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NEW R GENES IN *SOLANUM DEMISSUM* LINDL. AND THEIR COMPLEMENTARY RACES OF *PHYTOPHTHORA INFESTANS* (MONT.) DE BARY

JEAN F. MALCOLMSON and WILLIAM BLACK

Scottish Plant Breeding Station, Pentlandsfield, Roslin, Midlothian

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ABSTRACT

Three new major genes which control a hypersensitive reaction to certain races of *Phytophthora infestans* are recorded in potato seedlings bred from the wild species *Solanum demissum*. They are different from the six already established in that species and are designated R_7 , R_8 and R_9 . Nine new races of *P. infestans*, each capable of overcoming the hypersensitivity induced by at least one of the new genes, have been distinguished, and all originated from race 1, 2, 3, 4.

INTRODUCTION

The classification of specialised races of *Phytophthora infestans* (MONT.) DE BARY is based on the major genes responsible for hypersensitive resistance to blight in species of *Solanum*. An international scheme for nomenclature of such genes in *S. demissum* was proposed by BLACK, MASTENBROEK, MILLS and PETERSON (1953) and four genes, R_1 , R_2 , R_3 and R_4 , were recognised. This allowed for the identification of sixteen races of *P. infestans*, the most complex being race 1, 2, 3, 4. Since then, more complex races – race 1, 2, 3, 4, 5, race 1, 2, 3, 4, 6 and race 1, 2, 3, 4, 5, 6 – have been recorded (EIDE, BONDE, GALLEGLY, GRAHAM, MILLS, NIEDERHAUSER and WALLIN, 1959), indicating the existence of additional genes, R_5 and R_6 . Genes of a similar nature have been recognised in other species of *Solanum* (BLACK and GALLEGLY, 1957; SCHICK, SCHICK and HAUSDÖRFER, 1958; MCKEE, 1962).

GENES R_5 AND R_6 AND RACES IDENTIFIED BY THEM

The genes R_5 and R_6 were recorded in two derivatives of *S. demissum*, 3053-18 and XD2-21, from Canada, but a derivative of *S. stoloniferum*, 237/43, from Germany, was also credited with possessing the gene R_6 (SCHICK *et al.*, 1958). Therefore, according to their order of discovery, the genes of 3053-18, XD2-21 and 237/43 were tentatively designated R_5 , R_7 and R_8 respectively, by the Late Blight Investigations Committee of the Potato Association of America (EIDE, GALLEGLY, GRAHAM, MILLS, NIEDERHAUSER and WALLIN, 1960).

The original system of nomenclature (BLACK *et al.*, 1953) was established to accommodate R genes of *S. demissum* and, in order to maintain a comparatively simple system, the present authors favour placing genes from other species into separate series. Thus, the R gene possessed by clone 237/43 would be placed in the series for *S. stoloniferum*. The R gene of *S. demissum* derivative XD2-21 would then be designated R_6 in the *S. demissum* series, as has been practised by other authors (EIDE *et al.*, 1959; GRAHAM, DIONNE and HODGSON, 1961).

Races of *P. infestans*, capable of attacking either genotype R₅ (3053-18) or R₆ (XD2-21), or both, were observed in tests at Pentlandfield with cultures from the United Kingdom and overseas (Table 1). Such races proved to have a wide distribution and three isolates from Canada had the maximum host range, i.e. they were race 1, 2, 3, 4, 5, 6. The *S. stoloniferum* derivative 237/43 was included in the tests, and races which were identical according to the *S. demissum* differential hosts were differentiated by it (Table 1). The gene it possessed did not prove to be the same as any of those identified in the *S. demissum* series (Table 2).

