been shown to accumulate approximately 0.01 mg kaempferol per gram dry weight (DW) (Muir *et al.*, 2001). Even greater increases to 0.08 mg/g DW were obtained by coordinately expressing the two maize (*Zea mays*) transcription factor genes *Lc* and *C1* (Bovy *et al.*, 2002).

One phenolic antioxidant that is still produced in potato tubers at moderate levels is chlorogenic acid (CGA) (Lugasi *et al.*, 1999). Although about one-half of the ingested CGA is metabolized to products with lower antioxidant activity, such as hippurate (Olthof *et al.*, 2003), there are clear health benefits to consuming CGA-rich foods. These benefits include reduced incidences of atherosclerosis, diabetes and various types of cancer (Feng *et al.*, 2005; McCarty, 2005; Natella *et al.*, 2007). In tomato, over-expression of the hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase (*Hqt*) gene increased CGA levels by up to 1.8-fold (Niggeweg *et al.*, 2004).

In this article, a new approach is presented to increase the levels of kaempferol-rut (KAR) and CGA in potato. This method activates the flavonoid biosynthetic pathway, whilst blocking the conversion of dihydroquercetin to dihydromyricetin. Genetically modified lines produced tubers that not only contained an almost 100-fold increase in KAR (0.12 mg/g DW), but also accumulated fourfold increased levels of antioxidant phenolic acids.

Results

Activation of the flavonoid pathway

The complementary DNA (cDNA) of a novel MYB transcription factor gene, designated here as *StMtf1*, was amplified from a poly(A) mRNA-derived library of potato leaves. The encoded product contains a unique serine-rich N-terminus and typical tandem R2 and R3 domains implicated in binding the major groove of DNA (Figure 1a). The phylogenetic relationship between StMtf1 and members of the R2R3-MYB subgroup N09 (Stracke *et al.*, 2001; Jiang *et al.*, 2004), which include the tomato anthocyanin activator Ant1, is shown in Figure 1b.

An expression cassette carrying *StMtf1* fused to the promoter of the potato ubiquitin-7 (*Ubi7*) gene (pUbi7) (Garbarino *et al.*, 1995) was inserted between the T-DNA borders of pSIM401 (Rommens *et al.*, 2005) to create pSIM645 (Figure 1c). Stem explants of the potato variety Bintje were infected with an *Agrobacterium* LBA4404 strain carrying this vector. After 5 days, a limited number of individual plant cells started to accumulate purple pigments. This phenomenon was not observed on transformation with the control vector pSIM401, indicating that StMtf1 activates the part of the phenylpropanoid pathway that is associated with anthocyanin formation (Figure 2). The activity of the potato transcription factor was enhanced by substituting two nucleotides near the 5′-end of the corresponding open reading frame (A10T and T13C). This modification altered a putative regulatory serine-rich N-terminal domain (Kneissl *et al.*, 2008) from NH_2-MNSSP to NH_2-MNSTS. Replacement of *StMtf1* by the modified gene version (*StMtf1*^{M}) produced vector pSIM646. When used for transformation, the new vector increased the frequency of purple cells/explants by about 10-fold (Figure 3a,b). Transgenic potato plants derived from these cells produced stems and leaves that were intensely purple (Figure 3c), and also developed deeply purple flowers and anthers (Figure 3d).

After propagation in tissue culture, three plants of each of the lines 646-1, 646-12, 646-14, 646-15, 646-18 and 646-21 were grown for 1 month in a Percival growth chamber at 25 °C. All of these plants developed a dwarf phenotype and produced dark purple mini-tubers that were much smaller than those of control plants (Figure 3e). Surprisingly, the negative effect of *StMtf1*^{M} on plant stature and tuber yield was overcome by growing plants in either the glasshouse or the field, both environments with high light intensities (Figure 3f, and data not shown). The skin pigmentation of the harvested field-grown tubers was still very intense (Figure 3g), resembling that of some of the most deeply coloured Andean varieties, such as Moradita and Sallama (Ispizua *et al.*, 2007).

The skin-restricted pigmentation of transformed Bintje pSIM646 tubers was unexpected, given that the Ubi7 promoter supported β-glucuronidase activity across all tuber tissues (Figure 3h, and data not shown). Together, these results indicate that at least one essential gene for pigment formation, although expressed in the skin, is not functionally active in the tuber flesh of Bintje. The missing biosynthetic gene appears to be inducible by cold, as tubers developed small purple spots in their flesh after incubation for 1 month at 4 °C (Figure 3i).

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Page 1144

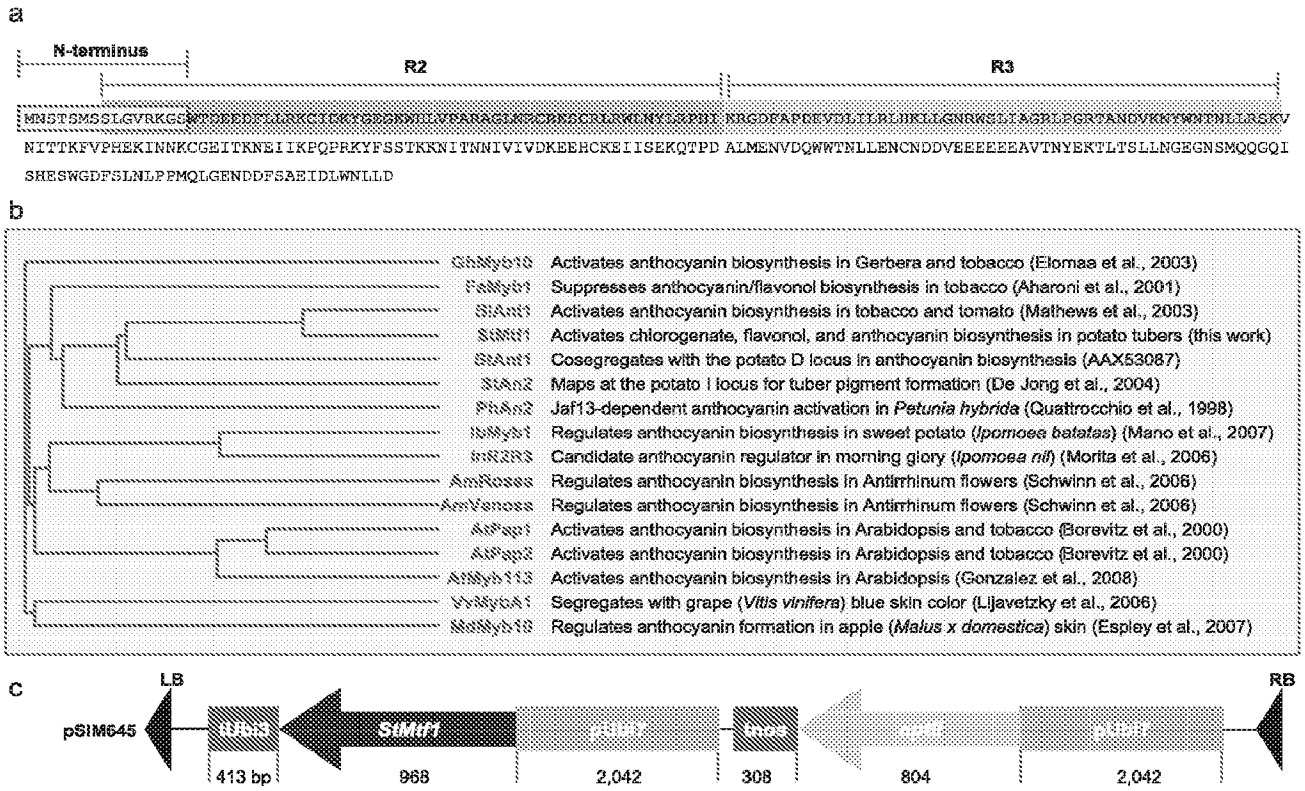

Figure 1 Potato MYB transcription factor gene and construct for its expression in plants. (a) Predicted protein sequence with serine-rich N-terminus and R2/R3 domains highlighted. (b) Cladogram of StMtf1 and some of its most closely related MYB transcription factors. (c) Diagrams of the transfer DNA of pSIM645. T-DNA borders are indicated by black triangles. LB, left border of the T-DNA; pUbi7, promoter of the *Ubi7* gene; *nptII*, neomycin phosphotransferase II gene; tnos, terminator of the *Agrobacterium* nopaline synthase gene; tUbi3, terminator of the potato *Ubi3* gene; RB, right border.

To correlate tuber phenotypes with the accumulation of phenolic compounds, secondary metabolite profiles were analysed by high-performance liquid chromatography (HPLC) combined with mass spectrometry (LC-MS) using electrospray ionization (ESI). As shown in Figure 4a, the epidermal pigmentation of 646-14 tubers was linked to reduced levels of the amino acids that function as flavonoid precursors. Control tubers contained 2.7 mg/g DW of phenylalanine, tyrosine and tryptophan, whereas tubers of line 646-14 showed a 40% reduction of these three amino acids, down to only 1.6 mg/g DW. Given the precursor role of aromatic amino acids in alkaloid biosynthesis, it was not surprising that the purple-skinned tubers also contained lower levels of the skin-produced glycoalkaloids α-solanine and α-chaconine (0.22 mg/g DW) than did pSIM401 controls (0.42 mg/g DW) (Figure 4a). Biosynthesis of the flavonols rutin (RUT) and quercetagin-rut (QUR) marked a third biochemical alteration. Trace amounts of these quercetin derivatives in control tubers contrasted with the accumulation of 0.0026 and 0.0118 mg/g DW, respectively, in tubers from line 646-14 (Figure 4b). However, the pigmented tubers still contained only wild-type amounts of the targeted health-promoting compound KAR (~0.0028 mg/g DW).

Although the metabolic change in flux did not alter the total amount of phenolic compounds (Figure 4c), it increased the tuber's antioxidant power from 353 μM equivalents of the vitamin E analogue Trolox (TE)/g for controls to 531 μM TE/g for the 646-14 tubers (Figure 4d). This finding suggests that the newly formed phenolic compounds are more effective in quenching cell-damaging free radicals than the original phytochemicals of the control plants. Together, our data

Figure 2 Simplified diagram of the biosynthetic pathway for flavonoids, caffeoylquinates and lignin. Enzymes abbreviated in red are as follows: ADH, arogenate dehydrogenase; ADT, arogenate dehydratase; AGA, anthocyanidin-3′-*O*-glycoside-6‴-*O*-acyl transferase; AGMT, anthocyanidin-glycoside-3′-*O*-methyl transferase; A3GT, anthocyanidin-3-*O*-glycosyl transferase; A5GT, anthocyanidin-5-*O*-glycosyl transferase; AS, anthocyanin synthase; ANS, anthranilate synthase; CAD, cinnamyl-alcohol dehydrogenase; CcOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; CHI, chalcone isomerase; CHS, chalcone synthase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate ligase; CM, chorismate mutase; DFR, dihydroflavonol reductase; FGT, flavonol glycosyltransferase; F3GT, flavonol 3-*O*-glycosyltransferase; F3H, flavonone-3-hydroxylase; F5H, ferulate

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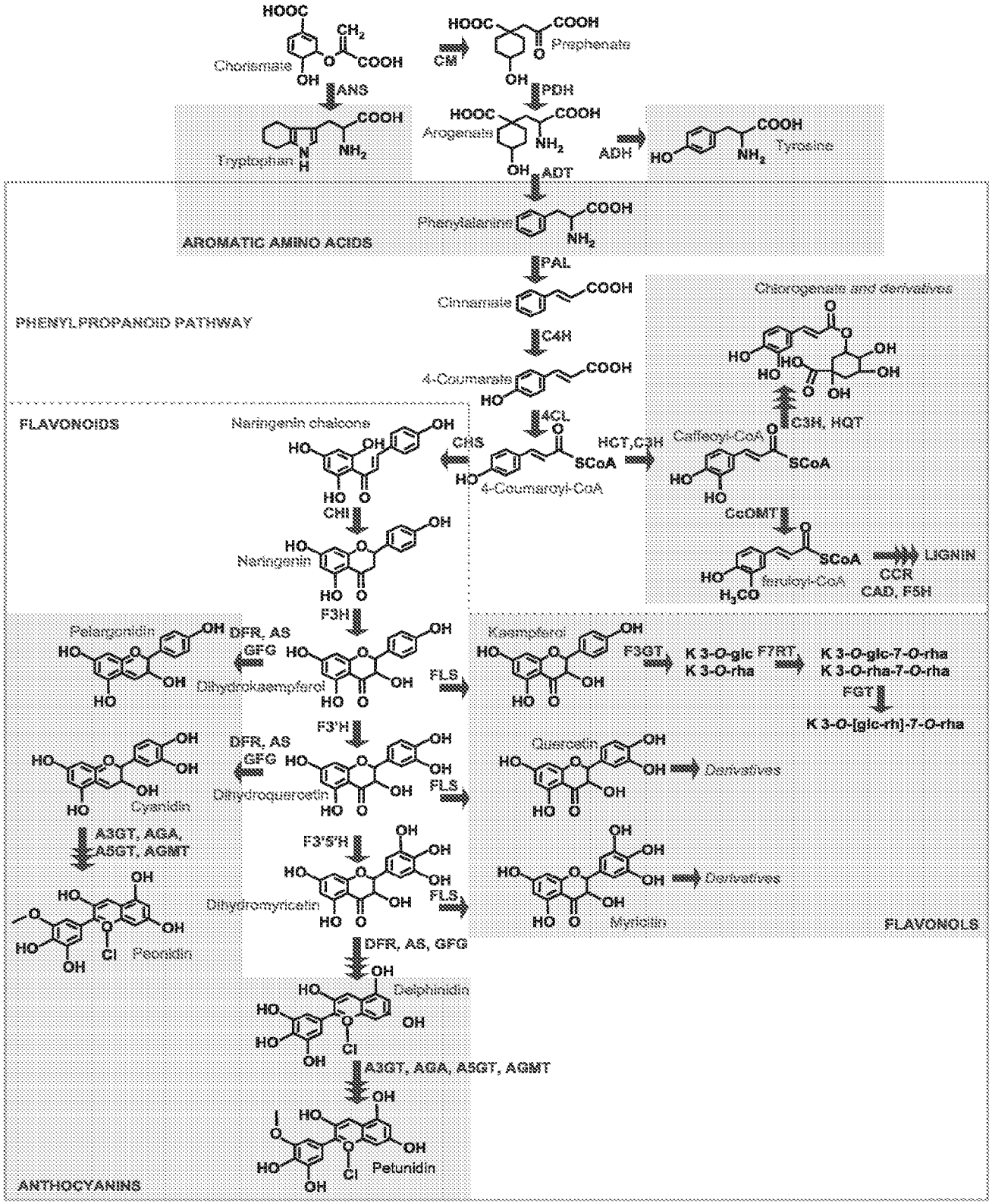
Page 1145



5-hydroxylase; F3'H, flavonoid-3'-hydroxylase; F3'5'H, flavonoid-3',5'-hydroxylase; FLS, flavonol synthase; F7RT, flavonol 7-*O*-rhamnosyltransferase; GFG, UDP-glucose 3-*O*-flavonoid glucosyltransferase; HCT, hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyl transferase; HQT, hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase; PAL, phenylalanine ammonia lyase; PDH, prephenate dehydratase.

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Page 1146

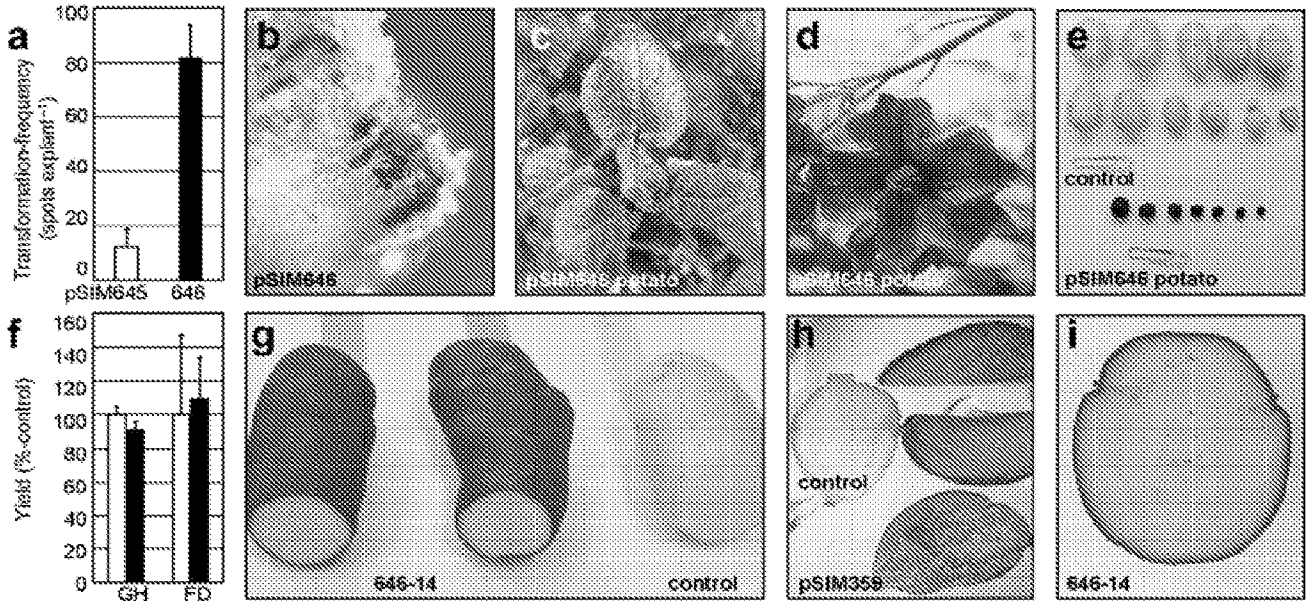

Figure 3 Pigment formation in transgenic Bintje potatoes. (a) Number of purple cells/stem explant, 1 week after infection with *Agrobacterium* strains carrying pSIM645 (white bar) and pSIM646 (black bar). (b) Potato stem explant, 2 weeks after infection with LBA4404/pSIM646. (c) Regenerated pSIM646 Bintje potato. (d) Typical flower of a pSIM646 potato plant. (e) Mini-tubers from pSIM401 control (top two rows) and pigment-producing pSIM646 (bottom row) plants, harvested 1 month after planting in soil. (f) Tuber yield for the control (white bars) and pSIM646 (black bars) plants that had been grown for either 3 months in a glasshouse (GH; 100% = 0.34 ± 0.17 g/plant) or 4 months in the field (FD; 100% = 2.05 ± 0.78 kg/plant). (g) Phenotype of mature cut Bintje tubers from line 646-14. (h) Histochemically stained Bintje pUbi7:*gus* mini-tubers from 6-week-old plants (right) and an untransformed control (left). (i) Phenotype of a cut pSIM646 Bintje tuber after 1 month of storage at 4 °C.

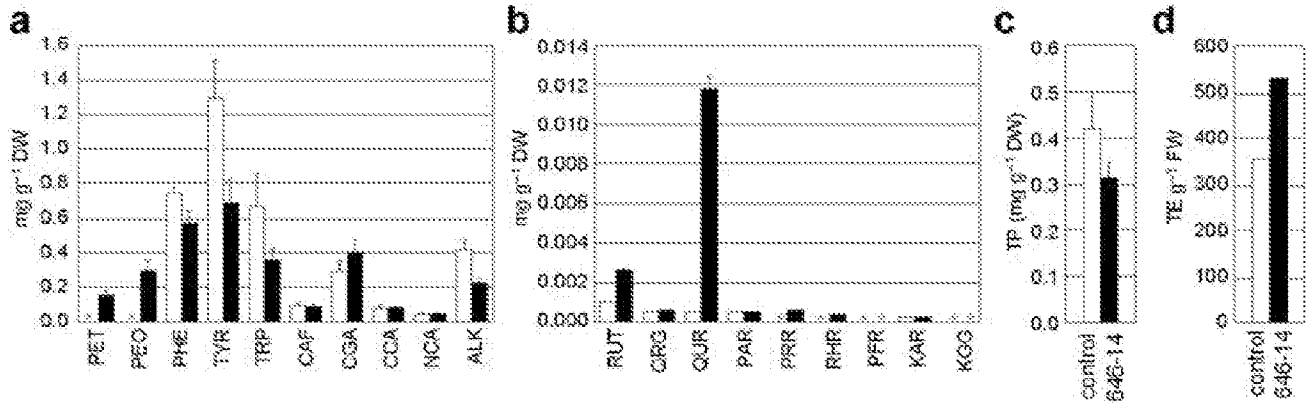

Figure 4 Biochemical analysis of pSIM646-14. (a) Accumulation of basic phenolic compounds and glycoalkaloids in control (white bars) and pSIM646 (black bars) tubers. Identified compounds were as follows: ALK, α-solanine and α-chaconin glycoalkaloids; CAF, caffeic acid; CCA, cryptochlorogenic acid; CGA, chlorogenic acid; PEO, peonidin glycoside; PET, petunidin glycoside; PHE, phenylalanine; NCA, neochlorogenic acid; TRP, tryptophan; TYR, tyrosine. (b) Content of various flavonol derivatives. Identified compounds were as follows: KAR, kaempferol-rut; KGG, kaempferol-glu-rut; PAR, patuletin-rut; PFR, patuletin-fer-glu-rha; PRR, patuletin-rut-rha; QRG, quercetin-rut-glu; QUR, quercetagin-rut; RHR, rhamnetin-rut; RUT, rutin. (c) Total phenolics (TP). (d) Oxygen radical absorbance capacity (ORAC) expressed in Trolox equivalents (TE). *Undetectable. DW, dry weight. FW, fresh weight. Data represent the average of three experiments ± standard deviation.

demonstrate that the pUbi7-driven expression of *StMtf1*^{M} triggers anthocyanin biosynthesis in above-ground tissues, roots and tuber skin, but not in tuber flesh. This modification was associated with both a decrease in the formation of toxic glycoalkaloids and an increase in antioxidant power.

Functional activity of StMtf1^{M} results in an increased production of CGA in tuber flesh

In an attempt to alter the tissue specificity of *StMtf1*^{M}-induced pigment formation, the Ubi7 promoter of pSIM646 was replaced

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Aardevo Exhibit 1010
Page 1147

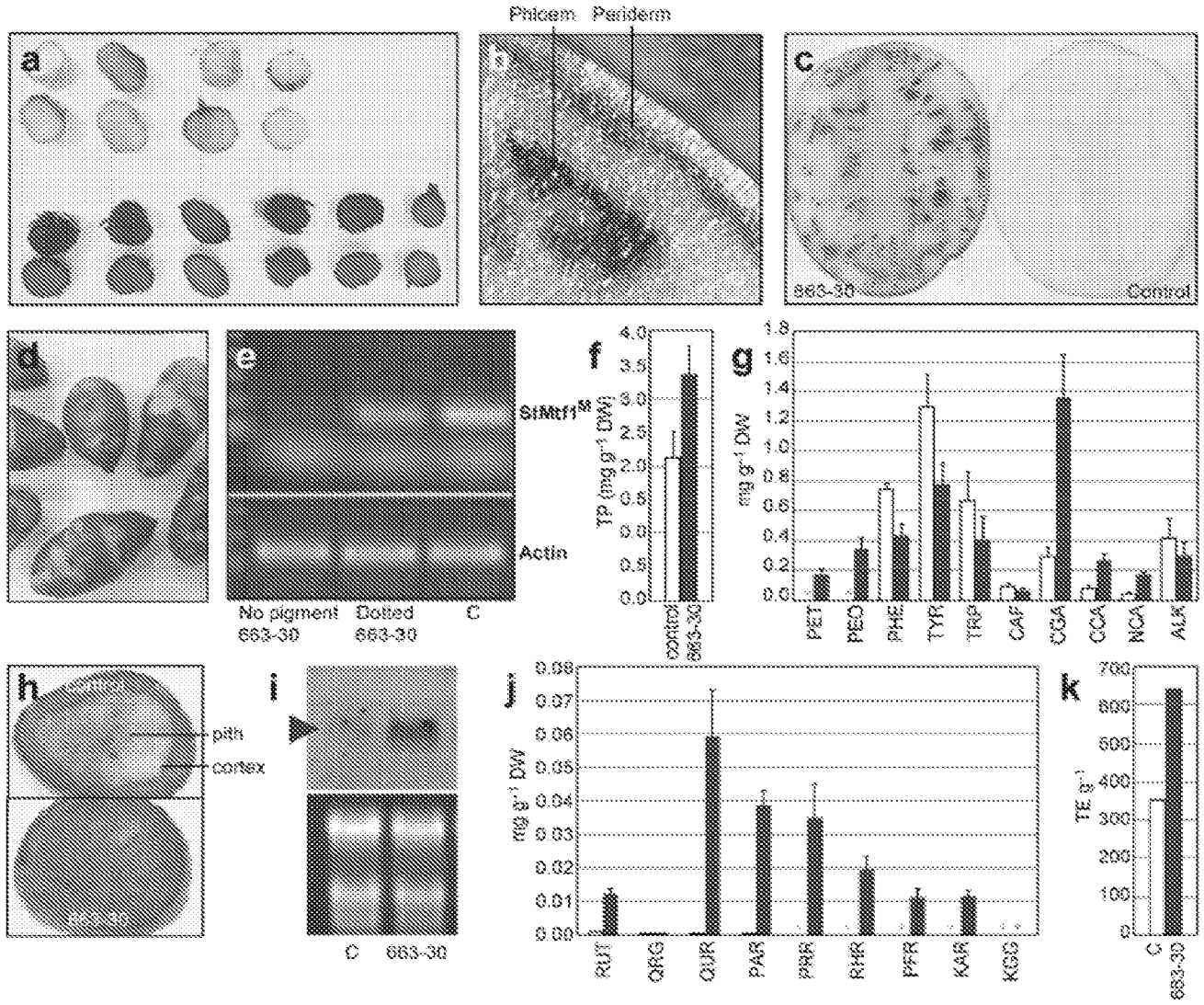

Figure 5 Analysis of pSIM663-30 Bintje tubers. (a) Phenotypes of histochemically stained untransformed control (top) and pGbss:*gus* (bottom) mini-tubers. (b) Close-up of pigment formation in periderm and phloem. (c) Cut tubers of a pSIM663 plant (left) and pSIM401 control (right). (d) Phenotypes of uncut pSIM663-30 tubers. (e) Relative expression levels of the *StMtf1*^{M} gene (top) and, as an internal control, the *actin* gene (bottom), as determined by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) in tubers of pSIM401 controls and either uncoloured or partially pigmented parts of pDIM663-30 tubers. (f) Total phenolics (TP) in pSIM401 control (white bar) and pSIM663-30 (black bar) tubers. (g) Accumulation of anthocyanins, simple phenolics and glycoalkaloids. Abbreviations are defined in the legend of Figure 4a. (h) Cut tuber surfaces, 24 h after incubation at room temperature for both control (top) and line 663-30 (bottom). (i) Transcript levels for the hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase (*Hqt*) gene, as visualized by RNA gel blot analysis (top). RNA loading is shown in the bottom panel. Arrow indicates the predicted position of the hydroxycinnamoyl transferase (Hct) transcript. (j) Accumulation of flavonols and flavonol derivatives. Abbreviations are defined in the legend of Figure 4b. (k) Oxygen radical absorbance capacity (ORAC), expressed in Trolox equivalents (TE), in pSIM401 control (white bar) and pSIM663-30 (black bar) tubers. Data represent the average of three experiments ± standard deviation. DW, dry weight.

by the promoter of the granule-bound starch synthase (*Gbss*) gene (pGbss). This alternative promoter is functionally active in all parts of the tuber, except for the epidermis (Figure 5a, and data not shown). The foliage of transgenic Bintje lines carrying the pGbss:*StMtf1*^{M} gene fusion (pSIM663) was phenotypically indistinguishable from that of control plants during glasshouse growth. Harvested pSIM663 tubers still resembled those of the controls in terms of yield and shape, but contained pigmented phloem and periderm cells that gave the tuber a mottled appearance (Figure 5b–d). Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) demonstrated that this tissue specificity was linked to *StMtf1*^{M} gene expression in both uncoloured tuber tissues and tissues that contained a combination of coloured and uncoloured cells (Figure 5e).

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Aardevo Exhibit 1010
Page 1148

At the biochemical level, pSIM663 tubers were found to contain greater amounts of phenolic compounds (3.3 mg/g DW) than controls (2.2 mg/g DW) (Figure 5f). More than 10% of these phenolics represented the anthocyanins petunidin glycoside (PET) (0.172 mg/g DW) and peonidin glycoside (PEO) (0.344 mg/g DW) (Figure 5g). The formation of these two pigments was correlated with an almost twofold decrease in aromatic amino acid concentrations and, unexpectedly, a fourfold increase in the combined levels of CGA, cryptochlorogenic acid (CCA) and neochlorogenic acid (NCA) (1.83 mg/g DW) when compared with controls (0.43 mg/g DW) (Figure 5g). The increase in caffeoylquinate formation was visualized by exposing cut tubers to air for several hours at room temperature. The resulting oxidation and precipitation of ferrous-CGA as a black ferric complex (Hughes and Evans, 1969) was restricted to the cortex region of the control tubers. In contrast, this darkening developed across the cut surface of 663-30 tubers, in both the cortex and pith (Figure 5h). Thus, the large amount of caffeoylquinate in pGbss:*StMtf1*^{M} tubers appears to be caused, at least in part, by a relaxed tissue-specific biosynthesis of these compounds.

It was theorized that the increased CGA levels in 663-30 tubers was triggered by the *StMtf1*^{M}-induced over-expression of the *Hqt* gene, which encodes a protein that is involved in the formation of CGA precursors by catalysing the conversion of coumaroyl-CoA into coumaroyl quinate (Niggeweg *et al.*, 2004). To test this hypothesis, RNA was isolated from tubers of untransformed and 663-30 lines, and used for RNA gel blot analysis. As shown in Figure 5i, it was found that the over-expression of *StMtf1*^{M} was associated with approximately fivefold increased Hqt transcript levels.

The transgenic 663-30 tubers also produced larger quantities of the flavonol derivatives that had first been identified in tubers of the line 646-14. Furthermore, they accumulated the new quercetin derivatives patuletin-rut (PAR), patuletin-rut-rha (PRR), rhamnetin-rut (RHR) and patuletin-fer-glu-rha (PFR) (Figure 5j). It was also found that 663-30 tubers contained small amounts of KAR (0.012 mg/g DW) (Figure 5j). This beneficial compound was of particular interest because of its scarcity in modern diets. A summary of the various novel flavonols is presented in Table 1. The combination of compositional changes was linked to an approximately twofold increase in antioxidant power, which is similar to that associated with the skin-specific pigmentation of pSIM646-14 tubers (Figure 5k). Collectively, our studies indicate that over-expression of the *StMtf1*^{M} gene in tuber flesh activated not only the anthocyanin biosynthetic pathway, but also at least one other branch of the phenylpropanoid pathway.

Flavonoid-3′,5′-hydroxylase (*F3′5′h*) gene silencing in *StMtf1*^{M}-expressing tubers triggers a massive production of kaempferol

In an attempt to further boost the biosynthesis of KAR, whilst maintaining high caffeoylquinate levels, a vector was developed containing an expression cassette designed to silence the *F3′5′h* gene (pSIM1166; Figure 6a). By retransforming line 663-30, we sought to capture the antioxidant potential offered by *StMtf1*^{M} over-expression, but to shunt the biochemical derivatives towards colourless flavonols. About two-thirds of the shoots regenerated from 663-30/pSIM1166 double transformants lost their ability to produce the deeply purple foliar anthocyanins typical of the 663-30 parental material (Figure 6b). Indeed, silencing of the purple pigment in leaves, stems and anthers was so strong that the light green, PCR-positive 663-30/pSIM1166 shoots could be readily distinguished from the faintly purple, untransformed Bintje shoots. In tubers, however, the ability to produce pigments was diminished, but not fully eliminated. As expected for *F3′5′h* gene silencing, remaining specks of pigment shifted from purple to a brick-red colour (Figure 6c), which marked the shift from delphinidin-type compounds to peonidin derivatives.

Table 1 Flavonol derivatives identified in Bintje 663-30 tubers by liquid chromatography-mass spectrometry (LC-MS). Compounds are listed in order of increasing mass. MS^2 product ions are listed in order of abundance

Compound	Retention time (min)	Molecular mass (g/mol)	$(M-H)^-$	MS^2 (*m/z* (% relative abundance))
Quercetagetin-rut	11.8	626	625	317 (100), 271 (27), 287 (8), 479 (1)
Kaempferol-glu-rut	11.9	756	755	593 (100), 285 (1), 634 (1), 255 (1), 447 (1)
Quercetin-rut-glu	12.1	772	771	301 (100), 591 (66), 609 (52), 505 (27), 271 (25), 255 (8)
Patuletin-rut	13.6	640	639	331 (100), 316 (22), 271 (8), 607 (5), 287 (2), 480 (2), 493 (1)
Rutin	14.1	610	609	301 (100), 271 (10), 255 (7), 343 (5), 591 (2), 463 (1)
Rhamnetin-rut	14.4	624	623	315 (100), 300 (24), 271 (12), 255 (5), 477 (< 1)
Kaempferol-rut	14.8	594	593	285 (100), 257 (5), 327 (4), 255 (3), 239 (1), 447 (1)
Patuletin-fer-glu-rha	15.1	816	815	639 (100), 653 (9), 331 (8), 329 (1), 621 (1)
Patuletin-rut-rha	15.4	786	785	639 (100), 331 (3), 316 (1), 621 (1), 493 (< 1)

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Aardevo Exhibit 1010
Page 1149

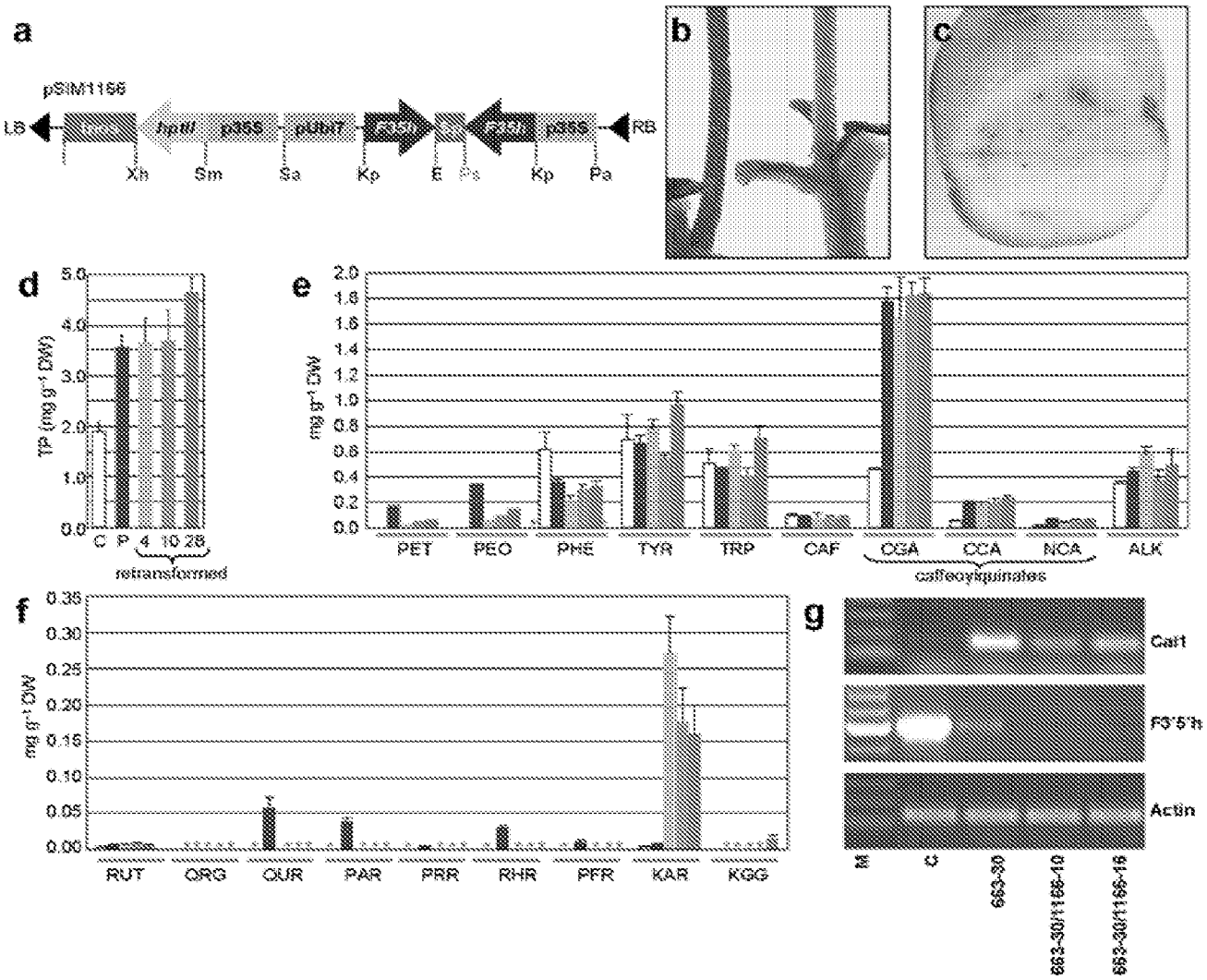

Figure 6 Inhibition of anthocyanin biosynthesis in *StMtf1*^{M}-expressing tubers silenced for the flavonoid-3′,5′-hydroxylase (*F3′5′h*) gene. (a) Diagram of the transfer DNA of pSIM1166. E, *Eco*RI; Kp, *Kpn*I; Pa, *Pac*I; Ps, *Pst*I; Sa, *Sac*I; Sm, *Sma*I; Xh, *Xho*I. (b) Regenerated untransformed shoot (left) and 663-30 shoot retransformed with pSIM1166 (right). (c) Typical tuber of a 663-30/1166 line. (d) Total amount of phenolic compounds (TP) in pSIM401 control (white bar) and pSIM663-30 (black bar) tubers, as well as tubers from retransformed lines 4 (light grey bar), 10 (medium grey bar) and 28 (dark grey bar). (e) Accumulation of simple phenolics and glycoalkaloids. Abbreviations are defined in the legend of Figure 4a. (f) Accumulation of flavonols and flavonol derivatives. Abbreviations are defined in the legend of Figure 4b. (g) Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) for the *StMtf1*^{M} (top panel), *F3′5′h* (middle panel) and *actin* (bottom panel) genes in tubers of untransformed, 663-30 and two 663-30/11664 lines. Data represent the average of three experiments ± standard deviation. DW, dry weight.

Biochemical analyses demonstrated that tubers from three independent 663-30/pSIM1166 lines contained similarly low levels of phenylalanine as tubers from the 663-30 parental line, most probably because of a higher utilization of this amino acid. The new tubers also contained higher levels of caffeoylquinates than did controls (Figure 6d,e). Most importantly, these tubers produced about 12-fold higher levels of the beneficial compound KAR (up to 0.12 mg/g DW) than did the original pGbss:*StMtf1*^{M} parent, which exceeded the control levels by almost 100-fold (Figure 6f). This massive increase in KAR coincided with both a disappearance of the various quercetin derivatives that accumulated at low levels in 663-30 tubers and a strong decrease in anthocyanins, especially delphinidin glycoside.

To correlate the new pigment phenotypes with expression levels for the *StMtf1*^{M} and *F3′5′h* genes, tubers were analysed by semi-quantitative RT-PCR. Figure 6g shows that the *StMtf1*^{M} gene was most strongly expressed in 663-30 tubers, and that this expression resulted in a partial suppression of the *F3′5′h* gene. Retransformed tubers contained a fully suppressed

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Aardevo Exhibit 1010
Page 1150

Table 2 Transcriptome analysis of 663-30, 663-30/1166-10 (Pokedot 1) and 663-30/1166-16 (Pokedot 2) lines compared with pSIM401 controls. The listed genes were differentially expressed ($P < 0.05$) in each of two independent microarray comparisons of the signal ratios for transcripts in a transgenic vs. untransformed tuber. The tentative contigs (TCs) associated with expressed sequence tags (ESTs) were obtained from The Gene Index Databases, Dana Farber Cancer Institute, Boston, MA, USA (URL: http://www.danafarber.org/)

Fold change					
663-30 (StMtf1^{M})	Pokedot 1	Pokedot 2	Tomato EST (TC)	Potato TC (% identity)	Annotation (EST)
Group 1. Transcription factor genes with known or putative regulatory roles in phenylpropanoid biosynthesis					
+8.3	+3.8	+3.4	AY348870		StMtf1^{M}
+9.0	+1.7	+1.3	X99134 (TC170130)	CV506186 (98%)	StMtf1^{M}-like MYB
+6.7	+6.1	+7.9	AI776994 (TC181588)	TC135093 (95%)	bHLH protein
+1.6	+1.6	+7.9	BG627387 (TC178386)	TC157693 (96%)	Homologue of bZIP transcription factor Bzi1
Group 2. Genes associated with the biosynthesis of chlorogenic acid and its derivatives					
+1.4	+4.3	+3.7	CK715539 (TC184397)	TC143161 (94%)	Chorismate mutase*
+3.0	+2.2	+2.7	BI923362 (TC172766)	TC134069 (95%)	Prephenate dehydratase*
+3.7	+2.6	+3.0	BI421300 (TC170174)	TC136412 (94%)	Hydroxycinnamoyl-CoA transferase
+2.0	+1.7	+1.4	AJ582652 (TC170212)	TC134780 (96%)	Hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase
Group 3. Genes associated with lignin biosynthesis					
+4.6	+4.8	NC	BT013041 (TC174902)	CV493179 (91%)	Ferulate-5-hydroxylase
–17.4	–16.6	–4.4	BG129820 (TC184549)	Not available	Cinnamyl alcohol dehydrogenase
Group 4. Genes involved (putatively) in flavonoid biosynthesis					
–1.9	–2.1	–3.0	BF097750 (TC170429);	TC135066 (96%)	Phenylalanine ammonia lyase*
–1.6	–1.8	–2.1	CN385790 (TC170429)	TC135066	
+9.6	+5.2	+9.6	U20596 (TC174169)	TC149768 (95%)	Putative flavanone 3-hydroxylase
+4.8	+4.8	NC	AJ785088	Not available	Flavonoid 7-*O*-glucosyltransferase
+2.2	+1.6	+2.1	BI205190 (TC175706)	CK860447 (98%)	Anthocyanin 7-*O*-glucosyltransferase
–3.9	–2.9	–3.2	CK716059 (TC171127)	TC134314 (95%)	Putative anthocyanin 5-aromatic acyltransferase
+3.6	+2.0	+1.5	AW031817 (TC170924);	TC133218 (96%)	*S*-Adenosyl-L-methionine synthetase
+2.8	+1.7	+1.5	Z24743 (TC170294)	TC133114 (95%)	
Group 5. Hormone biosynthetic and response genes					
+4.5	+3.8	+5.4	BG791226 (TC186460)	TC139639 (96%)	Diminuto
+4.6	+11.7	+10.7	BI209683 (TC176498)	CN214098 (84%)	Auxin-binding glutathione-*S*-transferase
NC	+1.6	+1.4	BI209735 (TC172132)	TC152381 (96%)	Auxin-response protein IAA1
NC	+1.7	+1.4	BT014412 (TC171221)	TC147866 (97%)	Auxin-response protein IAA8
NC	+1.4	+1.3	AF022020 (TC187912)	TC135441 (96%)	Auxin-response protein IAA9
–4.6	–2.0	–2.2	AB010991 (TC177967)	TC154307 (99%)	Gibberellin 3-β-hydroxylase
–15.9	–7.2	–16.2	AI895632 (TC188500)	Not available	Auxin-inducible gene
Group 6. Potential assembly of the major light-harvesting chlorophyll-*a*/*b* complex					
–7.9	–11.6	–7.9	CK715138 (TC170579);	TC148741 (96%)	Chlorophyll *a*/*b*-binding protein CP24
–4.3	–6.8	–6.6	CK714954 (TC170579)		
–5.0	–9.8	–7.0	BG628276 (TC179825)	TC159281 (96%)	Chlorophyll *a*/*b*-binding protein CP29
–4.2	–5.1	–4.4	AI775226 (TC178640);	TC133533 (97%)	Chlorophyll *a*/*b*-binding protein CP1B
–3.5	–3.2	–3.1	AI781554 (TC178640)		
–2.2	–2.2	–2.4	BG629407 (TC183219);	TC153607 (94%)	UV-B repressible protein
–3.9	–3.6	–3.4	BG125660 (TC183219)		
–4.7	–7.6	–4.3	BG628994 (TC171186);	TC133641 (97%)	33-kDa protein for oxygen-evolving complex
–6.8	–13.0	–10.6	BG629658 (TC170466)	TC133031 (96%)	
+2.3	+1.8	+1.6	AW930004 (TC170778)	TC133948 (96%)	B carotene hydroxylase
–1.8	–1.8	–1.6	L23424 (TC183738)	TC147009 (97%)	Phytoene synthase
Group 7. Other StMtf1^{M}-induced genes					
+9.2	+4.6	+6.4	BG626036 (TC185107)	TC152789 (95%)	Glycosyl-transferring cellulose synthase
+4.0	+2.6	+2.3	AY240230 (TC169925)	TC136516 (96%)	GAGA transaminase
+2.1	+1.9	+2.4	AJ784587 (TC182658)	TC154115 (91%)	Acyltransferase
+2.5	+2.3	+1.6	BI203183 (TC177758)	TC132871 (97%)	Disulphide isomerase

*The chorismate mutase (*Cm*), prephenate dehydratase (*Pdh*) and phenylalanine ammonia lyase (*Pal*) genes are involved in the biosynthesis of all phenylpropanoids.

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Aardevo Exhibit 1010
Page 1151

F3′5′h gene and also displayed reduced *StMtf1*^{M} gene expression. These results confirm the efficacy of the silencing construct employed, and indicate that an accumulation of flavonoid intermediates may negatively regulate *StMtf1*^{M} transcription. It can be concluded that simultaneous activation of the flavonoid pathway and partial suppression of anthocyanin biosynthesis trigger the formation of high levels of KAR in potato. Furthermore, *F3′5′h* gene silencing was frequently linked to a more comprehensive inactivation of the entire flavonoid biosynthetic pathway.

Gene expression profile in variegated tubers

The ability of StMtf1^{M} to activate anthocyanin biosynthesis in the tuber phloem and to trigger a more comprehensive formation of caffeoylquinates throughout the tuber led us to study the genome-wide changes in transcript levels that were induced, either directly or indirectly, by this transcription factor. To this end, Affymetrix microarrays containing 11 probe sets for more than 9200 tomato genes were hybridized with biotin-labelled cRNA probes from tubers of line 663-30 and two Pokedot lines (663-30/1166-10 and 663-30/1166-16), as well as from pSIM401 control tubers. The high degree of sequence conservation between the corresponding genes of the closely related plant species tomato and potato, together with the ability to obtain multiple independent measurements for the abundance of each transcript, meant that the cross-species hybridization experiments were likely to provide accurate and reproducible results (for a review, see Bar-Or *et al.*, 2007). All hybridizations were performed in duplicate, using independently isolated RNAs, to generate two independent data sets for signal intensity ratios. Gene expression levels in transgenic plants were only considered to be changed if the associated fluorescent signals were significantly different from those of controls ($P < 0.05$) for both of the microarray comparisons (Table 2).

The microarray analyses demonstrated that *StMtf1*^{M} expression altered the expression levels of seven groups of genes. One of the genes of the first group was the strongly up-regulated tomato *Ant1* gene, which is highly homologous to the *StMtf1*^{M} gene from potato. Confirming the earlier semi-quantitative RT-PCR results shown in Figure 5e, transcript levels for this gene were found to be about eightfold higher in 663-30 tubers than in tubers of control plants. This increase was halved in tubers of the two retransformed Pokedot lines, providing further evidence for the partial feedback inhibition of *F3′5′h* gene silencing on *StMtf1*^{M} gene expression.

In addition to *StMtf1*^{M}, several other transcription factor genes were identified as being over-expressed in transgenic tubers. A novel MYB transcription factor gene encoding a protein with 55% identity to StMtf1^{M} was strongly induced in 663-30 tubers. Sevenfold increased transcript levels were also found for a gene encoding a transcription factor with a helix–loop–helix domain that shares 46% identity with the sweet potato (*Ipomoea batatas*) regulator of anthocyanin biosynthesis (Streisfeld and Rausher, 2007). Furthermore, a homologue of the bZIP transcription factor Bzi1, which binds to the promoters of phenylpropanoid pathway genes (Heinekamp *et al.*, 2002), was induced in both 663-30 and Pokedot tubers.

Four "group 2" genes with increased transcript levels in all transgenic tubers were involved in the biosynthesis of caffeoylquinates. Apart from the hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyl transferase (*Hct*) gene, which had already been shown by RNA gel blot analysis to be induced in 663-30 tubers, 1.5–2.0-fold increased signal intensities were determined for probes associated with the *Hqt* gene. The product of this gene catalyses the last step in CGA formation by coupling caffeoyl-CoA to quinic acid. Expression levels were also increased for the chorismate mutase (*Cm*) and prephenate dehydratase (*Pdh*) genes involved in the formation of phenylalanine, the important precursor for caffeoylquinates and other phenylpropanoids.

The third group of genes was associated with the biosynthesis of caffeoyl-CoA-derived compounds other than caffeoylquinates. An approximately fivefold increase in transcript levels for the gene encoding ferulate-5-hydroxylase (*F5h*), which catalyses the rate-limiting step in the biosynthesis of syringyl lignin, suggests a possible shift in lignin monomer ratios. Furthermore, strongly reduced transcript levels for the cinnamyl alcohol dehydrogenase (*Cad*) gene, involved in the final step in the formation of all three different lignin monomers, may have resulted in a decrease in the overall lignin content in transgenic tubers. An assessment of the content and composition of lignin in *StMtf1*^{M}-expressing tubers is beyond the scope of the current work, but should be carried out to confirm these preliminary hypotheses.

Putative flavonoid biosynthetic genes with altered expression levels represented a fourth group of StMtf1^{M}-affected genes. Genes belonging to this group encode flavanone-3-hydroxylase and 7-*O*-glucosyltransferase. Over-expression of the latter gene has recently been shown to increase the accumulation of CGA and kaempferol in transgenic potato (Aksamit-Stachurska *et al.*, 2008). Several *S*-adenosyl-L-methionine synthase (*Sams*) genes, which participate in the formation of methyl groups for the 3′-*O*-methylation of flavonoids (Hahlbrock and Scheel, 1989), were also induced in transgenic plants. Interestingly, the increased accumulation

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Aardevo Exhibit 1010
Page 1152

of flavonoids (and caffeoylquinates) was not associated with up-regulated expression levels for upstream genes encoding cinnamate 4-hydroxylase (C4h), 4-coumarate:CoA ligase (C4l) and chalcone synthase (Chs). Indeed, phenylalanine ammonia lyase (*Pal*) gene expression levels were suppressed two- to threefold.

Members of a fifth group of induced or suppressed genes were associated with hormone production and response. The brassinosteroid biosynthetic gene *Diminuto* was up-regulated in all transgenic tubers, whereas various auxin early response genes were only induced in tubers of the Pokedot lines. Because the encoded proteins suppress the activity of auxin response factors, their induction may affect the tuber's responsiveness to auxin (for a review, see Teale *et al.*, 2006). The positive correlation between increased flavonoid levels and auxin/indole-3-acetic acid (*Aux/Iaa*) gene expression levels is in agreement with earlier studies in *Pinus pinaster* (Charvet-Candela *et al.*, 2002).

Multiple genes involved in the assembly of the major light-harvesting chlorophyll-*a/b* complex were down-regulated in transgenic plants, and were classified as a sixth group of StMtf1^{M}-affected genes. The possible consequences of these changes are not expected to affect the physiology of dark-grown tubers. A number of 'other' genes induced by StMtf1^{M} are shown in Table 2 as group seven. One of these genes encodes a protein with weak homology to an anthocyanin 5-*O*-glucoside malonyltransferase (Suzuki *et al.*, 2004), and is only induced in Pokedot tubers.