The R gene of *S. stoloniferum* derivative 237/43 (designated R₈ by SCHICK *et al.*, 1958) was used by BLACK (1960) to identify two isolates of *P. infestans* as race 1, 2, 4, 6 and race 2, 3, 4, 6. By using these races to test *S. demissum* derivative XD2-21, and breeding lines from *S. stoloniferum* respectively, BLACK (1960) and McKEE (1962)

TABLE 1. REACTION OF *Solanum demissum* DERIVATIVES 3053-18 (R₅) AND XD2-21 (R₆) AND *S. stoloniferum* DERIVATIVE 237/43 TO RACES OF *Phytophthora infestans*

Origin of culture	Designation of race by original <i>S. demissum</i> differentials*	Reaction** of <i>S. demissum</i> derivative		Present designation of race	Reaction** of <i>S. stoloniferum</i> derivative 237/43
		3053-18 (R ₅)	XD2-21 (R ₆)		
Italy	0	—	+	6	—
	4	—	+	4, 6	—
	2, 4	—	—	2, 4	+
	2, 3, 4	—	+	2, 3, 4, 6	—
Rhodesia	4	—	+	4, 6	—
	3, 4	—	+	3, 4, 6	—
	1, 2, 4	—	—	1, 2, 4	+
	1, 2, 4	+	—	1, 2, 4, 5	+
	1, 2, 4	—	+	1, 2, 4, 6	—
Brazil	1, 4	+	—	1, 4, 5	—
	1, 4	+	+	1, 4, 5, 6	—
	3, 4	—	—	3, 4	+
Netherlands	1, 2, 4	—	—	1, 2, 4	+
	1, 2, 4	—	+	1, 2, 4, 6	—
	1, 2, 4	—	+	1, 2, 4, 6	+
	2, 3, 4	—	+	2, 3, 4, 6	+
Germany	1, 2, 3, 4	+	—	1, 2, 3, 4, 5	—
	1, 2, 4	—	—	1, 2, 4	+
	1, 2, 4	—	+	1, 2, 4, 6	—
	1, 2, 4	—	+	1, 2, 4, 6	+
Canada	1, 2, 4	+	—	1, 2, 4, 5	+
	1, 2, 3, 4	—	+	1, 2, 3, 4, 6	+
	1, 2, 3, 4	+	+	1, 2, 3, 4, 5, 6	—
	1, 2, 3, 4	+	+	1, 2, 3, 4, 5, 6	+
United Kingdom	4	—	+	4, 6	—
	3, 4	—	+	3, 4, 6	—
	1, 2, 4	+	—	1, 2, 4, 5	—

* Differentials of BLACK *et al.* (1953)

** — = hypersensitive; + = susceptible

NEW R GENES IN SOLANUM DEMISSUM

TABLE 2. REACTION OF DIFFERENTIAL HOSTS TO ISOLATES OF *Phytophthora infestans*

Source of genes	Clone	<i>S. demissum</i> genes present	Reaction	Reaction* to isolates of type									
			to race 1, 2, 3, 4	E	C	H	D	K	A	Z	Y	X	
<i>S. demissum</i> Scotland	2070(54)	R ₁ R ₂ R ₃ R ₄	---	+	+	+	+	+	+	+	+	+	+
<i>S. demissum</i> Canada	3053-18	R ₅	---	+	---	---	---	---	---	---	+	+	---
<i>S. demissum</i> Canada	XD2-21	R ₆	---	---	+	---	---	---	+	---	+	+	---
<i>S. demissum</i> Scotland	2182ef(7)	R ₇	---	---	+	+	+	+	+	---	---	---	+
<i>S. demissum</i> Scotland	2424a(3)	R ₈	---	---	---	---	---	---	---	+	---	+	+
<i>S. demissum</i> Scotland	2573(2)	R ₉	---	---	---	---	---	---	---	---	---	---	---
<i>S. stoloniferum</i> Germany	237/43	---	---	---	---	---	---	+	---	---	---	---	---

* — = susceptible; + = hypersensitive

concluded that the R₆ was common to *S. demissum* and *S. stoloniferum*. In the present tests involving additional races it was evident that the *S. demissum* gene, R₆, was distinct from the gene R₆ of *S. stoloniferum* (Table 1) and that both genotypes were susceptible to the races 1, 2, 4, 6 and 2, 3, 4, 6 used by BLACK (1960) and MCKEE (1962). The earlier conclusion that the R₆ genes of *S. demissum* and *S. stoloniferum* were the same resulted from using two races which could not differentiate between them.