The conclusions deduced from the microarray experiments were verified by real-time quantitative PCR for the *Pdh*, *Pal*, *Hqt*, putative flavanone-3-hydroxylase (*pF3H*), *Sams2* and chlorophyll *a/b*-binding protein CP29 (*CP29*) genes. Figure 7 shows that the differences in relative expression levels for these six genes in transgenic tubers vs. pSIM401 controls were similar to those of the transcriptome analysis. Together, our results demonstrate that *StMtf1^{M}* gene over-expression in potato tubers increases the expression of genes involved in the biosynthesis of important precursors of phenylpropanoids, such as the *Pdh* and *Sams* genes, and triggers the modified expression of various genes involved in caffeoylquinate, lignin and flavonoid biosynthesis. Furthermore, it alters the expression levels of genes involved in both auxin response and chlorophyll biosynthesis. These pleiotropic effects do not affect the agronomic characteristics of genetically engineered tubers.

Flavonoid content and taste in processed foods derived from the modified tubers

The biochemical studies described above were performed on raw potato tubers. However, tubers are generally heat-processed before consumption. Although cooking enhances flavour, taste and palatability, it may also degrade the phenolic antioxidants, possibly obliterating the engineered quality improvements. Little is known about the heat stability of compounds such as CGA and KAR, and the few published reports have produced contradictory conclusions. Therefore, potato tubers were boiled for 20 min, and their flavonoid contents were compared with those of half-boiled (10 min) and raw tubers. Figure 8a shows that prolonged incubation at 100 °C did not lower the content of any of the phenylpropanoids tested. The genetically modified tubers were also incubated at 177 °C for 15 and 30 min. The resulting partially baked potatoes contained only slightly reduced flavonoid levels, whereas reductions were limited to approximately 25% in the fully baked product (Figure 8b).

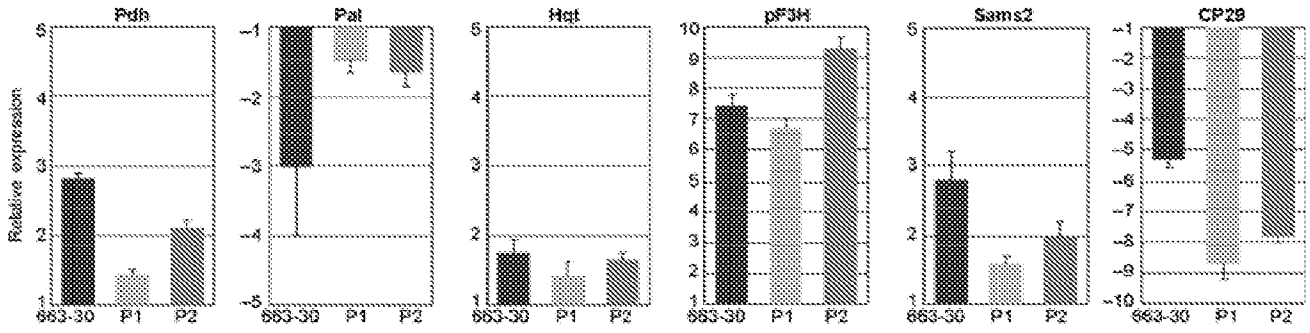

Figure 7 Confirmation of microarray results. Relative transcript levels for prephenate dehydratase (*Pdh*), phenylalanine ammonia lyase (*Pal*), hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase (*Hqt*), putative flavanone-3-hydroxylase (*pF3h*), *S*-adenosyl-L-methionine synthetase (*Sams2*) and chlorophyll *a/b*-binding protein CP29 (*CP29*) genes, as determined by quantitative polymerase chain reaction (PCR), in 663-30 tubers and the two lines 663-30/1166-10 (Pokedot-1, P1) and 663-30/1166-16 (Pokedot-2, P2), relative to control tubers. Values were normalized to actin, and the data shown are the mean values of three independent experiments.

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Aardevo Exhibit 1010
Page 1153

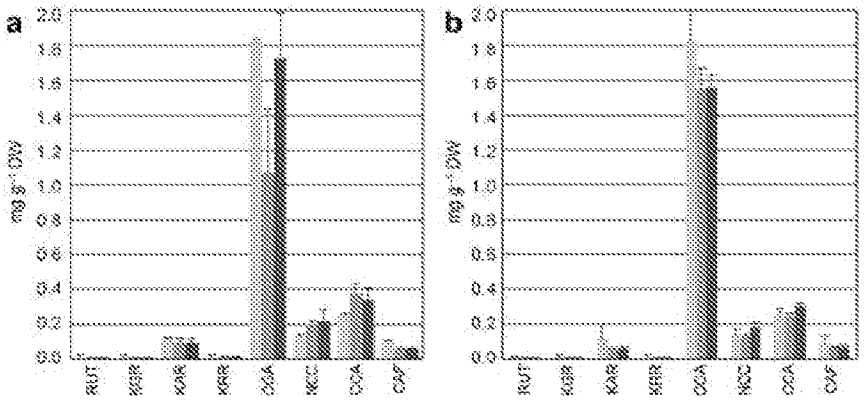

Figure 8 Evaluation of heat-processed tuber products. Abbreviations are defined in the legend of Figure 4. (a) Phenylpropanoid levels in raw (light grey bars), half-boiled (dark grey bars) and fully boiled (black bars) tubers. Results shown are the average of three measurements ± standard deviation. (b) Levels of phenolic compounds in raw (light grey bars), half-baked (dark grey bars) and fully baked (black bars) tubers.

Some reports have indicated that increased levels of CGA and other phenolic compounds may enhance the bitterness of processed food (Mondy *et al.*, 1971). This hypothesis was tested by evaluating the sensory characteristics of baked potatoes. A panel of eight food scientists assessed the food for various criteria, including aroma, taste, texture and mouth feel, and found no significant differences between Pokedot potatoes and their untransformed counterparts (data not shown). Collectively, it was concluded that high levels of phenolic antioxidants persist after cooking and do not appear to alter the taste or texture of processed products.

Discussion

Tissue specificity of StMtf1^{M}-mediated anthocyanin formation

One of the most interesting aspects of anthocyanin formation relates to the complex regulatory mechanisms that underlie its tissue specificity. Our study demonstrates that constitutive expression of the modified MYB transcription factor gene *StMtf1^{M}* results in the accumulation of anthocyanins in all tissues of potato, except for the interior of the tuber. Replacement of the strong constitutive Ubi7 promoter by the promoter of the *Gbss* gene, which is active in all tuber tissues except for the skin, consistently directs anthocyanin production towards the phloem and periderm. This new tissue specificity supports a role for sucrose as an anthocyanin signal, as both phloem and periderm contain much higher sucrose levels than the cortex and pith. Indeed, a similar link between sucrose signalling and pigment formation has been noted previously in studies of the *Arabidopsis* production of anthocyanin pigment 1/2 (*Pap1* and *Pap2*) genes (Teng *et al.*, 2005; Solfanelli *et al.*, 2006).

The failure to produce pigmented phloem or periderm cells in tubers of transgenic lines that constitutively express *StMtf1^{M}* suggests that epidermal anthocyanin biosynthesis inhibits the production of anthocyanins in other tuber cell types. There is a possible involvement of a mobile signal, produced in the epidermis and acting on anthocyanin regulators in the phloem and periderm. Alternatively, the tissue specificity of anthocyanin formation may be regulated by genes such as anthocyaninless2 (*Anl2*) and increased chalcone synthase expression 1 (*Icx1*), which have been shown to play an important role in the formation of anthocyanins in subepidermal and epidermal cells, respectively (Kubo *et al.*, 1999; Wade *et al.*, 2003). The activation of anthocyanin biosynthesis in StMtf1^{M} tubers resulted in increased expression levels of the 'upstream' phenylpropanoid biosynthetic genes *Cm* and *Pdh*, but also decreased expression levels of the *Pal* gene. Most probably, suppression of this gene is limited to tuber tissues that do not respond with pigment formation on expression of the *StMtf1^{M}* gene. Pigmented cells would be predicted to express the *Pal* gene at least at wild-type levels because the encoded protein represents the rate-limiting step in phenylpropanoid formation (Howles *et al.*, 1996). This hypothesis is currently being tested by performing transcriptome analyses of pigmented vs. unpigmented cells.

A second and much more thoroughly investigated level of anthocyanin regulation relates to transcriptional co-regulator genes encoding bHLH and WD-repeat proteins. The particular interactions between these proteins and their MYB protein counterparts determine whether transcriptional regulation of structural genes is positive or negative, and which flavonoids will be produced in specific organs and tissues. Each of the regulating genes involved may be induced by different environmental factors and in different tissue-specific manners. The transcriptional co-regulator of StMtf1^{M} has not yet been identified, but is likely to encode a bHLH protein (for a review, see Quattrocchio *et al.*, 2006). A candidate co-regulator is the gene corresponding to TC135093 (http://compbio.dfci.harvard.edu/tgi/plant.html), which is strongly induced in

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Aardevo Exhibit 1010
Page 1154

transgenic tubers expressing the *StMtf1*^{M} gene. It would be interesting to study the effect of TC135093 gene silencing in pSIM663 (StMtf1^{M}) plants.

In addition to the importance of signals and interacting transcription factors, it is also possible that differential activation of the anthocyanin pathway is controlled through the post-translational regulation of key proteins. For instance, our study demonstrated that the substitution of two amino acids at the N-terminus of StMtf1^{M} enhanced the activity of this protein in potato. Because the N-termini of MYB proteins are often highly divergent and rich in serine, it is possible that these proteins are differentially phosphorylated in a similar manner to that described for proteins such as phytochrome A (PhyA) (Kneissl *et al.*, 2008).

Potential relevance of Pokedot potatoes for consumption

Given the large amounts of potatoes that are consumed on a daily basis, estimated at 171 g per person, currently available processed potatoes already account for 15% of the CGA-type compounds that are ingested through the consumption of vegetables and fruits (Chun *et al.*, 2005; Mattila and Hellström, 2007) (Figure 9a). This percentage would nearly double on replacement of currently available commodity potatoes by varieties over-expressing *StMtf1*^{M}, because most

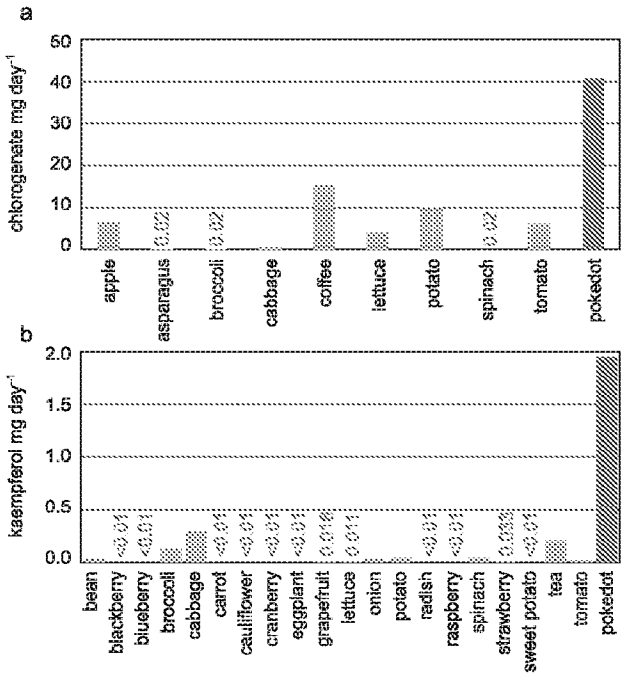

Figure 9 Potential impact of Pokedot potatoes on the estimated average daily intake of chlorogenic acid (a) and kaempferol (b).

or all of the CGA produced in potatoes is maintained on heat processing. Our study also demonstrated the heat stability of KAR, indicating that the consumption of processed Pokedot tubers would increase the intake levels of this compound even more substantially than those of CGA (Figure 9b).

In this study, it has been shown that the levels of phenolic antioxidants with health-promoting activities can be increased by simultaneously over-expressing the *StMtf1*^{M} gene and silencing the *F3′5′h* gene. This genetic modification does not alter tuber yields and has no effect on the sensory characteristics of processed food. However, microarray analyses have demonstrated that it results in altered expression levels of various groups of genes. The lower expression levels of the phytoene synthase-encoding carotenoid biosynthetic gene, together with increased expression levels of the metabolic carotene hydroxylase gene, suggest that Pokedot tubers may contain smaller amounts of carotenoids than tubers of the original Bintje variety. Given the abundance of carotenoids in frequently consumed foods, such as tomato, this possible disadvantage of reduced carotenoid content may be outweighed by the benefits associated with the strongly increased levels of kaempferol, a health-associated compound that is almost absent in the modern diet. Further tests need to be carried out to assess the commercial potential of Pokedot tubers. These tests will include, for instance, more rigorous field trials to confirm that the genetic modification does not negatively affect the plant's agronomic performance, analysis for new toxins or allergens, and a careful evaluation of nutritional composition.

Experimental procedures

Genes, constructs and sequence analysis

The primers used to amplify the potato *StMtf1* gene from potato DNA were 5′-ATGAACAGTTCACCTATGTCTTC and 5′-TTAATCAAGTAGATTCCATAAGTC. After confirming the integrity of the 5′ and 3′ terminal sequences by inverse PCR, *StMtf1* was deposited as GenBank accession number EU310399. The insertion of a *StMtf1* gene expression cassette into a pSIM401-derived binary vector (Rommens *et al.*, 2004) yielded pSIM646. Vector pSIM1166 was created by placing a silencing cassette designed to target the *F3′5′h* gene into a pCAMBIA1301-derived vector (AF234297). This silencing cassette contained two copies of the *F3′5′h* promoter (GenBank EU541414) as an inverted repeat between the two convergently orientated promoters from the potato *Ubi7* gene and the *35S* gene of cauliflower mosaic virus (p35S), respectively. On collision of transcription, non-processed and variably sized RNAs were produced that activated gene silencing at least as effectively as the double-stranded RNA produced with unidirectional gene silencing constructs (Yan *et al.*, 2006; Weeks *et al.*, 2007). Both the calculation of protein alignments and the visualization of cladograms were based on

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Aardevo Exhibit 1010
Page 1155

CLUSTALW (with penalties for opening and extending a gap set at 10.0 and 0.2, respectively).

Plant growth, transformation and genotyping

Stock plants were maintained in Magenta boxes containing 40 mL of half-strength M516 medium (PhytoTechnology, Shawnee Mission, KS, USA) with 3% sucrose and 2 g/L gelrite. Tobacco and potato were transformed as described previously (Rommens *et al.*, 2004). Isolated DNA was used as a template for amplification reactions with Eppendorf HotMaster Taq DNA Polymerase according to the manufacturer's recommendations (Brinkmann, Westbury, NY, USA). For mini-tuber production, plants were incubated in a growth chamber (Percival, Perry, IA, USA) under a 16-h photoperiod (300 μmol/m^2/s) at 25 °C for 1 month. Semi-mature tubers were obtained by growing plants in a temperature-controlled (18 °C minimum/27 °C maximum) and light-controlled (16-h photoperiod with an intensity of approximately 1500 μmol/m^2/s) glasshouse for 3 months. Field experiments were carried out by planting plantlets in the field in early June for tuber harvesting during early October.

Biochemical and histochemical analyses

Phenolic compounds were quantified by HPLC as described previously (Shakya and Navarre, 2006). Briefly, tubers were frozen in liquid nitrogen, freeze dried and samples were extracted with a 50% methanol buffer. LC-MS analyses of the extracts were conducted using an 1100 HPLC system (Agilent, Santa Clara, CA, USA) equipped with a quaternary pump, refrigerated autosampler and column heater, and employing a diode array detector (DAD) and MS ion trap detection. Typically, 5 μL of sample was injected on to an Onyx column (Phenomenex, Torrance, CA, USA) at a flow rate of 1 mL/min and column temperature of 35 °C. For MS analysis, ESI in negative mode was used. The source was operated using 350 °C drying gas (N_2) at 12 L/min, 379 000 Si unit Pa nebulizer gas (N_2) and the source voltage with a scan range of 50–1000 *m/z*. Data analysis was performed using Agilent Chemstation software and ACD/MS Manager software (Advanced Chemistry Development Inc., Toronto, ON, Canada). Compound identification was based on UV and mass spectrometric data and, in most cases, comparison with authentic standards, including tryptophan, phenylalanine, tyrosine, solanine, chaconine, CGA and caffeic acid. Flavonol identifications were based primarily on MS fragmentation with MS^2 and MS^3 analysis, using the two flavonol glucosides, quercetin-glu and RUT, together with kaempferol, naringenin, myricetin, quercetin and eriodictyol, as standards. Most potato flavonols are conjugated to sugars, and flavonols were quantified as RUT equivalents. The external standard method of calibration was used, and the identifications for flavonols for which standards were not available should be considered as tentative. Anthocyanin pigments were extracted from ground tubers with 2 mL of 2 M HCl for 30 min at 100 °C. After centrifugation for 2 min at 13 000 ***g***, the supernatant was mixed with 100 μL isoamylalcohol. Products in the organic phase were resolved by thin layer chromatography (TLC) using Silica Gel G plates (Sigma-Aldrich, St Louis, MO, USA) and a mobile phase comprising water (30%), formic acid (90%) and HCl (10%). Quantification was achieved by recording the absorbance at 520 nm. β-Glucuronidase activity was localized by incubating tuber segments in a solution containing 5-bromo-4-chloro-3-indoyl-β-D-glucuronide (X-gluc) (0.3% w/v), potassium ferricyanide (5 mM), potassium ferrocyanide (5 mM), sodium phosphate buffer (0.1 M; pH 7.0) and Triton X-100 (0.5% v/v) (Jefferson *et al.*, 1987).

RNA analyses

RNA was isolated from tubers using an RNAeasy Plant Mini Kit (No. 74904, Qiagen, Valencia, CA, USA) following the manufacturer's recommendations. cDNA was synthesized from 0.5 μg of total RNA in a 20-μL volume using an oligo dT primer and SuperscriptII RT (Invitrogen, Carlsbad, CA, USA) at 42 °C in 1 h. One microlitre of resulting cDNA was used as template in the following PCR: 25-μL volume containing 20 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 0.2 μM of each primer and 1.5 U Taq DNA Polymerase (Invitrogen). The cycling protocol was as follows: 3 min at 95 °C, followed by 33 cycles of 20 s at 95 °C, 20 s at 57 °C and 80 s at 72 °C, with a final step of 2 min at 72 °C. Nine microlitres of the amplification products were analysed by electrophoresis and visualized by ethidium bromide on a 1% agarose gel. Semi-quantitative PCR for the *F3′5′h* gene was carried out with 200 ng of RNA using the one-step RT-PCR kit from Qiagen (catalogue number 210212), according to the manufacturer's recommendations. Intron-spanning primers were 5′-CTTGTTCCTG-CTAGAGCTGGTCTGA and 5′-ACCAGCAATAAGTGACCATCTGTTG for *StMtf1*^{M} (31 cycles), 5′-AGTGGTCGTACCACCGGTATTGTG and 5′-ATGATCAGTGAGGTCACGACCTGC for *F3′5′h* (42 cycles), and 5′-AGTGGTCGTACCACCGGTATTGTG and 5′-ATGATCAGTGAG-GTCACGACCTGC for the internal control *actin* (35 cycles). Quantitative PCRs were performed using a TaqMan probe containing a reporter and quencher dye, designed to anneal between the forward and reverse PCR pair priming sites. During PCR, the probe anneals to the specific sequence location, and is then cleaved by the 5′–3′ exonuclease activity of the Taq DNA Polymerase. On cleavage, the reporter dye is displaced and its fluorescence is detected by an ABI Prism Sequence Detector 7000 (Applied Biosystems, Framingham, MA, USA). The resulting fluorescence emission data were collected and measured with each cycle. Primers for quantitative PCR were as follows: 5′-ACATAGTCGGCGAAGTCCAACT and 5′-CGAACGCCTGGAA-GAGCTAA for the *Pdh* gene; 5′-CACGTTATCCGATGGTCTCTCA and 5′-GGCCACGGGCGATGT for *Hqt*; 5′-CTGGAGTTTTTGGCAATGGAA and 5′-TGCCCTTGTTGCTGAATGTG for *Pal*; 5′-TTCACGAACCTC-CATTGGTA and 5′-GATTCTCCGGACCAACAAGT for *pF3H*; 5′-CAGGGTCCTTGTGAACATTG and 5′-GCATAGCCAAACATGTGACC for *Sams2*; 5′-TTCCCGGAGACTACGGTTAC and 5′-AAGCATAGC-CCATCTAGCGT for *Cp29*; and 5′-TTCAGGCTGTACTCTCACTGTATGC and 5′-CACCATCACCAGAGTCCAACAC for *actin*. The *Hqt*-derived probe for RNA gel blot analysis was obtained from reverse transcribed RNA with the primers 5′-GGGATGAACAAGGTAGAATTGAAG and 5′-GTGATCAGGATCTAGACAAACAGC.

For cross-species microarray hybridizations, GeneChip® Tomato Arrays (Affymetrix, Santa Clara, CA, USA) were used, each of which contained 11 probe sets for each of 10 038 probe sets to interrogate 9254 tomato transcripts (http://www.affymetrix.com/). Expression analyses were carried out according to the manufacturer's recommendations, and data were interpreted using Affymetrix's GeneChip® Operating Software User's Guide. Basically, signal intensities were calculated for each of the probe pairs of a probe set using Tukey's one-step biweight estimate, which yields a weighted mean that is

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Aardevo Exhibit 1010
Page 1156

relatively insensitive to outliers. Probe set intensities on the experimental array were then compared with their counterparts on the baseline array, and a 'change *P* value' was calculated using Wilcoxon's signed rank test to distinguish between an increase, decrease or no change in gene expression. The 'fs by taking a mean of the probe pair intensities across the two arrays.

Estimated average daily intake levels of CGA and kaempferol

Average dietary intake levels (mg/day) were calculated by multiplying the estimated content of CGA or kaempferol in a food (mg/g) by the estimated intake level of that food (mg/day) (Podsedek *et al*., 2000; Sakakibara *et al*., 2003; Chun *et al*., 2005; Farah and Donangelo, 2006). The content of CGA and kaempferol in processed potato products was estimated on the basis of the assumption that 85% and 75% of these phytochemicals, respectively, survive heat processing.

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Page 1157

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Page 1159

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A QTL for broad-spectrum resistance to cyst nematode species (*Globodera* spp.) maps to a resistance gene cluster in potato

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Abstract Broad-spectrum resistance in potato to the potato cyst nematode (PCN) species *Globodera rostochiensis* and *G. pallida* is commonly regarded as a polygenically inherited trait. Yet, by use of QTL analysis and a selected set of PCN populations, resistance to both PCN species could be ascribed to the action of locus *Grp1*. *Grp1* confers major resistance to *G. rostochiensis* line Ro_5-22 and *G. pallida* population Pa_2-D383 and partial resistance to *G. pallida* population Pa_3-Rookmaker. *Grp1* was mapped on chromosome 5 using previously characterized AFLP markers. Cleaved amplified polymorphic sequence (CAPS) markers available for RFLP loci GP21 and GP179 revealed that *Grp1* maps on a genomic region harboring other resistance factors to viral, fungal and nematodal pathogens. The present data indicate that *Grp1* is a compound locus which contains multiple genes involved in PCN resistance.

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Introduction

Characterization of complex genetic factors has been greatly facilitated by recent advances in DNA marker technology. Polymerase chain reaction (PCR)-based markers like amplified fragment length polymorphisms (AFLP™, Vos et al. 1995) are efficient tools to generate dense linkage maps. With these linkage maps, genomic regions containing quantitative trait loci (QTL) can be identified and accurately analyzed with the aid of statistical procedures which are applicable through the use of publicly available computer packages (Lander and Botstein 1989; Lincoln et al. 1992; Van Ooijen and Maliepaard 1996a,b). The ability to trace Mendelian loci of quantitatively inherited characters through their association with DNA markers has been used to study both complex agronomic traits like fruit mass, contents of soluble solids and fruit pH in tomato (Paterson et al. 1988), and polygenic disease resistances to, for example, bacterial wilt in tomato (Danesh et al. 1994), potato late blight (Leonards-Schippers et al. 1994) and rice blast (Wang et al. 1994).

The present study focuses on resistance to potato cyst nematodes (PCN: *Globodera rostochiensis* and *G. pallida*) which are severe pests in potato (*Solanum tuberosum* ssp. *tuberosum*). Several sources of monogenic resistance are available to potato breeders, either to *G. rostochiensis* [among others, genes *H1* (Huijsman 1955), *Gro1* (Barone et al. 1990) and *GroV1* (Jacobs et al. 1996)] or to *G. pallida* [*Gpa* (Kreike et al. 1994) and *Gpa2* (Rouppe van der Voort et al. 1997b)]. Interestingly, these resistance genes are clustered with loci involved in resistance to other plant pathogens (summarized in Leister et al. 1996). Quantitatively inherited resistance to *G. pallida* has also been found (e.g.

Goffard and Ross 1954; Ross 1986; Dale and Phillips 1982; Mugniéry et al. 1989). However, the aforementioned sources of resistance are only effective against a limited number of PCN populations.

To obtain broad-spectrum resistance, breeders have accumulated an unknown number of genes in breeding lines, which are derived from a range of wild *Solanum* species (Dellaert and Vinke 1987). Although these breeding lines confer complete resistance against a large number of PCN populations, the identification of resistant offspring clones is obscured by a continuous distribution in resistance levels; no distinct classes of resistant and susceptible clones are observed. This phenomenon is typical for various sources of PCN resistance. In addition, assessment of the level of quantitatively inherited resistance against PCN is complicated by the variation in multiplication rates of the nematodes in different tests. It has been shown that variation between tests is largely due to genotype-environment interactions (Mugniéry et al. 1989). Environmental factors, such as temperature and moisture, affect the infection capabilities and the sex ratio of the nematode (Mugniéry and Fayet 1984). Another difficulty in breeding for resistance against PCN is the choice of a proper test population. As with many other soil-borne pathogens, PCN have mosaic distribution patterns, and even in a small area populations may have distinct genetic structures (Folkertsma 1997). The current international pathotype scheme (Kort et al. 1977) reflects only a limited part of this variation (Bakker et al. 1993). To obtain insight in the genetic diversity of potato cyst nematodes, Folkertsma (1997), used molecular techniques to analyze more than 300 populations sampled all over Europe. At present, a diverse set of representatives of the PCN metapopulation is available to evaluate the resistance spectra of potato clones.