GENES R₇, R₈ AND R₉ AND RACES IDENTIFIED BY THEM

In the course of work with two isolates of race 1, 2, 3, 4 of *P. infestans* (one from Canada and one from The Netherlands) spreading blight lesions were occasionally observed on clones known to be hypersensitive to that race. Isolates from these lesions were tested on the standard series of differential hosts (possessing R₁, R₂, R₃ and R₄ alone or in combination) clones 3053-18 and XD2-21 (possessing R₅ and R₆ respectively) and clones 2182ef(7), 2424a(5) and 2573(2) (derivatives of *S. demissum* from Pentlandfield) which were hypersensitive to race 1, 2, 3, 4. The isolates separated into nine different types according to the reactions which occurred on this host series (Table 2).

Some isolates which caused a susceptible reaction on the clones possessing R₅ and R₆ induced a hypersensitive response in the clones 2182ef(7), 2424a(5) and 2573(2), as did race 1, 2, 3, 4 (Table 2). This indicated that the genes possessed by the three clones were different from the six already ascribed to *S. demissum*. In addition, the three clones did not show identical reactions to the new isolates (Table 2); therefore, the genes they possessed were all different. The number of new R genes they possessed was determined by testing with *P. infestans* the progenies of crosses involving the three clones (Table 3). When the progenies of clones 2182ef(7) and 2424a(5) crossed with recessive genotypes were tested with race 4 (progenies 3088a, 2943c, 2960a and 3010c) or with race 1, 2, 3, 4 (progeny 4365a) they segregated hypersensitive to susceptible in

the proportion 1:1; when the two clones were crossed with each other and the progeny tested with race 4 (progeny 3092d), a 3:1 segregation was obtained. These results indicate that the two clones 2182ef(7) and 2424a(5), each possessed only one R gene effective against the races used in the tests. Whether or not the gene R_4 was present in addition could not be determined since R_4 is not effective against the races which were used.

In tests with race 1, 2, 3, 4 of progenies (3392 and 3926c) bred from 2573(2) and a recessive genotype, hypersensitives and susceptibles segregated in equal proportions indicating that clone 2573(2) possessed only one gene effective against that race (Table 3). Again the race employed in the tests could not determine whether or not the R_4 was also present. However, the presence of three other genes was indicated by the segregations obtained with race 4 in tests of similar progenies (3927a and 4881) and of a progeny (3129b) bred from 2573(2) crossed with 2424a(5) – the clone possessing R_9 . Clone 2573(2) is thus credited with the genotype $R_1R_2R_3R_9$.

Since the new genes of the three clones were different from each other and from the six already established in *S. demissum*, the *S. demissum* series is continued with the genes of 2182ef(7), 2424a(5) and 2573(2) being designated R_7 , R_8 and R_9 respectively. It is of interest to note that R_7 and R_8 were derived from the same clone of *S. demissum* (C.P.C. 2172) as the four R genes already recognised in the standard series of differential hosts.

In these tests nine new races were derived from the clones inoculated with the two isolates of race 1, 2, 3, 4. They were all basically race 1, 2, 3, 4 since they could infect the $R_1R_2R_3R_4$ genotype (Table 3), and by means of the additional *S. demissum* hosts the different types of isolates (Table 2) may be designated as follows: E = race 1, 2, 3, 4, 5, 7; C = race 1, 2, 3, 4, 6, 7; H = race 1, 2, 3, 4, 7; D = race 1, 2, 3, 4, 7, 8; K = race 1, 2, 3, 4, 6, 7, 9; A = race 1, 2, 3, 4, 5, 7, 8; Z = race 1, 2, 3, 4, 5, 6, 7; Y = race 1, 2, 3, 4, 5, 6, 7, 8; X = race 1, 2, 3, 4, 6, 7, 8, 9. Race 1, 2, 3, 4, 5, 7 was the only derivative obtained from the isolate from The Netherlands but all nine races were obtained from the isolate from Canada. The Canadian culture thus appears to be much less stable than the one from The Netherlands.