In this paper, we report on the genetic analysis of broad-spectrum resistance against PCN. Following screening with diverse representants of the PCN metapopulation, we selected the tetraploid breeding line AM78-3778 as it confers broad-spectrum resistance and subjected it to a detailed genetic study. QTL analysis revealed one major locus which is part of a resistance gene cluster on chromosome 5. Contrary to the spectra of previously identified PCN resistance genes, this locus confers resistance to both PCN species.

Materials and methods

Plant material

The broadness of PCN resistance was tested in potato clones AM78-3778, 3778-14 and 3778-16. The tetraploid clone AM78-3778 contains resistance to both PCN species and is an interspecific hybrid between *S. tuberosum* and several wild potato species including *S. vernei* 24/20, *S. vernei* ssp. *ballsii* 2/1, *S. vernei* LGU 8, *S. oplocense* EBS 1786 and *S. tuberosum* ssp. *andigena* CPC 1673. Clones 3778-14 and 3778-16 are dihaploids ($2n = 2x = 24$) produced by prickle pollination of clone AM78-3778 with haploid inducer *S. phureja* clones (Hutten et al. 1994).

A mapping population of diploid potato was obtained from a cross between the clones 3778-16 × RH89-039-16. Clone 3778-16 is the resistant parent in our population and is referred to as AM. The susceptible male parent, clone RH89-039-16, will be referred to as RH. The mapping population F_1AM × RH consisted of 122 vigorous F_1 genotypes. Leaf material for DNA isolation was collected in the greenhouse at the seedling stage. Tubers for nematode tests were produced by first-year clones in the field.

Nematodes

The nematode populations used in this study are listed in Table 1. The code of the population is preceded by its pathotype designation. *G. rostochiensis* lines Ro_1-19 and Ro_5-22 are heterogeneous populations except that they are fixed for the alleles at the avirulence and virulence locus to the *H1* resistance gene, respectively. Lines Ro_1-19 and Ro_5-22 were selected from controlled single nematode matings as described (Janssen et al. 1990). Pathotype Ro_1 belongs to the most avirulent and Ro_5 to the most virulent category of *G. rostochiensis* populations (Kort et al. 1977). The *G. pallida* populations were originally sampled from heavily infested spots in a field. Population Pa_2-D383 is a relatively avirulent *G. pallida* population. Population Rookmaker, termed Pa_3-Rook, is one of the most virulent *G. pallida* populations found in the Netherlands and is currently used as test-inoculum for *G. pallida* resistance in several commercial breeding programs. The virulence characteristics as well as the molecular data of these *G. pallida* populations are extensively described in Bakker et al. (1992). Nematode populations were multiplied on susceptible cv 'Eigenheimer', inoculated with approximately 200 cysts and placed in a growth chamber at 18°C and 16-h daylength. PCN populations were stored at −80°C until use (Folkertsma et al. 1997).

Resistance testing and data collection

Preparation of the PCN inoculum was as described by Rouppe van der Voort et al. (1997b). The resistance spectrum assay was carried out in a closed container (Phillips et al. 1980) using 125-cc plastic containers filled with silversand. Per container, one tuber was added

Table 1 Broadness of resistance in AM78-3778-derived clones. The average numbers of cysts recovered in a closed container test are presented (*n.d.* not determined)

Potato clone	Ro1-19	Ro5-22	Pa2-D383	Pa2-D350	Pa2-HPL1	Pa3-1097	Pa3-Rook	Pa3-74.768.20
AM78-3778	2	10	0	0	2	2	2	4
3778-16	2	7	0	0	1	4	4	13
3778-14	n.d.	n.d.	1	9	7	135	108	73
RH89-039-16	189	98	101	132	146	240	153	166

Aardevo Exhibit 1010
Page 1161

and inoculated with nematodes to a final density of 5 eggs/J_2 per gram soil. The containers were maintained in the dark at approximately 20°C for at least 3 months.

The inheritance of the resistance to populations Ro_5-22, Pa_2-D383 and Pa_3-Rook was analyzed in mapping population F_1AMRH in three replications. Resistant standards were cv 'Multa' (resistant to Pa_2-D383), *S. vernei* hybrid cv 'Santé' (resistant to Pa_2-D383 and Ro_5-22) and AM78-3778 (resistant to Pa_2-D383, Pa_3-Rook and Ro_5-22). As susceptible standards cv 'Eigenheimer' and cv 'Maritta' were used. The tubers were inoculated with nematodes (final density of 5 eggs/J_2 per gram soil) in 900-g pots containing a mixture of silversand and a sandy loam fertilized with Osmocote (N-P-K granulates). Plants were arranged in a randomized block design and grown in a greenhouse with 15°C and 25°C as minimum and maximum temperatures, respectively.

After 3 months, cysts were recovered from the soil by elutriation and counted. In addition, the size of the root systems was classified on a scale of 0 to 3. Resistance data of a genotype were only recorded when at least 3 well-rooted plants of this genotype were available.

DNA marker analysis and linkage map construction

DNA isolation, AFLP analysis and data recording were done as described previously (Vos et al. 1995; Van Eck et al. 1995). From the segregation of 408 AFLP markers, generated by use of 11 primer combinations, separate genetic maps of the parental clones were constructed (Rouppe van der Voort et al. 1997a). The separate maternal AM and paternal RH maps consisted of 242 and 220 AFLP markers, respectively. These maps were aligned with the genetic map of potato by means of common AFLP markers which have been mapped relative to restriction fragment length polymorphism (RFLP) markers in a reference population (Van Eck et al. 1995). Common AFLP markers were visually recognized on autoradiogram images as co-migrating bands in fingerprints generated from different genotypes using the primer combinations E + AAA/M + ACG, E + AAC/M + CAC, E + AAC/M + CAG, E + ACA/M + CGT, E + AGA/M + CAT and E + ATG/M + CTA. Genetics maps are available from URL: http://www.spg.wau.nl/pv/aflp/catalog.htm.

The PCR primer sequences and the temperature cycle files of the CAPS markers (cleaved amplified polymorphic sequences; Konieczny and Ausubel 1993) for loci GP21 and GP179 were obtained from Meksem et al. (1995). Segregating AM alleles were detected after digestion of the amplification products using the restriction endonucleases *Dra*I for marker GP21 and *Rsa*I for marker GP179. The marker order for CAPS markers GP21 and GP179 was calculated using the software package JOINMAP 1.4 (Stam 1993). The primer sequences for locus CP113; CP113-5′1, CP113-3′1 and CP113-3′3* were obtained from Niewöhner et al. (1995). The following temperature cycle files were applied for these markers: CP113-5′1/CP113-3′1: 3 min at 93°C, followed by 35 cycles of 30 s at 93°C, 45 s at 60°C, 90 s at 72°C and finished by a 10 min-elongation at 72°C; CP113-5′1/CP113-3′3*: 3 min at 93°C, followed by 5 cycles of 30 s at 93°C, 45 s at 50°C and 90 s at 72°C, after which the annealing temperature was decreased to 48°C. This file was also completed by an elongation step at 72°C for 10 min.

Statistical analysis

Analysis of variance components was carried out on $^{10}\log(x + 1)$ transformed average cyst counts per plant genotype according to the following model:

$$\sigma^2_{tot} = \sigma^2_{plant} + \sigma^2_{rep}$$

where σ^2_{tot} is the phenotypic variance, σ^2_{plant} is the genetic variance among the plant genotypes and σ^2_{rep} is the environmental variance among the replications.

The broad-sense heritability was calculated according to the formulas:

$$\sigma^2_{gen} = (MS_{tot} - MS_{rep})/3$$

$$h^2 = \sigma^2_{gen}/\sigma^2_{gen} + \sigma^2_{rep}$$

where σ^2_{gen} is the genetic variance among the genotypes.

The data on marker segregation of both parents were included for QTL analysis using the program MAPQTL 3.0 (Van Ooijen and Maliepaard 1996a,b). The markers were transferred into a biallelic code according to the manual. Three different mapping methods were applied for QTL detection. The first method was a non-parametric rank-sum test of Kruskal-Wallis (see e.g. Sokal and Rohlf 1995) in which the non-transformed, average cyst counts were analyzed. A threshold value of $P < 0.0001$ was used for the individual marker tests. In the second option of MAPQTL, interval mapping for cross-pollinating species (CP) was applied. The likelihood that a QTL is present between two flanking marker loci is indicated by the LOD score (Lander and Botstein 1989). A LOD value of 3.0 was chosen as threshold value (Lander and Botstein 1989). As a third method, MQM mapping (Jansen 1993; Jansen et al. 1995) was applied. The QTL with the largest effect on the trait examined was used as covariate to enhance the power in the detection of putative other QTLs. The magnitude of the marker-associated phenotypic effect is presented by the coefficient of determination (R^2), which describes the percentage of the total variance explained for by the marker genotypes in the interval mapping procedure.

Results

Broadness of PCN resistance from AM78-3778

The broadness of PCN resistance present in clone AM78-3778 was assessed relative to the resistance present in the dihaploids 3778-14 and 3778-16 (AM). AM78-3778 as well as clone AM appeared to be resistant to all of the PCN populations tested (Table 1). Loss of resistance to the Pa_3 populations was observed in clone 3778-14. The diploid clone RH89-039-16 (RH) was susceptible to all of the populations tested.

Inheritance of the resistance

The average numbers of cysts developed on the parental genotypes are shown in Table 2. Analysis of variance on normalized cyst counts revealed that the genetic variance for both *G. pallida* and *G. rostochiensis* resistance was significant ($P < 0.0001$). No significant differences in cyst numbers were found among the blocks of replicates. The broad-sense heritabilities, listed in Table 2, indicate that the variation in cyst counts was barely affected by environmental factors and root-system development. It has been noted that the values for skewness and kurtosis sometimes deviate from the test criteria on normally distributed $^{10}\log(x + 1)$ transformed cyst numbers (Snedecor and Cochran 1967). The analyses on these data may therefore be slightly biased.

Figure 1A shows that in the progeny, the resistance to population Pa_2-D383 was correlated with resistance

Table 2 Results of the quantitative analysis of PCN resistance as measured by the number of cysts counted in the respective PCN populations. Skewness and kurtosis of $^{10}\log(x + 1)$ transformed cyst numbers, heritability of the specific resistance, the map location of the QTLs with their nearest marker, the *P* value of the nearest marker in a Kruskal-Wallis test, the LOD score and the R^2 at the QTL position are given

Trait	Average no. of cysts AM	Average no. of cysts RH	Skewness	Kurtosis	h^{2a}	Marker[b]	R^{2c}	*P* value	LOD
Pa2-D383	5	662	−0.45	−0.87	0.86	GP179	66%	< 0.0001	16.8
Pa3-Rook	30	1067	−0.70	0.19	0.83	GP179	45%	< 0.0001	11.7
Ro5-22	5	3961	−0.41	−1.32	0.83	GP21	77%	< 0.0001	17.9
Root system	–	–	–	–	0.086	–	–	–	–

[a] h^2 = Heritability
[b] Marker nearest to QTL
[c] R^2 = Percentage of the total variance explained by the marker genotypes

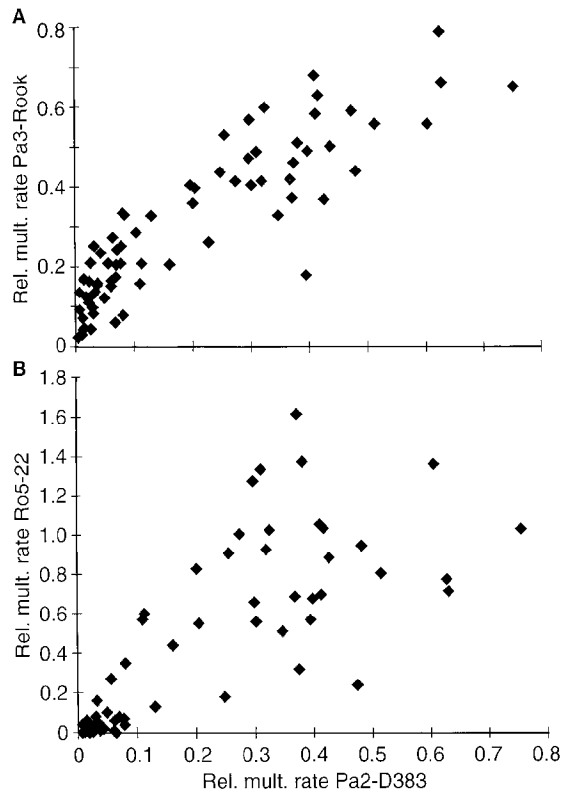

Fig. 1A, B Comparison of relative multiplication rates (rel. mult. rates) of different PCN inocula on F_1 genotypes. The relative multiplication rates on F_1 genotypes are expressed by the number of newly developed cysts divided by the number of cysts developed on the susceptible parent RH. **A** Comparison of rel. mult. rates on F_1 genotypes between populations Pa_2-D383 and Pa_3-Rook, **B** Comparison of rel. mult. rates between populations Pa_2-D383 and Ro_5-22

to population Pa_3-Rook. A decrease in the relative multiplication rate of population Pa_2-D383 on a F_1 genotype is associated with a decrease in the relative multiplication rate of population Pa_3-Rook. For example, the set of F_1 genotypes for which relative multiplication rates between 0 and 0.08 (actual cyst numbers between 0 and 56) for population Pa_2-D383 were found showed relative multiplication rates between 0 and 0.2 (cyst numbers between 0 and 140) for population Pa_3-Rook. Similarly, it is shown that the resistance to *G. pallida* Pa_2-D383 was correlated with resistance to *G. rostochiensis* line Ro_5-22 (Fig. 1B).

AFLP markers

By scoring resistance to Pa_2-D383 as a monogenic trait using the arbitrary criterion of genotypes containing fewer than 56 cysts as being resistant and genotypes containing more than 56 as being susceptible, we observed linkage (at LOD > 3.0) with 6 AFLP markers localized on the map of genotype AM. These AFLP markers reside on chromosome 5, as determined by their linkage with previously mapped common AFLP markers. Common AFLP markers can be visually recognized as co-migrating bands in fingerprints of potato genotypes which have also been analyzed with chromosome-specific RFLP markers (Rouppe van der Voort et al. 1997a). Pa_2-D383 resistance could not be precisely mapped on chromosome 5, apparently because the variance was too high to be explained by a monogenic analysis. A more profound analysis of the data was therefore performed by a QTL approach (see below). In addition, resistance to both Pa_3-Rook and Ro_5-22 could only be mapped on chromosome 5 by means of Pa_2-D383 resistance as the bridging marker. Nevertheless, monogenic analysis of the datasets indicates the presence of genetic factor(s) allocated on chromosome 5. A detailed description of the AFLP markers, used for alignment of potato maps are given at URL: http://www.spg.wau.nl/pv/aflp/catalog.htm.

CAPS markers

CAPS markers were tested to identify additional markers on both arms of chromosome 5. The loci GP21

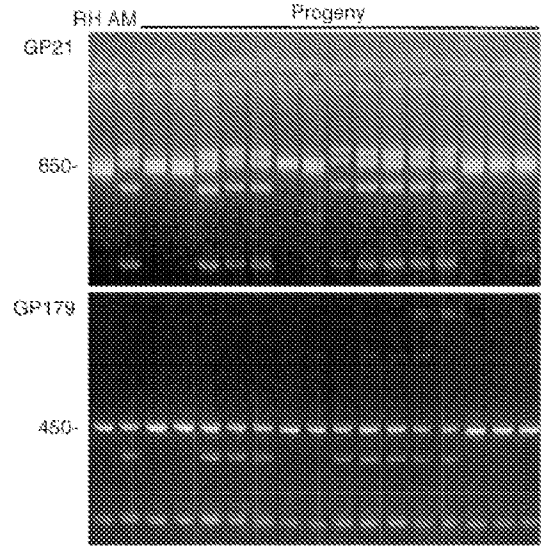

Fig. 2 CAPS analysis of RFLP markers GP21 and GP179. Linkage of both CAPS markers is shown by the profiles generated from the susceptible parent RH, the resistant parent AM and a subset of their progeny. The segregating AM alleles are linked in coupling phase with an allele conferring PCN resistance. Molecular weights of the DNA fragments are given on the *left*

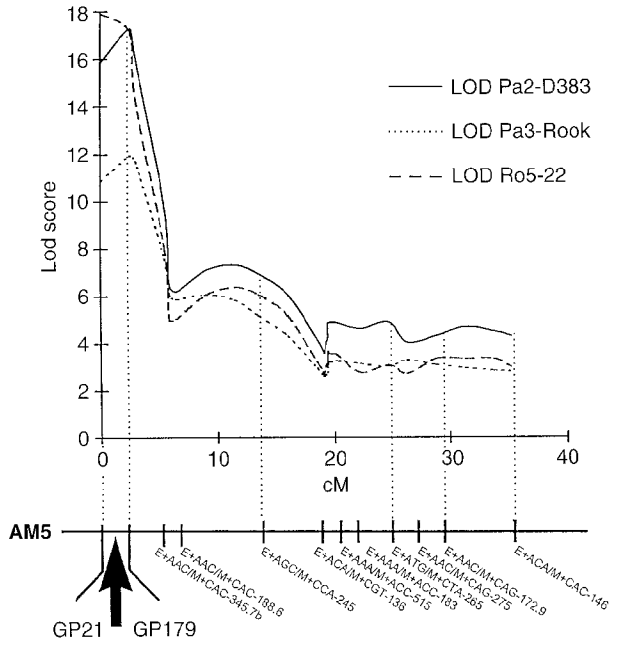

Fig. 3 LOD plot for PCN resistance on chromosome 5 of genotype AM. The position of the QTL on the map is indicated by an *arrow*

and GP179 (Gebhardt et al. 1991; Meksem et al. 1995) were detected after digestion of the amplification products using restriction endonucleases *Dra*I for marker GP21 and *Rsa*I for marker GP179 (Fig. 2). In agreement with the *S. tuberosum* map of Gebhardt et al. (1991), GP21 and GP179 mapped on chromosome 5 at 3 cM from each other. Unfortunately, locus CP113 could not be mapped as a CAPS marker in population F_1AM × RH. Both primer combinations (Niewöhner et al. 1995) produced a monomorphic amplification product even after digestion using a series of 12 four basepairs recognizing restriction endonucleases.

QTL mapping

The computer program MAPQTL (Van Ooijen and Maliepaard 1996a,b) was used to analyze both the resistant and the susceptible parental dataset. The Kruskal-Wallis test revealed significant associations between resistance against the *G. pallida* and *G. rostochiensis* populations and chromosome-5 markers segregating from clone AM. The highest significance levels were found at markers GP21 and GP179 for the three inoculum treatments (Table 2). Further on this chromosome, a second gradient in the Kruskal-Wallis test statistic was observed, presumably due to missing values for markers mapped on this region.

The LOD profiles of the interval mapping are presented in Figure 3. This figure shows high LOD values for markers GP21 and GP179 (3 cM), indicating a large effect on resistance for the three PCN populations tested (see also Table 2). From the interval mapping, it was unclear whether the high LOD values for other chromosome-5 intervals were the result of their linkage with the GP21-GP179 interval or a second QTL. However, the MQM mapping method revealed no additional statistically significant effects for PCN resistance at other genomic intervals.

Discussion

The results of the QTL mapping show that a locus with large effects on the resistance to both PCN species is localized on the genomic region possessing loci GP21 and GP179. This locus confers major resistance to *G. rostochiensis* line Ro_5-22 and *G. pallida* population Pa_2-D383 as well as partial resistance to *G. pallida* population Pa_3-Rookmaker. On the potato map, the GP21-GP179 region is known to contain a cluster of resistance genes encoding specificities to many different plant pathogens, for example, to the fungus *Phytophthora infestans*, (gene *R*1 and a major QTL; (Leonards-Schippers et al. 1992, 1994)), to potato virus X (extreme resistance *Rx*2, Ritter et al. 1991; hypersensitive resistance *Nb*, De Jong et al. 1997) as well as to *G. pallida* (locus *Gpa*, Kreike et al. 1994). Although mechanistically considered to be a different class of resistance, a QTL involved in trichome-mediated insect resistance (Bonierbale et al. 1994) resides also in this region.

The presence of both quantitative and qualitative resistance on the same chromosome region opens the possibility that resistance genes and the genes underlying QTLs are alleles of the same genetic locus. This assumption fits with the hypothesis that qualitative phenotypes are extreme, mutated allelic variants at a quantitative trait locus (Robertson 1985). Moreover, major resistance (*R*) genes cloned from several plant species share striking structural similarities despite their intimate interaction with a diversity of pathogen species. These major genes seem to be members of large multigene families that are arranged in large arrays of complex, evolutionary related but different loci having different specificities (reviewed in Baker et al. 1997).

In this context it seems likely that the QTL for PCN resistance mapped in this study is a compound locus containing different but related *R* genes for PCN resistance. The diploid clone AM used to map PCN resistance is derived from AM78-3778, a tetraploid clone which combines the resistance introgressed from many separate wild *Solanum* sources (Dellaert and Vinke 1987). As neither of these wild species contain resistance to both *G. rostochiensis* and *G. pallida*, the underlying genetic model for the locus identified in AM78-3778 probably includes more than one gene. Since these genes have been separately introgressed, the in-coupling linkage phase of these genes should be explained by the many generations involved in breeding clone AM78-3778, which may have resulted in fortuitous recombination events producing multiple *R* genes on the same homologous chromosome. We propose to name the PCN resistance locus *Grp1* (for *G. rostochiensis* and *G. pallida* resistance) until future research will enable us to ascribe the resistance of *Grp1* to the action of different genes.

The resistance to Pa_3-Rook seems to involve additional loci given the fact that 7 offspring clones harbor resistance levels comparable to that of parent AM. In addition, *R* genes which confer complete resistance at the level of the individual nematode may have the appearance of quantitatively inherited resistance genes when heterogeneous pathogen populations are used in the resistance test. The occurrence of the latter genetic model is not inconceivable given that resistance tests rely predominantly on screening with field populations. These field populations are often not homogeneous for virulence traits but are mixtures of virulent and avirulent genotypes. In case a PCN population is not homogeneous for the virulence trait examined, a single *R* gene operating on the basis of a classical gene-for-gene relationship will confer partial resistance against the population as a whole, whereas at the level of the individual the *R* gene will confer absolute resistance against the matching avirulent genotype. Formal proof for a gene-for-gene relationship has so far only been obtained for the interaction between *G. rostochiensis* and the *H1* gene from *S. tuberosum* ssp. *andigena* CPC1673 (Janssen et al. 1991), but it is likely that various other PCN resistance genes act in a similar way. It is therefore hypothesized that for complete resistance to Pa_3-Rook as observed in AM78-3778 only a few loci are involved. A precedent consistent with this hypothesis is that in the dihaploid genotype 3778-14, Pa_3 resistance was lost whereas the Pa_2 resistance was maintained. Thus, complete resistance can be obtained by accumulating *R* genes with specificities which match with the different avirulent genotypes in a heterogeneous field population.

The question of whether the resistance to Pa_2-D383 and Pa_3-Rook is mediated by the same *Grp1* allele remains unanswered by the results of this study. No recombination was found between Pa_2-D383 and Pa_3-Rook resistance among the three GP21/GP179 recombinant genotypes identified in population F_1AM × RH. Kreike et al. (1994) found recombination between Pa_2 and Pa_3 resistance as well as between markers in the same genomic interval. This indicates that *Gpa* may indeed be a compound locus. Partial overlap has been found in the resistance spectra of *Grp1* and *Gpa*; *Grp1* confers resistance to populations of both PCN species and only partial resistance to Pa_3-Rook, whereas *Gpa* confers resistance to all *G. pallida* populations tested so far but not to *G. rostochiensis* (Kreike et al. 1994; P. Wolters, personal communication). Therefore, *Grp1* should be considered as being different from *Gpa* while possibly having allele(s) in common with it.

In this report it is shown that by testing with a diverse set of representants of the PCN metapopulation, broad-spectrum PCN resistance in AM78-3778 can be determined by the action of different genes at a compound locus. The *Grp1* locus harbors resistance to both PCN species. Although it confers incomplete resistance to *G. pallida*, it is expected that complete resistance can be achieved by the introgression of *R* genes with complementary specificities. Current breeding strategies for PCN resistance rely on trial and error approaches. However, the availability of a representative set of pathotype populations and the development of MAS assays will allow a more directed approach towards achievement of complete PCN resistance in commercial cultivars by combining genes with complementary specificities.

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Development of a PCR-based selection assay for root-knot nematode resistance (*Rmc1*) by a comparative analysis of the *Solanum bulbocastanum* and *S. tuberosum* genome

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Abstract

A PCR-based assay has been developed for marker assisted selection of root-knot nematode resistance (*Rmc1*) in potato. To this end, a comparative genome analysis was carried out between *Solanum bulbocastanum* and *S. tuberosum* to identify PCR-based chromosome *11* alleles linked to *Rmc1*. The use of co-migrating AFLP markers, obtained by using primer combinations previously applied for AFLP analysis of the *S. tuberosum* genome, failed to align the AFLP map of the *S. bulbocastanum* genome with the *S. tuberosum* map. Apparently, the *S. bulbocastanum* genome is genetically too distantly related to the *S. tuberosum* genome for this type of analysis. Cleaved amplified polymorphic sequence (CAPS) markers were more readily applied for a comparative analysis within the region of interest. *Rmc1* could be localized within a 4 cM interval between markers CT182 and M39b. It is demonstrated that the resistance spectrum of *Rmc1* includes not only *Meloidogyne chitwoodi* and the related species *M. fallax* but also a genetically distinct population of *M. hapla*. The cost-efficiency of the CAPS markers applied for *Rmc1* renders this approach as an attractive alternative for screening large segregating populations of potato for root-knot nematode resistance.

Introduction

Molecular markers are of basic importance for linkage mapping of monogenic and polygenic traits and to clone genes on the basis of their map position (Tanksley et al., 1995). In addition, high resolution markers are a means to develop marker assisted selection (MAS) assays which allow efficient introgression and selection of individuals with specific characteristics (Tanksley et al., 1989).

Currently, the combined restriction fragment length polymorphism (RFLP) map of the potato and tomato genome comprises more than 1000 markers (Gebhardt et al., 1991; Tanksley et al., 1992). However, the development of MAS assays for tomato and potato lags behind the flurry of gene mapping studies which emerge from these high density maps. The principal reason for this is the technical complexity of RFLPs. For development of a MAS assay, RFLP markers should therefore be converted into a PCR-based marker and adapted for the genotypes under investigation. As some traits have a diverse genetic origin it remains obscure whether a marker which is diagnostic in a particular genomic background will be applicable in another background (Niewöhner et al., 1995). Moreover, a linkage map should be dense enough to provide either flanking markers for the selective trait or a marker close enough to permit its use in a reliable MAS assay.

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Aardevo Exhibit 1010
Page 1168

The development of high-density linkage maps has been facilitated by recent developments in PCR-based marker techniques. Among these, AFLP (Vos et al., 1995) is a most informative DNA fingerprinting assay. Applying this technique for potato resulted in a number of genetic maps which comprise of over 700 markers (Rouppe van der Voort et al., 1997a). These markers were mapped relative to 200 RFLP loci thereby providing a means for map alignment on the basis of shared (orthologous) AFLP alleles. In addition, a marker density can be increased in the region of interest by application of several powerful marker methods. One of these methods is bulked segregant analysis (BSA) which relies on the pooling of DNA's of genotypes having a particular phenotype in common and the subsequent identification of polymorphisms linked to the trait (Michelmore et al., 1991). A second DNA marker application is based on comparative genome analysis. Comparative genome analysis is a strategy that utilizes mapping information from one species to analyse a related species. For comparative mapping, the locus-specificity of RFLPs is particularly useful. By use of RFLPs, genetic maps have been aligned even across taxa (Ahn & Tanksley, 1993; Bonierbale et al., 1988; Brown et al., 1996; Kurata et al., 1994; Lagercrantz et al., 1996; Moore et al., 1995; Whitkus et al., 1992). However, the effectiveness of RFLPs for comparative mapping once converted into a PCR-based marker needs to be established.

This paper describes the development of a MAS assay by comparative mapping for root-knot nematode resistance in potato. Root-knot nematodes, *Meloidogyne* spp. are a major threat to potato growing due to their world-wide occurrence and their wide host range (*Meloidogyne* infestations are difficult to control by means of crop rotation). In temperate climates, the most harmful *Meloidogyne* species for potato are *M. chitwoodi* Golden et al. and *M. hapla* Chitwood. Recently, a deviant type of *M. chitwoodi* has been found which has been described as the new species *M. fallax* (Karssen, 1996). Only resistance to *M. chitwoodi* has been mapped on the potato genome. The *Rmc1* locus originates from the wild species *Solanum bulbocastanum* and is localized on chromosome *11* (Brown et al., 1996).

In the present study, two PCR-based approaches were followed to identify chromosome *11* specific *S. bulbocastanum* alleles closely linked to *Rmc1*. One strategy was based on the application of AFLP primer combinations in *S. bulbocastanum* which have previously been used to localize AFLP markers on the *S. tuberosum* map. This strategy has been proven to be an efficient means for mapping studies within *S. tuberosum* (Rouppe van der Voort et al., 1997b). As a second strategy, chromosome *11* specific CAPS primer sequences were applied, originally developed for fine mapping the R_{ysto} locus from *S. stoloniferum* conferring resistance to potato virus Y (Brigneti et al., 1997). Data are provided on the effectiveness of both marker systems for comparative mapping. In addition, the value of the MAS assay in breeding for *Meloidogyne* resistance in potato is discussed.

Materials and methods

Plant material

From a resistance screening trial (Janssen et al., 1996), three wild clones of the diploid species *S. bulbocastanum* have been selected for this study (Table 1). The resistant clone 93–D26–3 was derived from a selfing of accession PI275187 known to harbour the *Rmc1* resistance locus (Brown et al., 1996). Clone 93-D26–3 was crossed with the susceptible clones 93–57–15 and 93–60–10. Subsequently, two resistant genotypes were selected from the progeny of population M94–106 and (back)crossed to either 93–57–15 or 93–60–10 (Table 2). The progeny of the cross M94–106–2 × 93–60–10 (population M95–227), consisting of 67 genotypes, was used for further mapping studies on *Rmc1*.

BC_3 populations, generated by three times repeated backcrossing of a somatic *S. bulbocastanum*/*S. tuberosum* hybrid with *S. tuberosum,* were kindly provided by Dr. Brown, Prosser WA, USA. The pedigree of the BC_2 from which the clones BC_2-398.89 and BC_2-398.94 have been selected, has been described elsewhere (Brown et al., 1996).

Nematode resistance tests

Seedlings of hybrid and backcross populations of *S. bulbocastanum* were tested in a glasshouse for resistance to *M. chitwoodi*, population CHE and to *M. fallax*, population FB (Janssen et al., 1997b). Seeds were sown in a mixture of potting soil/silver sand and transplanted into square plastic tubes of 240 ml, filled with moist silver sand and NPK fertilizer. After three weeks, plants were inoculated with 400 juveniles of either *M. chitwoodi* or *M. fallax*. Plants were harvested eight weeks after inoculation and numbers of egg masses on roots were counted after staining with

Aardevo Exhibit 1010
Page 1169

Table 1. Origin of *S. bulbocastanum* genotypes used for intraspecific crosses and their resistance or susceptibility to *M. chitwoodi*, *M. fallax* and *M. hapla*. Levels of resistance are based on multiple resistance tests (Janssen et al., 1997a)

Genotype	Origin[a]	Synonym	*M. chitwoodi*	*M. fallax*	*M. hapla* (HSMB/HRO)
93–57–15	BGRC 8009	PI 275199	susceptible	susceptible	susceptible
93–60–10	BGRC 8006	PI 275194	susceptible	susceptible	susceptible
93-D26–3	BGRC 8002	PI 275187	resistant	resistant	resistant/susceptible
M94–106–2	93D26–3 × 93–57–15	–	resistant	resistant	resistant/susceptible
M94–106–4	93D26–3 × 93–57–15	–	resistant	resistant	resistant/susceptible

[a] Kindly obtained from the Dutch-German potato gene bank, Wageningen, the Netherlands. Originally identical accessions are present at the potato gene bank, Sturgeon Bay, Wisconsin, USA (PI numbers).

Phloxine B. For the genetic analysis, plants having no or one egg mass were regarded as resistant and those having more than one egg mass as susceptible (Janssen et al., 1997a).

Genotypes of the BC_3 populations were tested for resistance to *M. chitwoodi* CHE, to *M. fallax* FB and to *M. hapla*, populations HSMB and HRO (Janssen et al., 1997b) in 4 replications per nematode population. Genotypes were multiplied *in vitro* and plantlets were transplanted in 350 ml stone pots filled with moist silver sand. The experiment was further carried out as described for the seedling test. Data on numbers of egg masses were normalized by square root transformation and subjected to a *t*-test and ANOVA. Data analysis was based on numbers higher than one, since low numbers did not contribute to the overall variance.

DNA analysis

DNA isolation was performed on frozen leaf tissue (Van der Beek et al., 1992) or tubers. The AFLP procedure was essentially as described by Vos et al. (1995). Template DNA was prepared using the restriction enzymes *Eco*RI and *Mse*I and pre-amplified using the 'EcoRI-primer' E+0 and the 'MseI-primer' M+0 (Vos et al., 1995). For selective amplification the following combinations of primers were used: E+AAA/M+ACG, E+AAC/M+CAC, E+AAC/M+CAG, E+ATG/M+CTA, E+AGA/M+CAT. AFLP data were recorded as described (Van Eck et al., 1995). AFLP markers were designated by the primer combinations used, followed by the approximate sizes of the amplification products expressed in nucleotides. Comparisons of AFLP fingerprint images generated from genotype M94–102–2 and from diploid *S. tuberosum* genotypes are accessible via URL: http://www.spg.wau.nl/pv/aflp/catalog.htm.

Bulked segregant analysis was performed by constructing pools of AFLP template DNA from eight resistant and eight susceptible plants. Two separate resistant and two separate susceptible pools were analyzed and compared with the AFLP profiles from the parental clones.

CAPS markers were obtained from Brigneti et al. (1997) and applied on 100 ng of DNA using PCR temperature cycle files given in Table 4. Molecular weights were determined from a ϕx174/*Hinc*II digest (Sphaero Q, The Netherlands).

Mapping

Only the markers segregating from the female parent M94–106–2 were scored; consequently the *S. bulbocastanum* map was based on the recombination events in the female meiosis. The map was constructed using the computer program JoinMap 1.4 (Stam, 1993) and drawn using the program DrawMap (Van Ooijen, 1994).

Results

Effectiveness of Rmc1 *resistance*

The origin and the resistance levels of the parental clones of the *S. bulbocastanum* crosses are presented in Table 1. From the crosses with resistant clone 93-D26–3, most seedlings were found to be completely resistant to *M. chitwoodi* and *M. fallax*. The progeny from two resistant F_1 plants which were crossed with a susceptible clone, showed a segregation into a resistant and susceptible class of plants which fitted a 1:1 distribution. No reciprocal differences or differences between *M. chitwoodi* and *M. fallax* were observed (Table 2). The observed segregation patterns confirmed the origin of *Rmc1* being derived

Aardevo Exhibit 1010
Page 1170

Table 2. Segregation ratios of *M. chitwoodi* and *M. fallax* resistance in populations of *S. bulbocastanum*. Ratio R:S: segregation ratio resistant vs. susceptible

Population	Cross	Expected ratio R:S	*M. chitwoodi* ratio R:S	χ^2-prob	*M. fallax* ratio R:S	χ^2-prob
M94–106	93-D26–3 × 93–57–15	1:0	54: 2	–	54: 0	–
M94–105	93–57–15 × 93-D26–3	1:0	30: 0	–	31: 1	–
M94–81	93-D26–3 × 93–60–10	1:0	32: 1	–	54: 0	–
M94–150	93–60–10 × 93-D26–3	1:0	33: 1	–	54: 0	–
M95–224	M94–106–4 × 93–60–10	1:1	29:34	0.53	24:29	0.49
M95–225	M94–106–4 × 93–57–15	1:1	28:18	0.14	19:23	0.54
M95–226	M94–106–2 × 93–57–15	1:1	12:21	0.12	24:18	0.35
M95–227	M94–106–2 × 93–60–10	1:1	32:21	0.13	31:30	0.90

Table 3. Square root transformed numbers of egg masses of *M. chitwoodi* (CHE), *M. fallax* (FB) and *M. hapla* (HSMB and HRO) recovered from BC_3 genotypes generated by backcrossing a *S. bulbocastanum/S. tuberosum* hybrid with *S. tuberosum* (average of four replicates)

Nematode population	BC_3 genotypes analyzed									
	1	2	3	4	5	6	7	8	9	10
	BC_2-398.89 × A84118–3									
CHE	9.5	0.0	0.0	10.3	0.0	0.0	0.0	9.3	8.9	10.7
FB	9.5	0.0	0.0	9.9	0.0	0.0	0.0	9.5	9.1	10.1
HSMB	5.5	0.4	0.9	7.9	0.7	0.3	0.3	6.0	6.0	8.5
HRO	6.2	8.4	7.6	8.6	6.7	6.2	7.5	7.2	6.2	6.7
	BC_2-398.94 × Lemhi Russet									
CHE	0.3	9.2	0.0	9.6	0.0	0.0	6.1	8.6	7.6	9.6
FB	0.0	8.3	0.0	10.6	0.4	0.0	6.7	7.3	6.3	9.0
HSMB	0.9	6.9	0.9	6.5	0.9	0.4	5.6	8.5	5.9	7.4
HRO	8.0	7.3	6.8	6.0	7.5	7.2	5.4	6.7	5.4	6.4
	BC_2-398.89 × Ranger Russet									
CHE	0.0	9.5	0.0	0.0	0.3	0.0	10.5	10.1	0.0	
FB	0.0	9.4	0.0	0.0	0.0	0.0	10.2	8.4	0.0	
HSMB	0.9	9.8	0.7	0.6	0.3	0.9	8.5	7.8	1.2	
HRO	8.0	8.9	8.4	6.5	7.0	6.4	9.6	8.3	7.4	
LSD ($p < 0.05$) = 3.2										

from PI275187 (Brown et al., 1989), which would be present in a homozygous configuration in 93-D26–3. Population M95–227 was further analyzed.

The BC_3 genotypes segregated for resistance to *M. chitwoodi*, *M. fallax* and population HSMB of *M. hapla* (Table 3). A moderate to high level of resistance was expressed in the case of the resistant genotypes and no interactions were observed between these nematode populations. The resistance was not effective to population HRO of *M. hapla*.

Comparative genome analysis by common AFLP alleles

For identification of markers linked to *Rmc1*, five AFLP primer combinations were applied to analyze population M95–227. The AFLP primer combinations used have previously been applied to construct a dense AFLP map of *S. tuberosum*. Our aim was to identify *Rmc1* linked markers have previously been identified in *S. tuberosum* (accessible via http://www.spg.wau.nl/pv/aflp/catalog.htm) by identi-

Aardevo Exhibit 1010
Page 1171

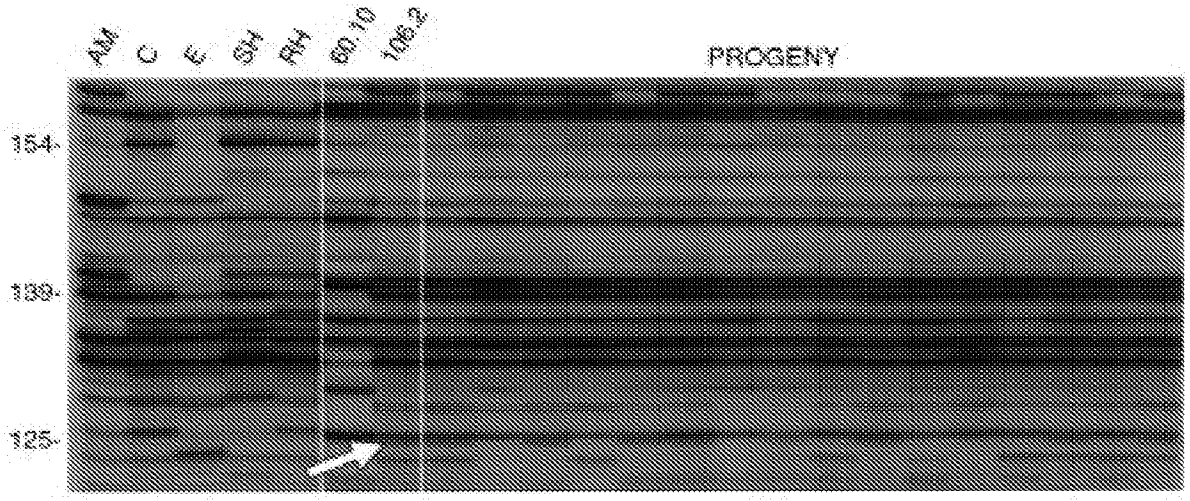

Figure 1. Part of an autoradiogram showing AFLP markers generated from the *S. bulbocastanum* genotypes M94 106 2 and 93 60 10 (designated by 106.2 and 60.10 respectively), and a series of offspring genotypes (designated by 'R' or 'S' indicating the resistant or susceptible phenotype respectively). On the left, five *S. tuberosum* reference genotypes 'AM', 'C', 'E', 'SH' and 'RH' (Rouppe van der Voort et al., 1997b). Marker E+AGA/M+CAT–125 is indicated by an arrow. The mobility of the markers indicated at the left was deduced by loading the profiles of the *S. tuberosum* genotypes next to a molecular weight marker.

fication of common AFLP markers for chromosome *11*. Common AFLP markers are generated by use of the same primer combination, have the same electrophoretic mobility and target the same genomic region on the maps of different genotypes. It is noted that for a proper identification of the genomic region by alignment with the potato map at least three co-migrating AFLP markers are required (Rouppe van der Voort et al., 1997a).

Large differences between AFLP fingerprints from *S. bulbocastanum* and those from *S. tuberosum* genotypes were observed (e.g., Figure 1). By use of five AFLP primer combinations, only 24% of the AFLP fragments of clone M94–106–2 had the same electrophoretic mobility as those of *S. tuberosum*. In comparison, the similarity among the five reference *S. tuberosum* genotypes was on average 58%. Thirty-four percent of the AFLP fragments identified segregated from clone M94–106–2 which indicates a lower level of heterozygosity as observed for *S. tuberosum* genotypes (Gebhardt et al., 1989; Rouppe van der Voort et al., 1997a).

Besides scoring of markers which have previously been mapped on chromosome *11* of *S. tuberosum*, all additional markers were scored and mapped in an attempt to investigate the genome synteny between *S. bulbocastanum* and *S. tuberosum*. For genotype M94–106–2, 119 segregating AFLP markers were identified of which 24 co-migrated with markers which were previously mapped in *S. tuberosum*. The M94–106–2 map consisted of 13 linkage groups with a total map length of 582 cM. The lengths of the linkage groups varied from 6 to 100 cM. However, no linkage groups were identified on which three or more shared AFLP alleles are localized which derive from the same chromosome. The *S. bulbocastanum* AFLP map could therefore not be aligned with the *S. tuberosum* map. Only marker, E+AGA/M+CAT-125 (Figure 1), was found to be linked with *Rmc1* at 7 cM distance to *Rmc1* (Figure 2). Details on the AFLP map of *S. bulbocastanum* are available upon request.

Marker E+AAC/M+CGA-170, linked to *Rmc1*, was found by bulked segregant analysis. This marker was identified after testing a total number of 25 primer combinations, thereby screening a total of about 2000 AFLP fragments for linkage with *Rmc1*. Marker E+AAC/M+CGA-170 maps 5 cM distant from *Rmc1* (Figure 2). Segregation analysis of the other four E+AAC/M+CGA markers segregating from M94–106–2 revealed two additional markers on chromosome *11*.

Comparative genome analysis by use of CAPS markers

As an alternative approach to identify PCR based markers in the *Rmc1* region, CAPS primer sequences

Aardevo Exhibit 1010
Page 1172

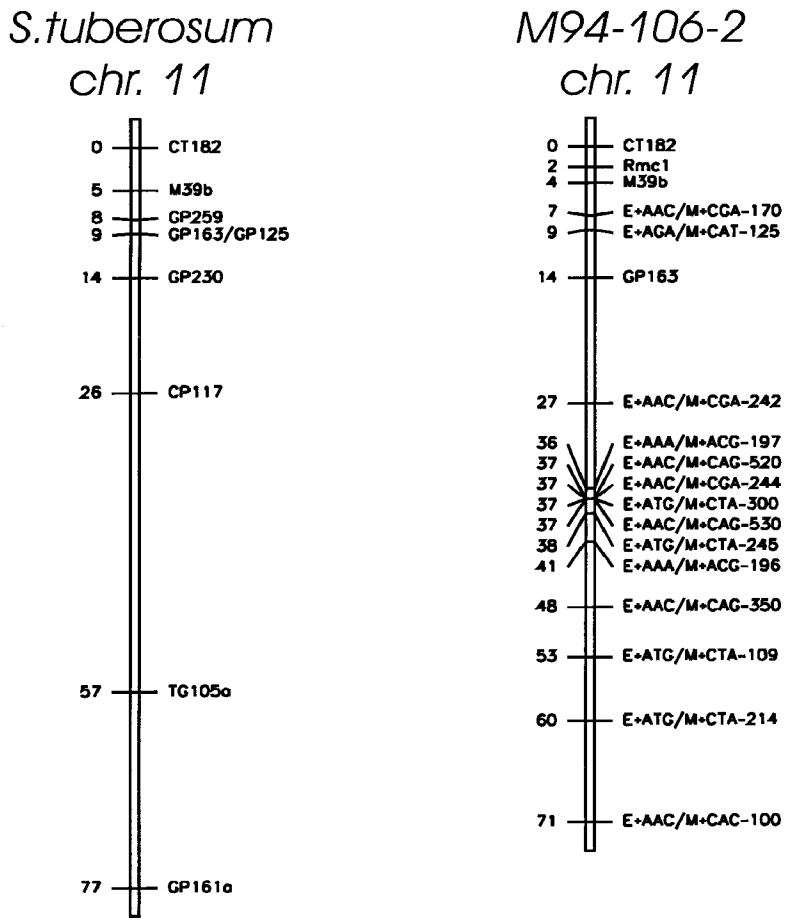

Figure 2. Position of the *Rmc1* locus on the linkage map of chromosome *11* of *S. bulbocastanum* clone M94–106–2 relative to the genetic map of *S. tuberosum* chromosome *11*. The map distances of the *S. tuberosum* map were derived from the potato map published by Gebhardt et al. (1991). The relative positions of the marker loci M39b and GP259 on the *S. tuberosum* map were deduced from Brigneti et al. (1997).

were applied to identify marker loci on the *S. bulbocastanum* map previously shown to be linked to the PVY resistance locus $R_y sto$ on chromosome *11* of *S. stoloniferum* (Brigneti et al., 1997). A considerable lack of polymorphisms in the region between markers CT182 and GP163 was observed in genotype M94–106–2; out of ten markers tested within this region only CT182, M39b and GP163 segregated by use of one restriction enzyme out of fifteen tested (Table 4). With these markers *Rmc1* was more precisely mapped in a 4 cM interval between markers M39b and CT182 (Figure 2). It is noted, that markers M39b and CT182 are the most distal markers at one end of chromosome *11* of potato (Tanksley et al., 1992, Brigneti et al., 1997).

The CAPS primer sequences could be more readily applied in a BC_3 of *S. bulbocastanum* with *S. tuberosum* as the recurrent parent. Diagnostic alleles in coupling phase with *Rmc1* were identified for markers CT182, M39b, GP259 (Figure 3) and GP163. Marker GP259 could not be mapped on chromosome *11* of *S. bulbocastanum* but was expected to be linked with *Rmc1* given its map position in the M39b-GP163 interval as determined by Brigneti et al. (1997). No recombinants were observed for the four markers among the 29 BC_3 clones tested. These CAPS markers seem thus be appropriate to follow the inheritance of *Rmc1* in a *S. tuberosum* background.

Aardevo Exhibit 1010
Page 1173

Table 4. Analysis of CAPS primers as putative linked markers to *Rmc1* in an intraspecific *S. bulbocastanum* population as well as in interspecific BC_3 populations of *S. bulbocastanum* backcrossed with *S. tuberosum*

Marker locus	Tm, $t_{extension}$	PCR result	Nature of polymorphism	
			S. bulbocastanum	BC_3 populations
CT182	54 °C, 30″	1 band of 500 bp in repulsion with *Rmc1*	allele specific	allele specific
M33	61 °C, 45″	1 band of 250 bp	not informative	not informative
M39b	50 °C, 45″	1 band of 1000 bp	*Rsa*I[a] site	*Dde*I site
GP259	50 °C, 45″	1 band of 750 bp	not informative	*Rsa*I, *Dde*I sites
GP163	55 °C, 30″	1 band of 600 bp	*Hha*I[b] site	*Hha*I[c] site

[a] Band in repulsion phase with *Rmc1*.
[b] Band in coupling phase with *Rmc1*.
[c] Polymorphic band is close to non-polymorphic band.

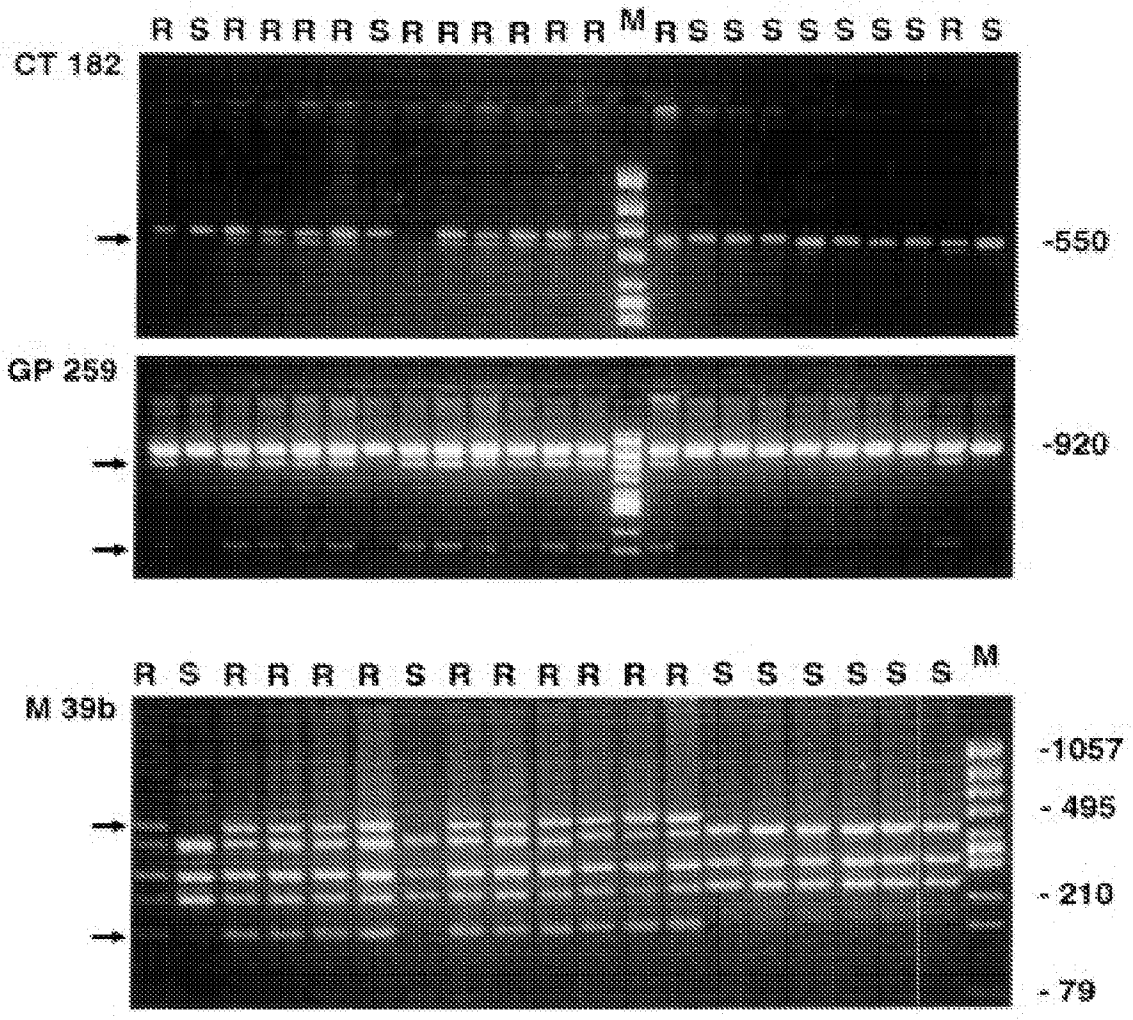

Figure 3. Images of CAPS profiles of markers CT182, GP259 and M39b on a BC_3 progeny of *S. bulbocastanum* having *S. tuberosum* as the recurrent parent. Molecular weights are indicated at the right.