TABLE 3. SEGREGATIONS OBTAINED IN PROGENIES INVOLVING GENES R_7 , R_8 AND R_9

Ref. No of progeny	Parentage	Race employed in testing progeny	Segregation		Major genes indicated in parents
			Observed R:r	Theoretical R:r	
4365a	2182ef(7) × M109-3	1, 2, 3, 4	71:79	1:1	R_7 × Recessive
3088a	2182ef(7) × 2288a(2)	4	50:46	1:1	R_7 × Recessive
2943c	Craigs Defiance × 2424a(5)	4	12:12	1:1	Recessive × R_8
2960a	Craigs Royal × 2424a(5)	4	13:11	1:1	Recessive × R_8
3010c	Saskia × 2424a(5)	4	24:24	1:1	Recessive × R_8
3092d	2182ef(7) × 2424a(5)	4	39: 9	3:1	R_7 × R_8
3392	2573(2) × M109-3	1, 2, 3, 4	38:34	1:1	R_9 × Recessive
3926c	2573(2) × M109-3	1, 2, 3, 4	23:25	1:1	R_9 × Recessive
3927a	2573(2) × M109-3	4	80: 0	15:1	$R_1R_2R_3R_4$ × Recessive
4881	M136-6 × 2573(2)	4	38: 2	15:1	Recessive × $R_1R_2R_3R_4$
3129b	2573(2) × 2424a(5)	4	95: 1	31:1	$R_1R_2R_3R_9$ × R_8

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M.R. Marano · I. Malcuit · W. De Jong
D.C. Baulcombe

High-resolution genetic map of *Nb*, a gene that confers hypersensitive resistance to potato virus X in *Solanum tuberosum*

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Abstract *Nb* is a single dominant gene in potato that confers hypersensitive resistance to potato virus X (PVX) isolates from strain groups 1 and 2. Genetic and molecular analyses showed that *Nb* is located on the upper arm of chromosome V and forms part of a cluster of resistance genes encoding specificities to many different pathogens. We describe the genetical localisation of molecular markers tightly linked to the *Nb* locus and the development PCR-based markers suitable for isolation of the *Nb* resistance gene by positional cloning. A bulked segregant approach was applied to identify polymorphic AFLP markers tightly linked to the *Nb* locus. These markers were mapped in a population of segregating S1 progeny (1,300 plants) from a self-pollinated potato cultivar, Pentland Ivory. From this analysis, *Nb* was placed in an interval of 0.76 cM, flanked by the AFLP markers GM339 and GM637. Recombinant PVX strains carrying different combinations of avirulence genes were used in biological assays to show that *Nb* was also present in potato cv. Cara but was masked by the extreme PVX resistance conferred by the *Rx* gene. PCR-based screening of a Cara genomic BAC library with markers closest to the *Nb* locus identified a new marker tightly linked to *Nb*.

Keywords *Nb* resistance gene · *Solanum tuberosum* · PVX strains · Amplified fragment length polymorphism · Map-based cloning

Introduction

Genetic control of resistance to pathogens in plants is often determined by simple gene-for-gene interactions. The resistance response is induced only if the pathogen encodes a strain-specific avirulence (*avr*) gene and the plant carries the corresponding disease resistance (*R*) gene (Flor 1971; Keen 1990). It has been proposed that *R* genes are members of multigene families encoding putative receptors that recognise specific molecules (ligands) derived directly or indirectly from pathogen *avr* genes. In many cases, this recognition is manifested as a hypersensitive response (HR) which is associated with a programmed cell death at the initial site of infection (Morel and Dangl 1997).