Aardevo Exhibit 1010
Page 1174

Discussion

The resistance based on *Rmc1* appeared to be highly effective against *M. chitwoodi* and the supposedly closely related species *M. fallax*. However, the resistance showed a clearly different response to the two populations of *M. hapla* tested. *M. hapla* has a large genepool and has been found in temperate regions all over the world (Eisenback & Triantaphyllou, 1991). Also in the case of other South- and Central American *Solanum* sources of resistance, differential responses to Dutch populations of *M. hapla* have been observed (Janssen et al., 1997b). In addition, populations of *M. chitwoodi* able to overcome the *Rmc1* resistance from *S. bulbocastanum* have been identified (Mojtahedi & Santo, 1994). The specific interactions between *Rmc1* and *M. chitwoodi* at the one hand and *M. hapla* populations at the other hand is remarkable since the first and latter species may have diverged millions of years ago and species boundaries are evident (Esbenshade & Triantaphyllou, 1987). This indicates a multiple specificity for *Meloidogyne* resistance at the *Rmc1* locus. Multiple specificities at resistance loci have been found in *Arabidopsis thaliana* (Grant et al., 1995) in tomato (Kaloshian et al., 1995) and potato (Rouppe van der Voort et al., 1998).

Rmc1 could not be targeted on the *S. bulbocastanum* genome by means of common AFLP markers. The comparative mapping of *S. bulbocastanum* and *S. tuberosum* genome was obstructed by the dissimilarity in AFLP fingerprints of *S. bulbocastanum* as compared to the fingerprints obtained from the *S. tuberosum* genotypes. Among the sparse number of co-migrating bands, the common *S. tuberosum* markers which were localized on similar chromosome regions (Rouppe van der Voort et al., 1997a), appeared to be unlinked at the *S. bulbocastanum* genome. The differences in AFLP profiles between *S. bulbocastanum* and *S. tuberosum* seem to reflect a relatively high level of sequence divergence between the two species. This is corroborated by the taxonomic classification of both species which considers the diploid Central American species *S. bulbocastanum* (series *Bulbocastana*) as being genetically distinct from the more advanced South American species *S. tuberosum* (series *Tuberosa*) (Hawkes, 1990). Some loss of genome synteny has been observed between the *S. bulbocastanum* and the *S. tuberosum* genome (Brown et al., 1996). However, as stated by Brown et al. (1996) as well as by the linkage of CAPS markers CT182, M39b, GP259 and GP163 to *Rmc1*, the genetic similarity between *S. bulbocastanum* and *S. tuberosum* is sufficient for interspecific comparative mapping using CAPS markers.

The CAPS markers applied bestowed more flexibility to align genetic maps of different species. The difference between both marker types for interspecific comparative mapping probably reflects the difference in the ability of the two methods to detect divergent alleles. Within the same stretch of DNA, the AFLP assay will detect more polymorphic sites since the number of informative nucleotides is about threefold higher as compared to the CAPS assay. In addition, CAPS markers are based on detection of homologous sites in the genome instead of a perfect match which is the case with AFLP markers. Prior to the digestion of the amplified product, nucleotide differences may be tolerated in the PCR step of the CAPS assay since longer primers and less stringent temperature profiles are applied as compared to the AFLP protocol.

MAS on the basis of markers CT182, M39b and GP259 may be a valuable tool for introgressing *Rmc1* into cultivated potato. From an economical point of view, a molecular test for *Rmc1* resistance seems favorable since the estimated costs for a CAPS test are estimated to be four to eight times cheaper as compared to a nematode resistance test. Additional advantages of a molecular assay are speed, accuracy, and the ability to perform a selection in the seedling stage before a tedious resistance test will be performed. The diagnostic value of the detection of the CAPS allele(s) in potato genotypes depends on 1) the level of recombination between *Rmc1* and the marker loci and 2) the origin of the potato genotypes analyzed. Previously, the development of a PCR assay for the *H1* resistance gene in potato revealed a poor predictiveness of this assay in existing cultivars (Niewöhner et al., 1995). This can be explained by the loss of linkage of the markers with the resistance during the relatively long breeding history of *H1* in potato (Ross, 1986; Niewöhner et al., 1995). The use of both CAPS markers in concert with breeding material descending from the BC_3 progeny having genotype PI275187 in its pedigree seems therefore most favorable.

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Aardevo Exhibit 1010
Page 1175

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Aardevo Exhibit 1010
Page 1176

Aardevo Exhibit 1010
Page 1177

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Two additive QTLs conferring broad-spectrum resistance in potato to *Globodera pallida* are localized on resistance gene clusters

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Abstract Broad-spectrum resistance in potato to the potato cyst nematode (PCN) is commonly regarded as a complex inherited trait. Yet, in this paper we show that, by use of a selected set of PCN test populations, broad-spectrum resistance to the species *Globodera pallida* can be fully ascribed to the action of two loci: *Gpa5* and *Gpa6*. These loci were readily mapped by means of a strategy based on two steps. Firstly, the chromosomal localization of both loci was assessed by use of an online catalogue of AFLP markers covering a substantial part of the potato genome (http://www.spg.wau.nl/pv/aflp/catalog.htm). Subsequently the chromosomal regions of both loci were identified by means of CAPS markers based on RFLP insert sequences. Locus *Gpa5* explains at least 61% of the genetic variation. This locus maps to chromosome *5* on a region which has previously been shown to harbor resistance factors to viral (*Nb, Rx2*), fungal (*R1*) and nematodal (*Gpa, Grp1*) pathogens. The *Gpa6* locus exhibits a minor effect on the resistance (24%) and acts additively to *Gpa5*. Interestingly, the *Gpa6* locus maps to a region on chromosome *9* where, in the homoeologous tomato genome, the virus resistance gene *Sw-5* resides as part of a resistance gene cluster. In potato, resistance to potato virus X has been reported in the vicinity of this region. The map location of *Gpa6* indicates the presence of a resistance gene cluster at the end of the long arm of chromosome *9* of potato.

Key words AFLP · CAPS · *G. rostochiensis* · *Solanum tuberosum* · QTL mapping

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Introduction

Quantitative resistance calls up the image of complex, polygenic inheritance patterns with multiple genes having small additive effects on the resistant phenotypes. According to Van der Plank (1982) these minor genes may be equally effective to different pathogen isolates (pathotypes) and may be relatively durable in the field. However, quantitative resistance based on several minor genes is less desirable for breeding purposes given its complexity to introgress this type of resistance in existing cultivars. Breeders rely therefore on major genes for resistance. Monogenic resistance is usually referred to as resistance which complies with a gene-for-gene interaction. It contrasts to quantitative resistance by its pathotype specificity; for each major gene in the plant host there exists an avirulence gene in the pathogen (Flor 1956, 1971). Major resistance (*R*-) genes are often clustered in the genome and molecular analyses have shown that the gene-for-gene model holds true for several plant-pathogen systems (reviewed in Hammond-Kosack and Jones 1997). In these systems, the cloned *R*-genes are thought to encode receptor molecules which perceive a pathogen (avirulence) signal, whereupon a resistance response is triggered (Baker et al. 1997). As a result of the strong selective force of monogenic field resistance, the site of recognition in the pathogen may be altered in a short time span. Qualitative resistance is therefore considered to be less-durable than quantitative resistance.

The image of multiple minor genes underlying quantitative resistance is inconsistent with the present outcomes of most quantitative trait locus (QTL) mapping studies in different plant-pathogen combinations. Although some examples exist where quantitative resistance is explained by more than five QTLs (Geiger and Heun 1989; Bubeck et al. 1993; Leonards-Schippers et al. 1994; Wang et al. 1994; Qi et al. 1998) it is more common to find two to five loci (summarized in Young et al. 1996) underlying complex disease resistance. In these cases, quantitative resistance is polygenic in the sense that a few genes have large effects on the phenotype. Evidence is accumulating that a wide variety of molecular and genetic mechanisms may underly quantitative resistance. For example, insect resistance in potato is mediated by the glandular secretions of two types of leaf trichromes. QTLs associated with the resistance are co-localized with loci linked with phenol oxidase, trichrome density and sucrose ester production (Bonierbale et al. 1994). The expression of a resistance phenotype may also be developmentally regulated; e.g. while a single gene was found to control resistance to the spot blotch pathogen in the seedling stage, two QTLs were detected for resistance in the adult plant stage (Steffenson et al. 1996). Quantitative resistance may also fit in a classical gene-for-gene relationship. A major *R*-gene which triggers a specific HR response upon recognition of the appropriate avirulence gene product (Baker et al. 1997) may have a reduced effect once a mutated version of this avirulence gene product is encountered. This phenomenon has been observed for the rice resistance gene *Xa4* which acts as a QTL against a virulent strain of the fungus *Xanthomonas oryzae* pv *oryzae* (Li et al. 1999).

In the present study, quantitative resistance in potato to the potato cyst nematode *Globodera pallida* can be fully ascribed to two additive QTLs, which are likely to be explained by a gene-for-gene mechanism in which two distinct avirulence determinants in the nematode population interact with two *R*-genes. The potato cyst nematodes (PCN) *Globodera rostochiensis* and *G. pallida* are serious pests in potato and especially *G. pallida* is a threat for West-European potato culture. Broad-spectrum monogenic resistance to this species is not available and the supposed heterogeneity of the species would obstruct resistance breeding efforts (Schnick et al. 1990; Folkertsma et al. 1996a, b). By use of a selected set of PCN test populations (Folkertsma 1997), in the present study quantitative resistance to *G. pallida* is dissected into the resistance loci *Gpa5* and *Gpa6*. These resistance loci map to two *R*-gene clusters in potato and one of these clusters appears to be novel for the potato genome. Although it is likely that these *R* loci act in a pathotype-specific way, it is argued that, as a result of the population genetics of potato cyst nematodes, the combination of *Gpa5* and *Gpa6* may confer durable resistance to *G. pallida*.

Materials and methods

Plant material

The broadness of PCN resistance was tested in potato clones AM78–3704, 3704–76 and 3704–27. The tetraploid clone AM78–3704 contains resistance to both PCN species and is an interspecific hybrid between *Solanum tuberosum* and several wild potato species including *Solanum vernei* 24/20, *S. vernei* ssp. *ballsii* 2/1, *S. vernei* LGU 8, *Solanum oplocense* EBS 1786, *S. tuberosum* ssp. *andigena* CPC 1673 and *Solanum spegazzinii* 440. Clones 3704–76 and 3704–27 are di-haploids ($2n = 2x = 24$) produced by prickle-pollination of clone AM78–3704 with haploid inducer *Solanum phureja* clones (Hutten et al. 1994).

A mapping population of diploid potato was obtained from a cross between the clones 3704–76×RH89–039–16. Clone 3704–76 is the resistant parent in our population and is referred to as JP. The susceptible male parent, clone RH89–039–16, will be referred to as RH. The mapping population F_1JP × RH consisted of 103 vigorous F_1 genotypes. Leaf material for DNA isolation was collected in the greenhouse at the seedling stage. Tubers for nematode tests were produced by first year clones on the field.

The origin of the resistance segregating from genotype JP was compared with the resistance segregating from genotype 3778–16. With the exception of *S. spegazzinii* 440, genotype 3778–16 has a similar wild-species background. Locus *Grp1* conferring resistance to *G. rostochiensis* and *G. pallida* has been identified by analysing a population derived from a cross between clones 3778–16 and RH (Rouppe van der Voort et al. 1998b).

Nematodes

The nematode populations used in this study are listed in Table 1. *G. rostochiensis* and *G. pallida* populations are indicated by a "Ro" and "Pa" prefix respectively, followed by the pathotype designation and the code of the population. Resistance screening of the segregating potato population was carried out by use of the populations "Pa_2-D383" and "Rookmaker". Population Pa_2-D383 is unrelated to population Rookmaker (which is referred to as population Pa_3-Rook). Population Pa_3-Rook is selected out of a set of 226 *G. pallida* populations found in the Netherlands as being a representative test population for the majority of these populations (Bakker et al. 1992; Folkertsma et al. 1996a, b). This population appeared to contain the highest number of virulent genotypes when tested with a range of resistant clones and cultivars.

Table 1 Level of PCN resistance in the tetraploid genotype AM78–3704 and the AM78–3704 derived di-haploid clones. Presented are average numbers of cysts recovered from closed container tests

Potato clone	Ro1-19	Ro5–22	Pa2-D383	Pa2-D350	Pa2-HPL1	Pa3–1097	Pa2-Rook	Pa3–74.768.20
AM78—3704	1	86	0	1	0	2	4	2
3704–76	6	42	0	2	0	1	2	2
3704–27	n.d.[a]	n.d.	8	4	3	130	109	52
RH89–039–16	189	98	101	132	146	240	153	166

[a] n.d.: not determined

Resistance testing and data collection

Preparation of the PCN inoculum was as described by Rouppe van der Voort et al. (1997a). The resistance spectrum assay was performed in a closed-container test (Phillips et al. 1980) using 125 cc plastic beakers filled with silver sand using one tuber per assay and inoculated with nematodes to a final density of five eggs/J_2 per g of soil. The containers were maintained in the dark at approximately 20°C for at least 3 months.

The inheritance of the resistance to populations Pa_2-D383 and Pa_3-Rook was analysed in mapping population F_1JP × RH in three replications. Resistant standards were cv Multa (resistant to Pa_2-D383), *S. vernei* hybrid cv Nika (resistant to Pa_2-D383) and cvs Florijn and Seresta (resistant to Pa_2-D383 and Pa_3-Rook). As a susceptible standard cv Eigenheimer was used. Plant growth conditions and the design of the experiment were as in Rouppe van der Voort et al. (1997a). Resistance data were only recorded when at least three well-rooted plants of a single plant genotype were available.

DNA-marker analysis and linkage-map construction

The isolation of genomic DNA, the generation of AFLP markers and data analysis were done as described previously (Van Eck et al. 1995; Vos et al. 1995). A genetic map was constructed only from the resistant genotype JP. This map was constructed on the basis of segregating AFLP markers which were generated by use of the AFLP primer combinations E+AAA/M+ACG, E+AAC/M+CAC, E+AAC/M+CAG, E+ACA/M+CAC, E+AGA/M+CAT and E+ATG/M+CTA (the restriction enzymes *Eco*RI and *Mse*I, abbreviated "E" and "M" in the primer designation were used for template preparation). These primer combinations have previously been used to build up a catalogue of chromosome-specific AFLP markers for a set of different potato genotypes (Rouppe van der Voort et al. 1997b, 1998a). Alignment of the JP map was achieved by the identification of common AFLP markers between any of the potato genotypes included in the catalogue. The genetic reference maps are available from URL: http://www.spg.wau.nl/pv/aflp/catalog.htm.

CAPS markers (cleaved amplified polymorphic sequences; Konieczny and Ausubel 1993) for potato RFLP loci GP21 and GP179 (Meksem et al. 1995), GP186 and TG432 (De Jong et al. 1997), and CT220 (Ganal et al. 1998) were applied to determine the chromosomal regions of the QTLs. Segregating JP alleles were detected after digestion of the amplification products using the restriction endonucleases *Dra*I for marker GP21, *Rsa*I for marker GP179 and *Nci*I for GP186 and TG432. The primer sequences for locus CT220 were derived from Folkertsma et al. (1999) and were designed from the published tomato cDNA sequence of tomato RFLP probe CT220 [Ganal et al. 1998; sequences can be retrieved via the Solgenes database (Paul et al. 1994) which can be accessed via http://probe.nalusda.gov:8300/cgi-bin/browse/solgenes]. Primers CT220F (AAGCGAATTATCTGTCAAC) and CT220R (GTTCCTGACCATTACAAAAGTAC) were used to amplify a genomic DNA fragment of approximately 220 bp from potato DNA by applying the following temperature cycle file: 3 min 93°C followed by 30 s 93°C, 45 s 60°C, 90 s 72°C for 35 cycles and finished by 10-min elongation at 72°C. The amplification product was digested with *Mse*I for identification of a segregating JP allele

The grouping of markers and the determination of marker orders were calculated using the software package JoinMap 1.4 (Stam 1993). Maps were drawn by means of the graphical package Drawmap (Van Ooijen 1994).

Statistical analysis

Analysis of variance (ANOVA) were carried out on $\log^{10}(x+1)$-transformed average cyst counts per plant genotype according to the model: $\sigma^2_{tot}=\sigma^2_{plant}+\sigma^2_{rep}$. The phenotypic variance (σ^2_{tot}), the genetic variance among the plant genotypes (σ^2_{plant}) and the environmental variance among replications (σ^2_{rep}) were estimated using Genstat 5 release 3.2 (Payne et al. 1987), and the broad-sense heritabilities were estimated based on the appropriate mean squares from the ANOVA. QTL analyses were performed by using the program MapQTL (Van Ooijen and Maliepaard 1996a, b). Both the non-parametric rank-sum test of Kruskal-Wallis (see e.g. Sokal and Rohlf 1995) and two parametric methods, interval mapping (Lander and Botstein 1989) and multiple-QTL mapping (MQM) (Jansen 1993; Jansen and Stam 1994) were applied. These procedures are implemented in the MapQTL software. Threshold values for assigning a QTL to a map position are P <0.001 for the Kruskal Wallis test and a LOD score of 3.0 for interval and MQM mapping. The percentage of the total variance explained by a marker genotype (R^2) was calculated by $(\sigma^2_m-\sigma^2_{res})/\sigma^2_m$ where σ^2_m represents the variance between the two marker-genotype classes (i.e. presence or absence of a particular marker) and σ^2_{res} the residual variance.

Results

Broadness of PCN resistance from AM78–3704

The level of PCN resistance present in the tetraploid genotype AM78–3704 and two AM78–3704 derived di-haploid clones 3704–76 and 3704–27 was assessed by a resistance screen with a diverse set of PCN populations. These PCN populations are known to harbour differences in molecular as well as virulence characteristics (Folkertsma et al. 1996b; Folkertsma 1997). Comparison of the tetraploid genotype with the di-haploid clones showed that resistance against *G. pallida* has been retained in clone 3704–76 (Table 1). However, loss of resistance to the Pa_3 populations was observed in clone 3704–27. No resistance to *G. rostochiensis* Ro_5-22 was found. The diploid clone RH89–039–16 was susceptible to all populations tested. From these data it was decided to analyse a segregating population derived from the cross 3704–76 × RH89–039–16 (F_1JP × RH). Since large differences exist in the molecular and virulence characteristics of *G. pallida* populations Pa_2-D383 and Pa_3-Rook, these populations were used for further resistance testing.

Inheritance of resistance

The average numbers of cysts developed on the parental genotypes as assessed in pot tests for *G. pallida* populations Pa_2-D383 and Pa_3-Rook are shown in Table 2. The average number of newly developed cysts in the progeny ranged from 0 to 1361 for population Pa_2-D383 and from 9 to 1047 for population Pa_3-Rook. For population Pa2-D383 susceptible plants could be clearly distinguished from plants assigned to the resistant class. The numbers of cysts developed on resistant plants varied from 0 to 42, whereas these numbers ranged from 202 to 1361 on the susceptible plants.

The Pa_3-Rook cyst numbers showed a continuous distribution among the plant genotypes; no clear distinction between a resistant and susceptible class could be made. Analysis of variance on normalized cyst counts showed

Table 2 Results of the quantitative analysis of PCN resistance as measured by counted cyst numbers of the respective PCN populations. Presented are the heritability, the most flanking marker to the resistance loci, the test values indicating the appropriate map position, and the R^2 at the QTL position

Trait	#Cysts[a] JP	# Cysts RH	h^{2b}	Marker[c]	Chrom. location[d]	*P* value[e]	R^{2f}	LOD[g]
Pa2-D383	1	856	–	TG432	Chr. 5	< 0.0001	–	21
Pa3-Rook	23	635	0.91	TG432	Chr. 5	< 0.0001	61%	12
				CT220	Chr. 9	< 0.001	24%	3

[a] # Cysts: average number of cysts,
[b] h^2 : heritability,
[c] Marker : nearest marker to resistance locus,
[d] Chrom. location: chromosomal localization of resistance locus,
[e] *P* value of the nearest marker in a Kruskal Wallis test,
[f] R^2 : percentage of the total variance explained by the marker genotypes,
[g] LOD: logarithm of odds score which indicates the likelihood that two loci are linked (Lander and Botstein 1989)

significant differences in the level of resistance among the plant genotypes ($0.001 < P < 0.0001$). No significant differences in cyst numbers were found among the blocks of replicates. The broad-sense heritability, listed in Table 2, indicated that the variation in the numbers of cysts developed was genetically determined. Skewness and kurtosis values were – 0.68 and 0.07 respectively showing that the assumption on normally distributed $\log^{10}(x+1)$-transformed cyst numbers was justified (Snedecor and Cochran 1967).

AFLP mapping

A genetic map of genotype JP was produced in order to the find the association between molecular markers and the resistance trait(s). The AFLP map of genotype JP consisted of 149 AFLP markers and spanned a total map length of 603 cM. The sizes of the linkage groups varied between 13 cM (for chromosome *4*) and 69 cM (for chromosome *5*). Comparison of the positions of common AFLP markers present on the JP map and the maps of five other potato genotypes revealed that parts of the maps of chromosomes *1, 4, 8, 10* and *11* were not fully covered with AFLP markers. It was therefore anticipated that QTLs may be unresolved as a result of the relative incompleteness of the JP map.

Mapping of Pa_2-D383 resistance

Linkage analysis showed a significant association between the resistance to Pa_2-D383 and AFLP markers which were localized at the top of chromosome *5*. This map position is well known for its presence of a complex *R*-gene cluster in potato harboring resistance to different pathogen species (summarized in Leister et al. 1996). Therefore CAPS markers GP186, GP21, TG432 and GP179, residing on the short arm of potato chromosome *5*, were tested in population F_1JP×RH for a more precise mapping of this locus. Three (GP186, GP21 and TG432) of the four markers segregated in F_1JP×RH. Marker TG432 showed the highest linkage with the resistance locus (recombination fraction=3/102; LOD=21). As two different resistance loci to *G. pallida* have previously been assigned to this map position (*Gpa*, Kreike et al. 1994 and *Grp1*, Rouppe van der Voort et al. 1998b) the origin of the resistance was further investigated.

Mapping of Pa_3-Rook resistance

The presence of the TG432 marker allele in the plant genotypes also appeared to be associated with a reduced number of Pa_3-Rook cysts. A total of 32 out of 42 plant genotypes which harbored the TG432 allele contained <100 cysts, whereas among the remaining 61 genotypes (without the TG432 allele) 58 genotypes were found which contained >100 cysts (Fig. 1). Subsequent analysis using MapQTL software revealed a significant effect of marker TG432 on the resistance, both in the Kruskal Wallis test and by interval mapping; the highest significance levels were found at marker locus TG432 (Table 2, Fig. 2). This QTL explained about 61% of the total variance which is in the same order of magnitude as the amount of variation in Pa_3-Rook resistance explained by locus *Grp1* (Rouppe van der Voort et al. 1998b). Interestingly, the LOD profiles for Pa_3-Rook resistance of *Grp1* and the present QTL coincide, indicating that a similar gene(s) underlies *G. pallida* resistance in genotypes 3778–16 and JP. However, *Grp1* differs from the JP QTL at chromosome *5* as it confers resistance to both species of the potato cyst nematode. By analogy with the nomenclature of other *G. pallida* resistance loci in potato (Kreike et al. 1994; Rouppe van der Voort et al. 1997a) we propose to name the QTL on chromosome *5*, *Gpa5*.

An additional effect on Pa_3-Rook resistance was found at AFLP markers on the long arm of chromosome *9*. The position of this locus could be more precisely defined by application of the most-distal RFLP marker CT220 at this chromosomal region as a CAPS marker. Locus CT220 yielded the highest significance level for Pa_3-Rook resistance (Fig. 3). Since CT220 is localized at the end of a linkage group only one neighboring marker was used in the interval mapping. The addition of more neighboring markers (to a maximum of 5) in the analysis showed an increase in the LOD score to a maximum of 9.1 at this end of chromosome *9* but resulted in a less-precise localization of the QTL.