Over the last decade more than 20 *R* genes against pathogens as diverse as viruses, bacteria, fungi, insects and nematodes have been isolated by map-based cloning or transposon tagging in different plant species (reviewed by Meyers et al. 1999). It is currently unknown how many different types of *R* genes there are in plants. Analysis of the predicted protein sequences of these genes revealed that the most common types are proteins containing a nucleotide binding site (NBS) and leucine-rich repeats (LRRs) of various lengths which are potentially a ligand-specific recognition domain (reviewed by Ellis et al. 2000). These proteins can be divided into two major classes, depending on the presence or absence of an amino terminal toll/interleukin-1 like receptor motif (TIR) (Michelmore 2000). In the non-TIR class of proteins, the TIR domain is often replaced by a coiled-coiled (CC) structure, which in some cases has the features of a leucine zipper (LZ) (Young 2000). It is thought that the TIR and CC/LZ motifs are involved in signal transduction (Young 2000). These conserved structural motifs in *R* proteins suggest that plants have common

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M.R. Marano and I. Malcuit contributed equally to this work

M.R. Marano · I. Malcuit · W. De Jong · D.C. Baulcombe (✉)
The Sainsbury Laboratory, John Innes Centre, Colney Lane,
Norwich, NR4 7UH, UK
e-mail: baulcombe@sainsbury_laboratory.ac.uk
Fax: +44-1603-450011

Present addresses:

M.R. Marano, Instituto de Biología Molecular
y Celular de Rosario (CONICET), Departamento Microbiología,
Facultad de Ciencias Bioquímicas y Farmacéuticas,
Universidad Nacional de Rosario, Suipacha 531, 2000 Rosario,
Argentina

W. De Jong, Department of Plant Breeding, 405 Bradfield Hall,
Cornell University, Ithaca, NY 14853, USA

signal transduction pathways for different defense responses. However, the molecular basis of the *R-avr* recognition and the signalling programme activated immediately following this event is still largely unknown.

Two types of resistance to potato virus X (PVX) have been identified in *Solanum tuberosum*: hypersensitive resistance controlled by the genes *Nb* and *Nx* and extreme resistance (ER) conferred by the *Rx1* and *Rx2* genes. *Rx1* and *Rx2* are located on chromosome XII and V, respectively (Bendahmane et al. 1997; Ritter et al. 1991), and belong to the CC/LZ-NBS-LRR class of resistance genes (Bendahmane et al. 1999, 2000). *Nx* maps to chromosome IX (Tomniska et al. 1998), and *Nb* is located on chromosome V (De Jong et al. 1997) in a region where other loci, (*Rx2*, *R1*, *Gpa*, *Gpr1*) conferring resistance to many different pathogens have been mapped (Ritter et al. 1991; Kreike et al. 1994; Leonards-Schippers et al. 1992; Roupe van der Voort et al. 1998). For two of the *R* genes present in this region, the corresponding pathogen *avr* gene has been characterised. Induction of the *Nb*-mediated HR in potato cells is dependent upon the expression of the PVX 25-kDa viral movement protein (Malcuit et al. 1999), whereas the *Rx2*-mediated resistance is elicited by the viral coat protein (Bendahmane et al. 1995). In this paper we describe the localisation of molecular markers tightly linked to the *Nb* locus from potato cv. Pentland Ivory. These markers were generated using amplified fragment length polymorphism (AFLP) technology and during the screening of a potato BAC library from potato cv. Cara. The development of polymerase chain reaction (PCR) based markers suitable for the detection of *Nb* in practical breeding programmes will be useful for isolation of this gene by positional cloning and the genetical characterisation of the cluster of *R* genes on chromosome V.

Materials and methods

Plant material, virus strains, PVX cDNA clones and resistance assays

Tetraploid potato cv. Pentland Ivory carrying *Nb* in the simplex condition (*Nb nb nb nb*) (De Jong et al. 1997) was self-pollinated to produce an S1 population. Tetraploid potato cv. Cara carrying *Rx1* in duplex condition (*Rx1 Rx1 rx1 rx1*) has been described previously (Kanyuka et al. 1999).