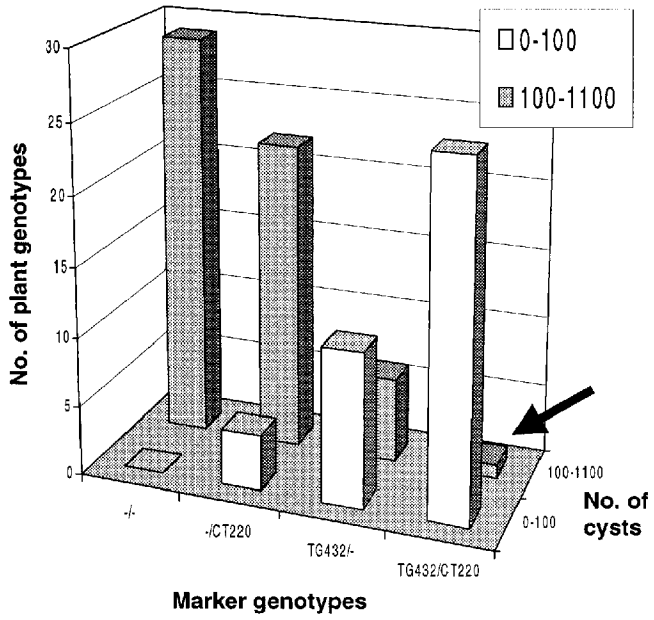

Fig. 1 Resistance to Pa_3-Rook is conferred by the combined action of *Gpa5* and *Gpa6*. Shown is the distribution of the number of *G. pallida* Pa_3-Rook cysts recovered within the TG432/CT220 offspring classes TG432/CT220 (presence of both marker alleles), TG432/- (presence of the TG432 allele, absence of the CT220 allele), -/CT220 (absence of the TG432 allele, presence of the CT220 allele) and -/- (absence of both marker alleles). The genotype indicated by an *arrow* most likely harbours a recombination event between TG432 and *Gpa5* as this genotype lacks all subsequent distal marker alleles in coupling with the TG432 allele

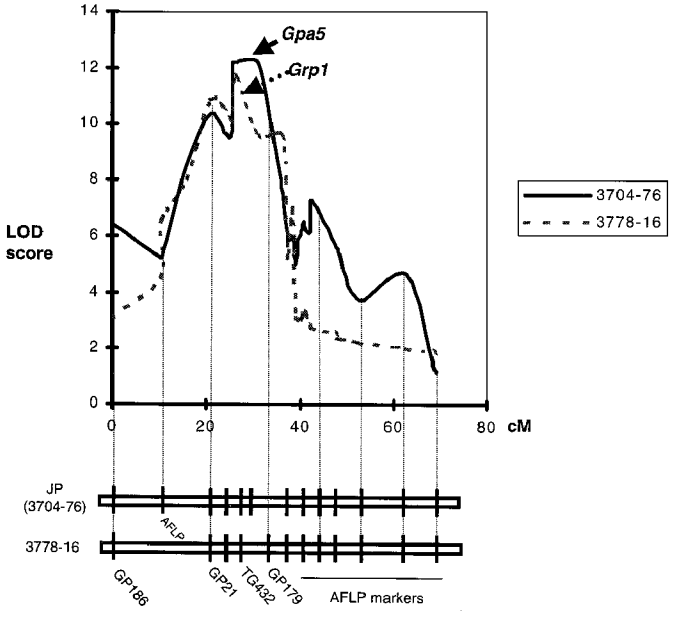

Fig. 2 LOD plots for *G. pallida* Pa_3-Rook resistance on chromosome *5* in genotypes JP and 3778–16 which show that the effect on Pa_3-Rook resistance is localized on the same map region. The Pa_3-Rook resistance locus on chromosome *5* segregating from clone JP is referred to as *Gpa5* (see text). The PCN resistance in 3778–16 is ascribed to the action of locus *Grp1*. For reasons of simplicity, only schematic maps are drawn. More details on the AFLP markers mapped on chromosome *5* are presented in Fig. 4

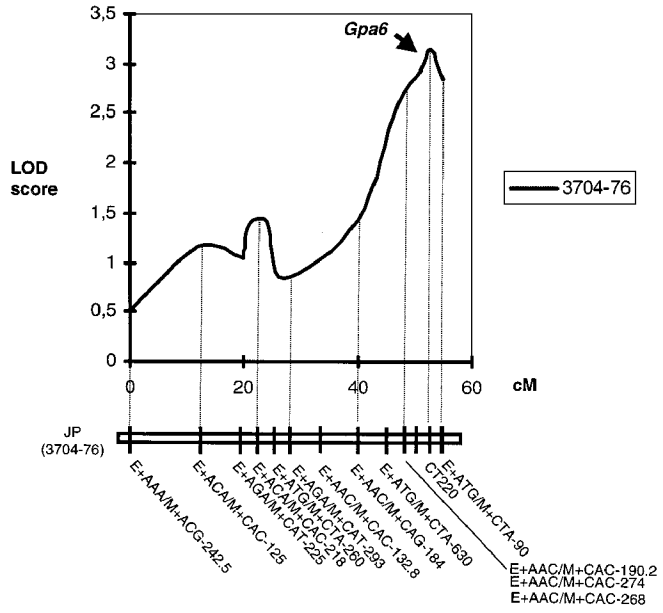

Fig. 3 LOD plot for *G. pallida* Pa_3-Rook resistance on chromosome *9*. This resistance locus is referred to as *Gpa6* (see text)

The QTL at chromosome *9* explained 24% of the total variance (Table 2) and acts addititively with *Gpa5*. The additive effect of the chromosome-*5* and chromosome-*9* loci is shown in Fig. 1. A significant reduction in the numbers of Pa_3-Rook cysts was observed in the class of plants harboring the TG432 and CT220 alleles as compared to the three other marker genotype classes. In the TG432/CT220 class, one plant genotype was found with an average number of 550 cysts. This plant most-likely lacks *Gpa5* as a result of a recombination event in the TG432 – E+AAA/M+ACG-244.0 region. The respective plant genotype contains all JP marker alleles in the GP186 – TG432 region and lacks all subsequent JP alleles onwards which are in coupling with *Gpa5* resistance. Since no resistance specificity to PCN species has been assigned to chromosome *9*, we propose to name the QTL at chromosome *9* *Gpa6*.

Gpa5 may be part of the *Grp1* resistance locus

To address the question whether the same gene(s) underlies the *G. pallida* resistance conferred by *Gpa5* and *Grp1*, we compared the occurrence of common marker alleles linked in coupling with the QTLs at chromosome 5 of clones JP and 3778–16. The result of this analysis is shown in Fig. 4. At the short arm of chromosome *5*, identical marker alleles were identified for CAPS loci GP186, GP21 and TG432, and AFLP loci E+ATG/M+CTA-350, E+AGA/M+CAT-88.9. This similarity is lost from marker GP179 onwards. Marker GP179 and all subsequent AFLP markers which were identified in clone 3778–16 were either absent in JP or present in repulsion with *Gpa5*. The occurrence of two sets of common marker alleles (intervals GP21 –TG432 and

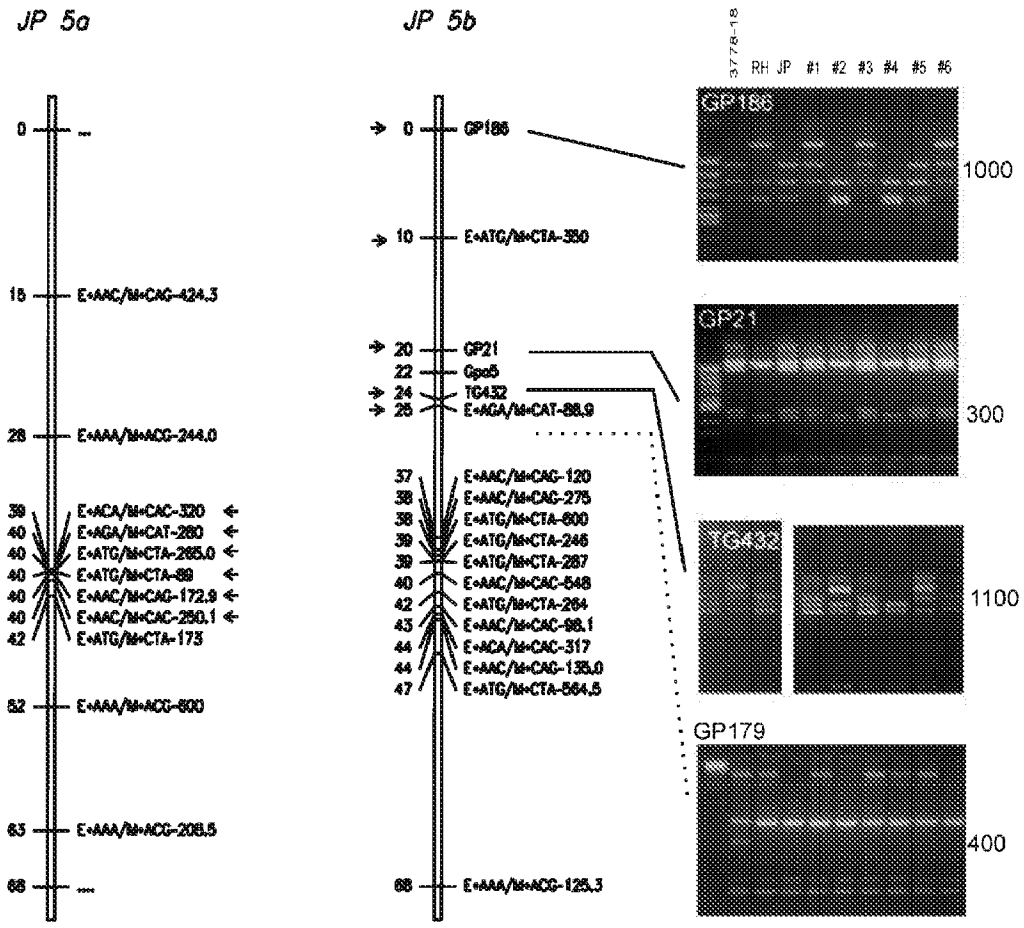

Fig. 4 Comparison of the presence of common marker alleles between clones JP and 3778–16 indicates that *Gpa5* is likely to be introgressed by a common ancestor of both parental clones. Shown are the maps of the two homologous chromosomes *5* of clone JP which harbors linkage blocks of marker alleles which are also present in 3778–16 (indicated by arrow heads). The comparative analysis is exemplified by the profiles of the CAPS markers localized on top of chromosome *5*. Next to the marker lane, amplification products of clones 3778–16, RH, JP and a series of six progeny clones are loaded. Markers GP186, GP21 and TG432 show the similar profiles of clones 3778–16 and JP. For marker GP179 (shown is the profile which contains the mapped 3778–16 allele which is identified after *Rsa*I digestion) no segregating JP allele is identified even after the use of a series of 20 restriction enzymes. The corresponding map position of locus GP179 on the potato map is indicated by a *dashed line*. The *numbers* to the right indicate the molecular weights (in bp) of the segregating alleles

E+ACA/M+CAC-320 – E+AAC/M+CAC-250.1) indicate the presence of similar introgression segments in clones JP and 3778–16. Within these intervals, the DNA sequences between both clones may be highly similar. Since *G. pallida* resistance has been an important trait for selection to generate both breeding lines, we argue that the same gene(s) underlies the *G. pallida* resistance. However, the linkage between TG432 and GP179 and all subsequent marker alleles on the 3778–16 chromosome harboring *Grp1* may have been lost during the course of the breeding process of clone JP. We therefore hypothesize that the recombination between loci TG432 and GP179 in a progenitor of clone JP has been associated with loss of *G. rostochiensis* resistance in clone JP.

Discussion

This study has shown that the map position of the two resistance loci *Gpa5* and *Gpa6* coincides with two resistance gene clusters in potato. *Gpa5* maps close to marker TG432 in a region where resistance to potato virus X (*Rx2*, Ritter et al. 1991; *Nb*, De Jong et al. 1997) and to the fungus *Phytophthora infestans* (*R1*; Leonards-Schippers et al. 1992; Pi-QTL Leonards-Schippers et al. 1994) have been mapped. In addition, two resistance loci to PCN are located in this region. *Gpa* which acts against *G. pallida* (Kreike et al. 1994), and *Grp1* which confers resistance to both PCN species (Rouppe van der Voort et al. 1998b), reside within the GP21 – GP179 marker interval. This prompted us to the question whether the resistance mapped in clone JP could be ascribed to either of these loci. However, combining various data revealed that *Gpa5* is different from *Gpa* and *Grp1*. Locus *Gpa* has been mapped in an intraspecific population of the wild potato species *S. spegazzinni*, and this wild species is not included in the pedigree of the resistant genotype JP. Moreover, the specificity of *Gpa* extends to that of *Gpa5*; *Gpa* confers complete resistance to the population Pa_3-Rook (Wolters, personal communication) whereas 61% of the Pa_3-Rook resistance is explained by *Gpa5*. Locus *Gpa5* can also be distinguished from *Grp1* as it confers only partial resistance to *G. pallida*. Since the level of resistance of both loci is in the same order of magnitude, it may be possible that the *G. pallida* resistance is mediated by the same gene(s). This hypothesis is corroborated by the fact that the pedigrees of the parental clones JP and 3778–16 share the major part of the progenitors which have been used to introgress wild species resistance. In addition, it has previously been argued that *Grp1* is a compound locus since *G. rostochiensis* and *G. pallida* diverged millions of years ago (Bakker et al. 1992) and the multiple specificity of *Grp1* is unlikely to be the result of the action of a single gene (Rouppe

van der Voort et al. 1998b). Circumstantial evidence for the occurrence of *Gpa5* in the genome of 3778–16 was found by a comparative analysis of the maps of both genotypes. On the basis of common marker alleles within a 5-cM interval on the short arm of chromosome *5* (Fig. 4), it can be argued that the same gene(s) underlies the *G. pallida* resistance. Recombination in the TG432 – GP179 interval during the process of generating clone JP may thus be associated with loss of *G. rostochiensis* resistance in clone JP.

Locus *Gpa6* explains only a minor part of the genetic variation of the resistance to population Pa_3-Rook. However, the finding of *Gpa6* is significant because the combined action of *Gpa5* and *Gpa6* results in the same level of resistance as has been transmitted by clone JP to the progeny. *Gpa6* is not able to resist the population Pa_2-D383, which indicates that the resistance at *Gpa6* acts in a pathotype-specific way. The pathotype specificity of *Gpa6* indeed suggests that a major gene(s) underlies the resistance of *Gpa6*. The involvement of a major gene(s) in *Gpa6* resistance is further corroborated by the map location of this locus. *Gpa6* maps close to marker CT220, an RFLP marker locus which targets a resistance-gene cluster in tomato containing the *Sw-5* resistance gene (Brommonschenkel and Tanksley 1997; Folkertsma et al. 1999). The *Sw-5* gene confers broad-spectrum resistance to tomato spotted wilt virus and belongs to the class of resistance genes which encodes a putative gene-product harboring a leucine zipper (LZ), a nucleotide binding site (NBS) and a leucine-rich region (LRR) (Brommonschenkel et al. 1998; Folkertsma et al. 1999). The gene cluster to which the *Sw-5* gene belongs is composed of at least five resistance gene analogues (Folkertsma et al. 1999; Folkertsma and Prins, personal communication) and the location of *Gpa6* suggests the presence of a similar cluster on the homoeologous potato genome.

On the potato genome, no additional resistance specificities have been mapped in the vicinity of marker CT220. The closest resistance locus to marker CT220 is the Nx_{phu} locus which confers resistance to potato virus X (Tommiska et al. 1999). This locus maps at 11 cM from marker CT220 and the map positions of these loci should therefore be considered different. The question of whether any molecular relationship exists between *Gpa6*, Nx_{phu} and *Sw-5* can only be resolved when more information is available on the genes which underly *Gpa6* and Nx_{phu} resistance.

The partial resistance phenotype of *Gpa5* and *Gpa6* does not conflict with the hypothesis that major genes are responsible for both resistance specificities, considering that the *G. pallida* test population Pa_3-Rook is likely to be heterogeneous at the respective (a)virulence loci. In case avirulence alleles are not fixed in a population, a single *R* gene which operates on the basis of a gene-for-gene relationship, will confer partial resistance against the population as a whole whereas, at the level of the individual, the *R* gene will confer absolute resistance against the matching avirulent genotype. The potato cyst nematode is an obligate outbreeding species and field populations, such as Pa_3-Rook, are often composed of a mixture of virulent and avirulent genotypes (Bakker et al. 1993). Formal proof for the occurrence of a gene-for-gene relationship has so far only been demonstrated for the interaction between *G. rostochiensis* and the *H1* gene from *S. tuberosum* ssp. *andigena* CPC1673 (Janssen et al. 1991), but evidence is accumulating that various other PCN resistance genes operate on the same basis. For example, the *Gpa2* gene which is part of a resistance gene cluster on chromosome *12* of potato (Kanyuka et al. 1999), and which encodes a LZ-NBS-LRR type of protein (Van der Vossen et al. 1999), confers specific resistance against population Pa_2-D383. At the molecular level this population is clearly separated from all other *G. pallida* populations which are found in the Netherlands (Folkertsma et al. 1996b). Also the *H2* gene (Dunnet 1961), whose map location is presently unknown, confers resistance against a set of *G. pallida* populations pathotyped "Pa_1" which are well-defined at the molecular level (Blok et al. 1997; Folkertsma 1997).

The availability of broad-spectrum resistance against *G. pallida* is of practical importance. For example, combining *Gpa6* with *Grp1* brings complete resistance against both PCN species into focus. Compared to major gene resistance against various other pathogen species, it is expected that resistance against potato cyst nematodes may be relatively durable. PCN species are endemic in the Andean region of South-America and are thought to have been introduced into Europe last century. Only a limited part of the genetic variation (and thus the variation at virulence loci also) present in their centre of origin has been introduced. Because the multiplication rate of PCN is low, their active spread is limited and the time between generations is often 2–4 years in a normal crop rotation, it seems highly unlikely that the broad-spectrum resistance obtained by combining *Gpa5* and *Grp1* is broken down rapidly.

In summary, this study reports on the QTL mapping of the resistance loci *Gpa5* and *Gpa6* in potato. These QTLs coincide with the map location of other resistance factors which are known to be involved in gene-for-gene relationships. On the basis of the occurrence of common motifs in previously cloned *R*-genes, it is hypothesized that *R*-genes are evolutionary related components of a recognition system and that the mechanisms generating variation are entailed with a clustered distribution on the genome (Baker et al. 1997). Recent advances in the identification and cloning of resistance-gene candidates (RGCs) has shown that these types of sequences occur abundantly in plant genomes (Botella et al. 1997; Shen et al. 1998). With the aid of an increasing amount of sequence information, different RGCs in the genome will be discriminated and resistance specificities will be assigned to the genes. This may eventually lead to a detailed dissection of the resistance gene clusters harbouring *Gpa5* and *Gpa6* and may confirm our hypothesis that these two additive QTLs operate on the basis of a classical gene-for-gene relationship.

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Molecular cloning and sequencing of sucrose synthase cDNA from potato (*Solanum tuberosum* L.): preliminary characterization of sucrose synthase mRNA distribution

(Recombinant DNA; starch biosynthesis; gene evolution; phage λ vector; homology; divergence)

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SUMMARY

A cDNA library constructed from poly(A)$^+$ RNA from potato tuber in λgt11 was screened for sucrose synthase using a maize sucrose synthase cDNA probe. The longest 2.7-kb insert was sequenced. An ATG located within the sequence CTGCAATGG starts an open reading frame of 805 codons. The nucleotide sequence, when compared to the maize sucrose synthase cDNA sequence, exhibits about 70% identity and the deduced amino acid sequence about 75%. Three amino acid regions are about 90% homologous and two of them could be important for the protein function. Expression studies show that transcription of the potato sucrose synthase gene is at least ten fold higher in tubers compared to photosynthetically active tissues.

INTRODUCTION

In plants, sucrose is an energy and carbohydrate source. Sucrose is synthetized in photosynthetically active tissues and transported to other plant organs where it is used after cleavage to monosaccharides or derivatives. Sucrose synthase is one of the enzymes involved in sucrose breakdown and catalyse the reaction: UDP + sucrose ↔ UDPglucose + fructose. Sucrose synthase gene expression has been investigated in great detail in maize (Springer et al., 1986; McCarty et al., 1986). The shrunken gene is highly expressed in the endosperm of developing kernels. A rather high transcription rate has been found in the etiolated shoots and primary roots of germinating kernels. Moreover, the transcript level can be induced, in these tissues, by anaerobic stress. The second sucrose synthase gene seems to be transcribed constitutively at a low level in every tissue. Sucrose synthase studies in potato are of great interest due to the role of this enzyme in starch biosynthesis. In addition the major way of reproduction is vegetative and uses tubers. One can therefore assume that tuber formation might undergo selection pressure contributing to potato species evolution.

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Abbreviations: aa, amino acid(s); bp, base pair(s); cDNA, DNA complementary to mRNA; ds, double stranded; kb, kilobase(s) or 1000 bp; MES, morpholinoethane sulfonic acid; ORF, open reading frame; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl – 0.015 M Na · citrate, pH 7.0; TBE buffer, 0.089 M Tris · borate; 0.089 M boric acid; 0.01 M EDTA.

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We describe here the molecular cloning and sequencing of a cDNA derived from the putative sucrose synthase gene from potato. Comparison with the published sucrose synthase sequence from maize indicates that this cDNA encodes the sucrose synthase of potato. Aspects of expression of this gene in different tissues have been studied. Evolution of this gene is also discussed.

MATERIAL AND METHODS

(a) Plant material

Potato tubers (*Solanum tuberosum* L. var. Sirtema 2n = 4x) were kept for one and a half year at 4°C, then grown in the dark until new tubers appeared. Leaves and stems were cut off plants five weeks after tuber germination.

(b) RNA preparation

Washed young tubers were frozen in liquid nitrogen and stored at −70°C. Frozen tubers were ground with dry ice in a coffee grinder, homogenized with 3 ml/g of a guanidinium·HCl buffer (8 M guanidinium·HCl; 20 mM MES pH 7; 20 mM EDTA; 50 mM β-mercaptoethanol). After centrifugation the supernatant was phenol/chloroform-extracted and precipitated with 1/20 vol. of acetic acid and 0.7 vol. of ethanol (2 h at −70°C). The precipitate was washed twice in 70% ethanol, once in 3 M sodium acetate, once again in 70% ethanol and resuspended in water (Eckes et al., 1985; S. Rosahl, personal communication). Poly(A)$^+$ RNA was isolated with two cycles of oligo(dT)-cellulose affinity chromatography (BRL). RNA was stored in water at −70°C.

(c) RNA blot hybridization

RNAs were denatured with formaldehyde, run on a 1.2% agarose gel containing formaldehyde (Lehrach et al., 1977; Goldberg, 1980) and subsequently blotted on either nitrocellulose or GeneScreen Plus paper. Hybridization was performed according to Southern (1975).

(d) cDNA library

A cDNA library was established from poly(A)$^+$ RNA of potato tubers from the tetraploid variety, Sirtema. The ds-cDNA was synthesized using the Amersham cDNA synthesis system. After *Eco*RI methylation and addition of *Eco*RI linkers the ds-cDNA was sized on a Sepharose 4B column. A fraction corresponding to the longest ds-cDNA was ligated into *Eco*RI-cut and dephosphorylated arms of λgt11. Bacteriophages obtained by in vitro packaging were grown on *Escherichia coli* strain 1106 and screened for homologous sequences by standard methods (Maniatis et al., 1982).

(e) Phage DNA preparation and insert isolation

Large-scale DNA preparation of bacteriophage was performed according to the protocol of Yamamoto (1970). cDNA inserts were isolated by *Eco*RI digestion, followed by low-melting-temperature agarose gel electrophoresis.

(f) Subcloning and plasmid DNA preparation

The pBR322 (Bolivar et al., 1977) and M13tg130 (Kieny et al., 1983) vectors were used to transform *E. coli* HB101 and BMH71-18, respectively. Plasmids were isolated as described by Birnboim and Doly (1979).

(g) Nucleotide sequencing and computing program

The dideoxy chain-termination method of Sanger et al. (1977) was employed. Ordered deletions were created by random DNase I cleavage as described by Hun-Chi Lin et al. (1985). Sequence analyses were performed with the computer program of UWGCG package on a VAX 750 computer.

RESULTS AND DISCUSSION

(a) Cloning of potato sucrose synthase cDNA

A cDNA library established from poly(A)$^+$ RNA from potato tubers was screened for sequences homologous to cDNA of the maize sucrose synthase

gene. To avoid cross-hybridization with the galactosidase gene present in both vectors λgt11 and pWW110/1, the *Hin*dIII-*Bgl*II fragment from plasmid pWW110/1 (Werr et al., 1985) was cloned into pBR322 cut with *Hin*dIII + *Bam*HI. The ^{32}P-labeled *Hin*dIII-*Sal*I fragment from the resulting plasmid was used to screen the library for clones containing cDNA sucrose synthase inserts. Fifty clones homologous to the probe were isolated. DNA from 20 of these was prepared, and after *Eco*RI digestion, fractionated on agarose and polyacrylamide gels.

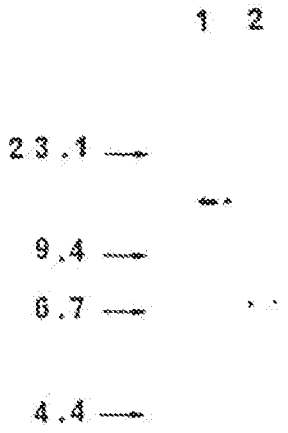


Fig. 1. Southern blot analysis of potato genomic DNA. Total DNA (5 μg) extracted from haploid potato plant was digested by *Hin*dIII (lane 1) and *Eco*RI (lane 2) and transferred to nitrocellulose filter (according to the manufacturers' specifications) after separation by 0.7% agarose gel electrophoresis in TBE buffer. The autoradiogram shows the hybridization pattern obtained using the nick-translated [α-^{32}P]dCTP 1.8-kb *Eco*RI probe from λ10a. Hybridization was performed in 6 × SSC, at 68°C and washed in 0.1 × SSC at 50°C (2 × 30 min). Size of the markers is indicated in kb on the left.

Sizes of inserts ranged between 1.5 and 2.7 kb; the longest insert, composed of five *Eco*RI fragments (1.8, 0.5, 0.12, 0.11, 0.08 kb), was named λ10a.

(b) DNA analysis

DNA of haploid potato (n = x) (originated from the Max Planck Institute of Cologne) was digested by either *Hin*dIII or *Eco*RI, fractionated on a 0.7% agarose gel, transferred to a nitrocellulose paper and hybridized under stringent conditions (6 × SSC, 68°C for hybridization and 0.1 × SSC, 50°C for washing) with a ^{32}P-labeled 1.8-kb *Eco*RI fragment derived from the cDNA of the potato sucrose synthase clone λ10a (Fig. 1). For both digestions one major signal was detected. The presence of additional bands larger than the major signal in the *Hin*dIII digest and their absence in the *Eco*RI digest is difficult to explain and does not permit an exact estimation of the number of copies of this gene in potato. It was not confirmed whether the extra bands present in the *Hin*dIII pattern were due to partial digestion of the DNA. However, between one and three copies of this gene in potato would be a probable order of magnitude.

(c) Expression of the sucrose synthase gene

To investigate how many transcripts hybridizing to the potato sucrose synthase gene are detectable in tuber tissues, Northern blot analysis was performed. Poly(A)$^+$ RNA from tuber were fractionated in duplicate on a 1.2% formaldehyde agarose gel, blotted, hybridized with 1.8-kb *Eco*RI fragment from λ10a, and washed either under moderately stringent conditions (2 × SSC, 50°C) or under stringent conditions (0.1 × SSC, 50°C). The resulting autoradiogram is shown on Fig. 2. Only one 2.8-kb transcript was detected in both cases. This result is consistent with the expression of only one gene encoding the potato sucrose synthase in tubers but it is also possible that sucrose synthase is encoded by several genes producing transcripts of the same size.

The sucrose synthase gene is highly expressed in growing tubers. To determine if this gene is tuber-specific or also expressed in green tissues total RNA from leaves and stems was isolated two months after tuber germination. A Northern blot analysis was performed and the 1.8-kb *Eco*RI fragment derived

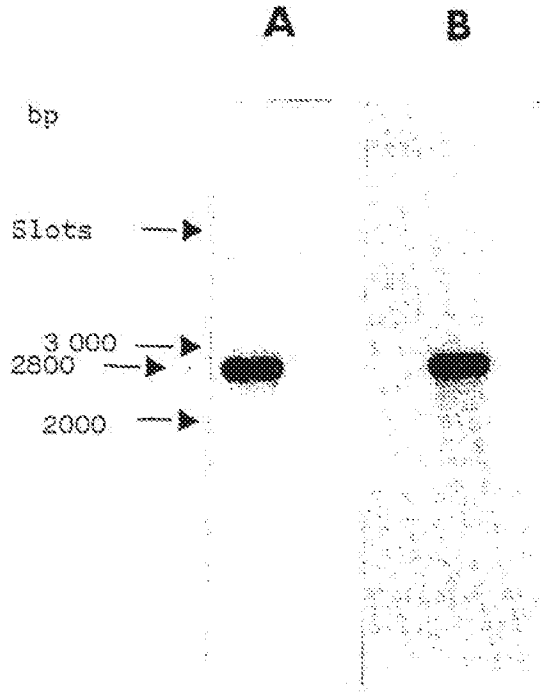


Fig. 2. Northern blot analysis. RNA was isolated from growing tuber tissues. 1 μg of poly(A)$^+$ RNA isolated with two oligo(dT) cycles were transfered to Genescreen plus filter after separation by a 2.2 M formaldehyde, 1.2% agarose gel electrophoresis. The hybridization pattern was obtained using the 1.8-kb *Eco*RI probe from λ10a under stringent washing condition (0.1 × SSC, 50°C) (**A**) or non-stringent washing conditions (2 × SSC, 50°C) (**B**). Labeling of the probe and hybridization conditions were the same as described in Fig. 1. Size of the markers is indicated in bp on the left.

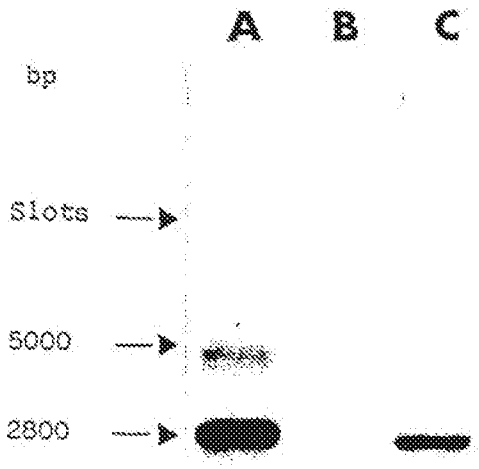
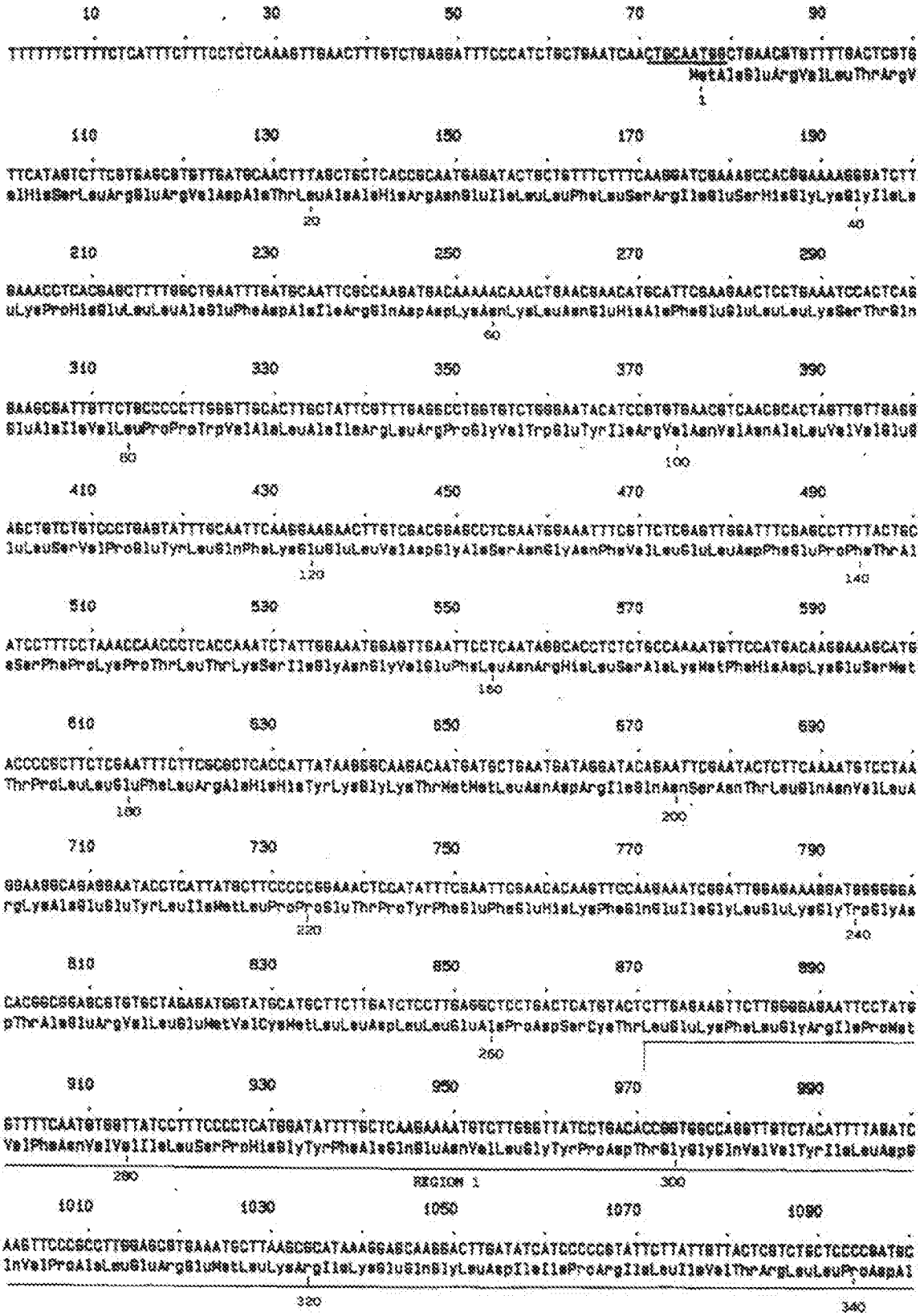


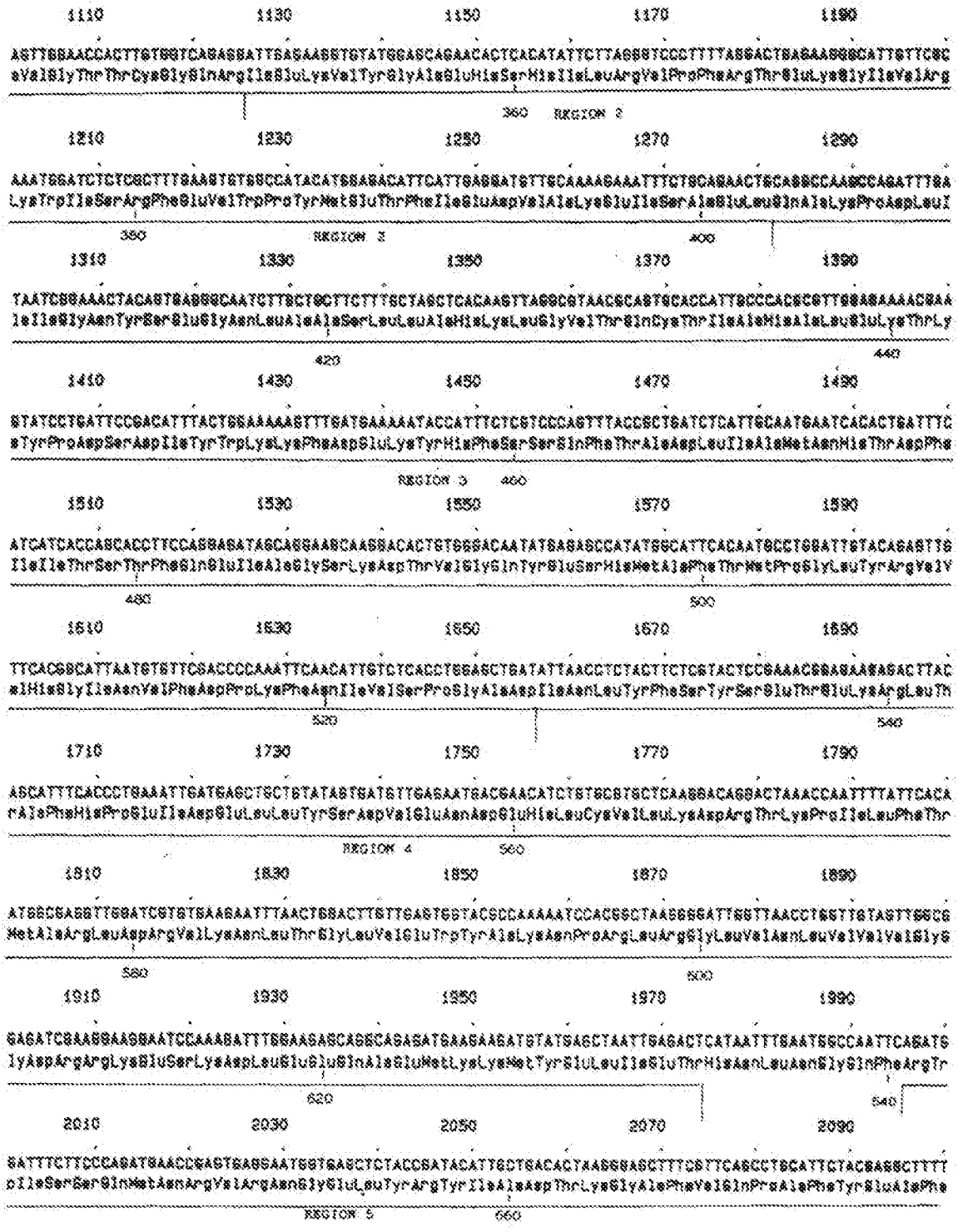
Fig. 3. Expression of the sucrose synthase gene in different tissues. Northern blot with 0.5 μg of total RNA extract from growing tubers (**A**), 5 μg of total RNA from leaves (**B**), and from stems (**C**). Leaves and stems were harvested two months after planting. The probe used for hybridization was the 1.8-kb *Eco*RI fragment of the cDNA clone λ10a. Experimental details are described in Figs. 1 and 2. Sizes are indicated on the left.

from the potato sucrose synthase cDNA was used as a probe. The result is shown in Fig. 3. In tuber two bands were found, a major one at around 2.8 kb and another at around 5 kb. The origin of the 5-kb band is unknown but this band can be removed by two cycles of oligo(dT) as shown in Fig. 2 [the intensity of the band was already reduced after one cycle of oligo(dT)]. This band was not found in the stem of the plant. In the following discussion only the 2.8-kb band will be taken into account. As expected from the putative role of this enzyme in starch biosynthesis a strong signal was obtained in tuber. Less than 10% of the level of transcript detected in tuber was found in stems. No transcript was found in leaves (the sensitivity of the experiment should permit the detection of around 2% of the level of transcription found in tuber). These results show that the expression of the sucrose synthase gene in tuber is at least ten times higher than in the photosynthetically active tissues analysed.

(d) Nucleotide sequence analysis

The 2714-bp-long cDNA clone λ10a was sequenced from both strands. An ORF of 805 aa found in this sequence starts with an ATG in bp position 76 and is terminated by a TGA stop codon downstream. The ATG is located within the sequence CTGCAATGG which resembles other eukaryotic translational start sites (Kozak, 1984). This ORF




1110 1130 1150 1170 1190
AGTTGGAACCACTTGTGGTCAGAGGATTGAGAAGGTGTATGGAGCAGAACACTCACATATTCTTAGGGTCCGTTTTAGGACTGAGAAGGGCATTGTTCGC
eValGlyThrThrCysGlyGlnArgIleGluLysValTyrGlyAlaGluHisSerHisIleLeuArgValProPheArgThrGluLysGlyIleValArg
360 REGION 2
1210 1230 1250 1270 1290
AAATGGATCTCTCGCTTTGAAGTGTGGCCATACATGGAGACATTCATTGAGGATGTTGCAAAAGAAATTTCTGCAGAACTGCAGGCCAAGCCAGATTTGA
LysTrpIleSerArgPheGluValTrpProTyrMetGluThrPheIleGluAspValAlaLysGluIleSerAlaGluLeuGlnAlaLysProAspLeuI
380 REGION 2 400
1310 1330 1350 1370 1390
TAATCGGAAACTACAGTGAGGGCAATCTTTGCTGCTTCTTTGCTAGCTCACAAGTTAGGCGTAACGCAGTGCACCATTGCCCACAGCGTTGGAGAAAACGAA
IleIleGlyAsnTyrSerGluGlyAsnLeuAlaAlaSerLeuLeuAlaHisLysLeuGlyValThrGlnCysThrIleAlaHisAlaLeuGluLysThrLy
420 440
1410 1430 1450 1470 1490
GTATCCTGATTCCGACATTTACTGGAAAAAAGTTTGATGAAAAATACCATTTCTCGTCCCAGTTTACCGCTGATCTCATTGCAATGAATCACACTGATTTC
sTyrProAspSerAspIleTyrTrpLysLysPheAspGluLysTyrHisPheSerSerGlnPheThrAlaAspLeuIleAlaMetAsnHisThrAspPhe
REGION 3 460
1510 1530 1550 1570 1590
ATCATCACCAGCACCTTCCAGGAGATAGCAGGAAGCAAGGACACTGTGGGACAATATGAGAGCCATATGGCATTCACAATGCCTGGATTGTACAGAGTTG
IleIleThrSerThrPheGlnGluIleAlaGlySerLysAspThrValGlyGlnTyrGluSerHisMetAlaPheThrMetProGlyLeuTyrArgValV
480 500
1610 1630 1650 1670 1690
TTCACGGCATTAATGTGTTCGACCCCAAATTCAACATTGTCTCACCTGGAGCTGATATTAACCTCTACTTCTCGTACTCCGAAACGGAGAAGAGACTTAC
alHisGlyIleAsnValPheAspProLysPheAsnIleValSerProGlyAlaAspIleAsnLeuTyrPheSerTyrSerGluThrGluLysArgLeuTh
520 540
1710 1730 1750 1770 1790
AGCATTTCACCCTGAAATTGATGAGCTGCTGTATAGTGATGTTGAGAATGACGAACATCTGTGCGTGCTCAAGGACAGGACTAAACCAATTTTATTCACA
rAlaPheHisProGluIleAspGluLeuLeuTyrSerAspValGluAsnAspGluHisLeuCysValLeuLysAspArgThrLysProIleLeuPheThr
REGION 4 560
1810 1830 1850 1870 1890
ATGGCGAGGTTGGATCGTGTGAAGAATTTAACTGGACTTGTTGAGTGGTACGCCAAAAATCCACCGCTAAGGGGATTGGTTAACCTGGTTGTAGTTGGCG
MetAlaArgLeuAspArgValLysAsnLeuThrGlyLeuValGluTrpTyrAlaLysAsnProArgLeuArgGlyLeuValAsnLeuValValValGlyS
580 600
1910 1930 1950 1970 1990
GAGATCGAAGGAAGGAATCCAAAGATTTGGAAGAGCAGGCAGAGATGAAGAAGATGTATAGAGCTAATTGAGAGCTCATAATTTGAATGGCCAATTCAGATG
lyAspArgArgLysGluSerLysAspLeuGluGluGlnAlaGluMetLysLysMetTyrGluLeuIleGluThrHisAsnLeuAsnGlyGlnPheArgTr
620 640
2010 2030 2050 2070 2090
GATTTCTTCCCAGATGAACCGAGTGAGGAATGGTGAGCTCTACCGATACATTGCTGACACTAAGGGAGCTTTCGTTCAGCCTGCATTGTACGAGGCTTTT
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REGION 5 660

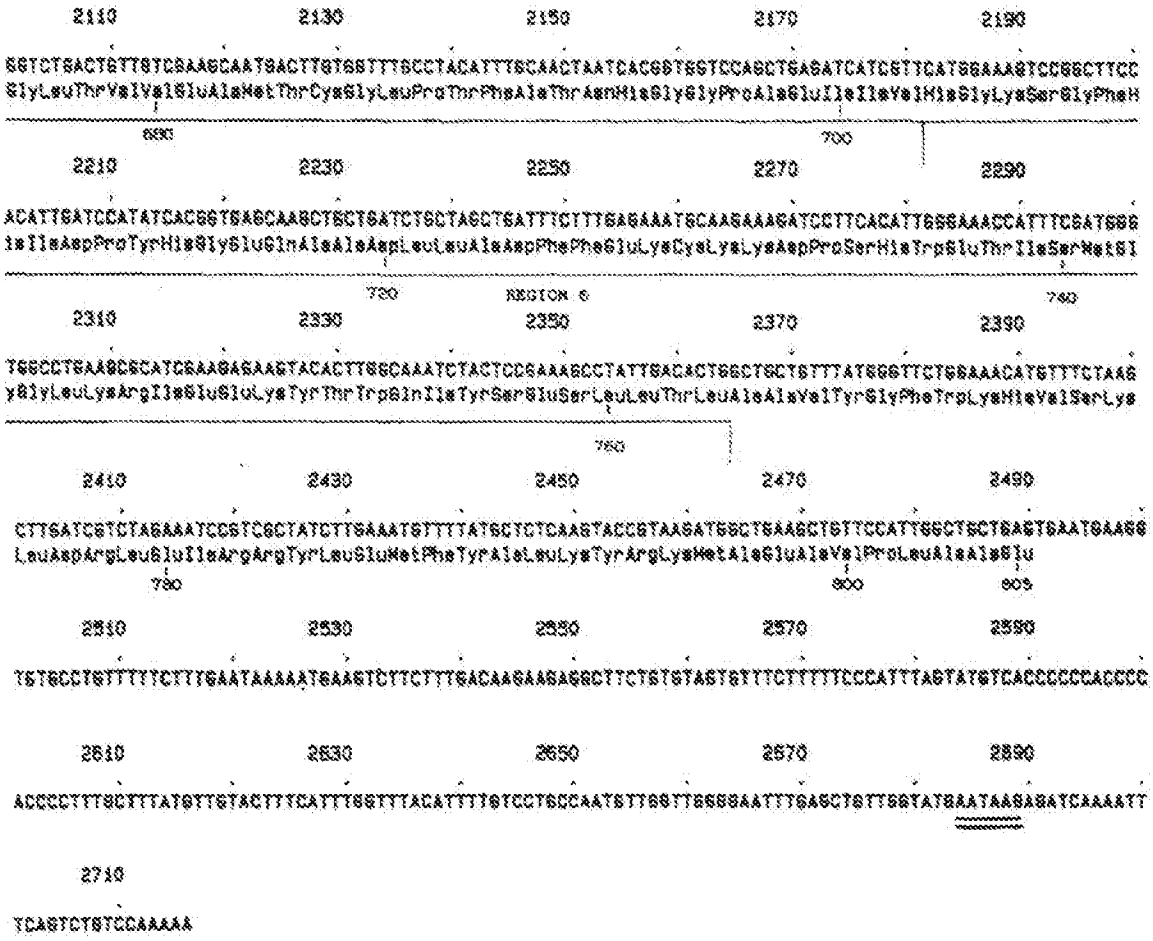

Fig. 4. Nucleotide sequence of potato sucrose synthase cDNA. The coding region has been translated into the corresponding amino acids. Numbers above each line refer to the nucleotide positions. Numbering starts with the first bp of the cDNA clone λ10a. Numbers below each line refer to the amino acid position. Numbering begins with the ATG start codon. The positions of the regions are indicated by horizontal brackets. The CTGCAATGG sequence containing the start codon and the AATAAG polyadenylation site are underlined with single and double lines, respectively.

predicts an M_r for the protein of around 90 000. This ORF is preceded by an untranslated region of 75 bp between the 5′ end of the clone and the AUG start codon and followed by another 3′ untranslated region of 223 bp between the TGA stop codon and the poly(A) tail. The complete sequence is shown in Fig. 4. At 23 bp and 187 bp upstream from the poly(A) tail, the sequences AAUAAG and AAUAAA, respectively, are found. These sequences resemble the AAUAAA signal found in front of the polyadenylation site of most non-histone eukaryotic mRNAs (Proudfoot and Brownlee, 1976). The location of the AAUAAG sequence 23 bp upstream from the polyadenylation site is in agreement with the general location of the AAUAAA signal in higher eukaryotes, but the substitution of the canonical A by a G in the signal is surprising. Indeed, besides the predominant AAUAAA signal some minor modifications have been described, but the AAUAAG yields 3′ ends at a very much reduced level (Birnstiel et al., 1985). The use of the sequence AATAAG as a polyadenylation signal had already been described for the proteinase inhibitor gene in tomato (Graham et al., 1985), in potato (Sanchez-Serrano et al., 1986) and has been used for the expression of a potato proteinase inhibitor II gene in transgenic tobacco plants (Sanchez-Serano et al., 1987). Taken together these results indicate that, in at least Solanaceae,

AATAAG is an efficient signal for mRNA 3' end formation. The role of the AATAAA sequence upstream is unknown, but the presence of such a signal around 150 bp upstream from the poly(A)-addition signal has been already described for the potato proteinase inhibitor II (Sanchez-Serrano et al., 1986).

(e) Evolutionary aspects

(1) Codon preferences

In maize, codon usage has been shown to deviate from random, for codons where the second base is a T, and where A and G are used synonymously in the third position. In these cases G is preferred strongly over A (valine GTG/A: 17/2; leucine TTG/A: 16/0, CTG/A: 34/3) (Werr et al., 1985). In the sucrose synthase gene of potato such a strong deviation is not found: valine GTG/A: 10/3; leucine TTG/A: 21/5; CTG/A: 17/9. This might indicate that the selection pressure leading to this preference in maize, is weaker or absent in potato.

(2) Comparison between maize and potato sucrose synthase genes

A comparison among maize and potato sucrose synthase cDNA sequences reveals conserved and divergent regions. Regions flanking the ORF are highly divergent.

(i) Untranslated regions. The 5'- and the 3'-untranslated regions share very little homology between maize and potato. No meaningful matching was obtained in any cases.

(ii) The open reading frame. The ORF of the potato sucrose synthase gene is 3 aa longer than the homologous maize sucrose synthase gene. The additional 3 aa are located in the first 260 bp of the ORF. The nucleotide sequence of the ORF of the potato gene exhibits 70% homology when compared to the ORF of the maize gene. At the amino acid level the homology is not clustered to special regions as shown in Fig. 5a by a plot analysis using a stringency comparable to the homology between the two genes (14 out of 21). If the stringency is increased up to 18

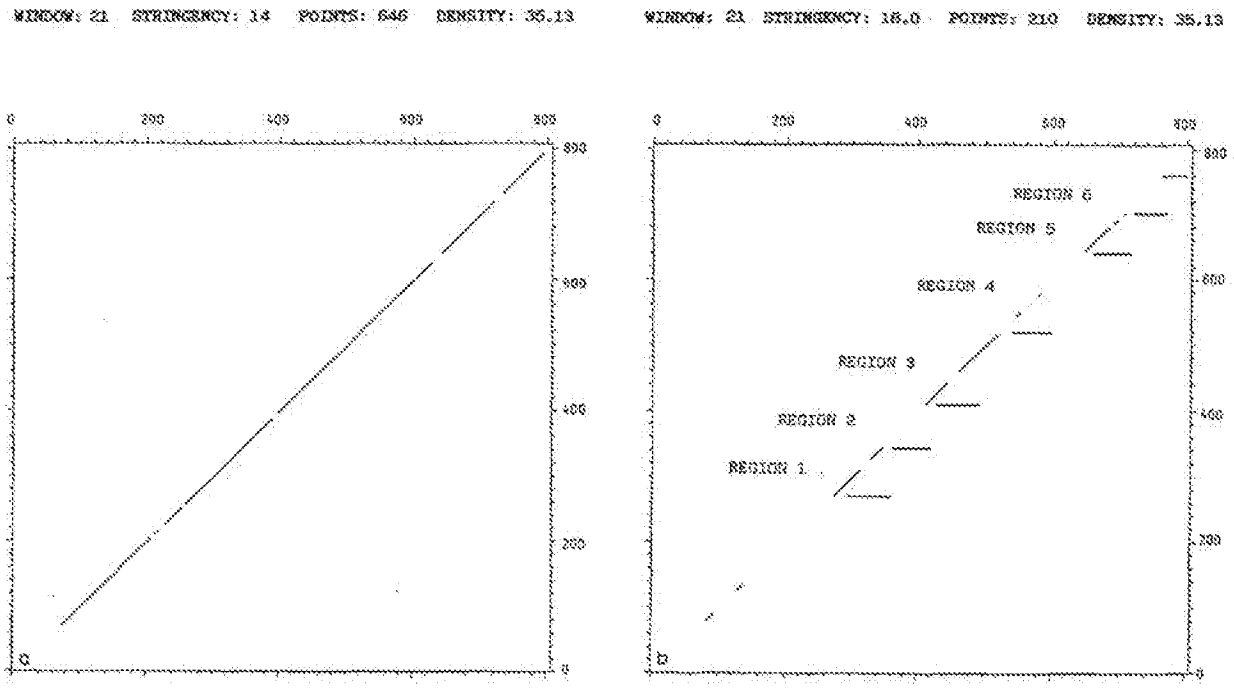

Fig. 5. Plot analysis comparing maize and potato sucrose synthase gene at the amino acid sequence level. A window of 21 aa is moved along the amino acid sequence (e.g., from aa residues 1 to 21, then from aa residues 2 to 22 etc..) If the number of matching amino acids is equal or superior to the stringency a point is drawn in the corresponding position on the diagram. (a) Calculation performed with a window of 21 aa and a stringency of 14/21. (b) Calculation performed with a window of 21 aa and a stringency of 18/21.