PVX strains ROTH1, CP2, UK3 and HB have been described previously (Malcuit et al. 1999; Orman et al. 1990; Kavanagh et

al. 1992; Querci et al. 1993). PVX strains ROTH1, CP2 and UK3 belong to PVX strain groups 1, 2 and 3, respectively, based on the classification of Cockerham (1955). The plasmid pUIHB (Fig. 3) was constructed by substituting the *ApaI-AvrII* fragment from pUK3-Ile6 (Malcuit et al. 1999), which contains an avirulent version of the 25-kDa protein, into the Ti plasmid vector pGR103 (R. Lu, unpublished data), which carries the genomic sequence of UK3 with a coat protein derived from PVX strain HB (Fig. 3). The PVX cDNA is under the control of the CaMV 35S promoter.

Plants were tested for resistance to PVX avirulent strains by graft inoculation (De Jong et al. 1997) and/or particle bombardment of detached leaves (Malcuit et al. 1999; Marano and Baulcombe 1998). Plants were considered resistant (*Nb*) if they displayed HR (necrotic lesions) on leaves inoculated with PVX avirulent strains ROTH1 or CP2 and if no virus was detected on systemic leaves. Plants were considered susceptible (*nb*) if after challenging with ROTH1 or CP2 the plants became systemically infected with the virus. To monitor virus accumulation in infected plants, we carried out ELISA tests with polyclonal (ELISA kit for detection of PVX; Boehringer, Mannheim) and monoclonal antibodies MAC58 or MAC72 (Torrance et al. 1986) prepared against the PVX coat protein.

Histological assay

Aniline blue staining for the presence of callose deposition in potato leaves undergoing HR was performed as described previously (Dietrich et al. 1994). Leaves were examined and photographed under UV light with a Zeiss Axiophot epifluorescence microscope.

PCR-based screening of the mapping population and AFLP analysis

Genomic DNA for AFLP and PCR-based analyses was isolated according to Bendahmane et al. (1997). Primers, PCR conditions and restriction enzymes used for the markers GP21, SPUD839, TG432 and SPUD237 have been described previously (De Jong et al. 1997). To isolate DNA for the resistant (R) and susceptible (S) bulked segregant pools, equivalent amounts of leaf material from individuals in each class were pooled before extraction (Michelmore et al. 1991). Template DNA from the R and S pools as well as from each individual recombinant plant was prepared for AFLP as described previously (Thomas et al. 1995; Vos et al. 1995), using the restriction enzymes *PstI* and *MseI*. For selective amplification, 741 primer combinations were analysed: 13 *PstI*+2 primers (two selective nucleotides) and 57 *MseI*+3 primers (three selective nucleotides). AFLP reactions were performed essentially as described by Thomas et al. (1995). Selective amplification products were separated on a 4.5% polyacrylamide sequencing gel run at 100 W for 2.5 h. After electrophoresis, gels were vacuum-dried onto 3 MM Whatman paper (Whatman, Maidstone, UK) and exposed to X-ray film (BioMax MR, Kodak) overnight. Bands of interest were cut out of the gel with a scalpel and incubated in 150 µl of TE (10 mM Tris pH 7.5, 1 mM EDTA pH 8.0) overnight

Table 1 PCR-based markers linked to the *Nb* locus. For each marker, the sequence of the primers and the PCR conditions are shown

Marker ^a	Primer (5'→3')	PCR conditions ^b (35 cycles)
GM339	GGT AGT TGG ACG AGC ATA T CTC ACT TTT AGA CCA GAT TT	94 °C, 15 s; 52 °C, 15 s; 72 °C, 40 s
GW339	GGC TGG TAC TAA CAG CAA GCC AAT GGG CTG GTA TTC AAA AAT CGT CTC	94 °C, 20 s; 52 °C, 15 s; 72 °C, 60 s
GM637	GCA GAA GAT CGG ATA GCA AAC GTA ACG AGT TGA AGT TAC TGA	94 °C, 15 s; 55 °C, 15 s; 72 °C, 40 s
98L	GAA TGA TGA AGA AAG ACA TC GTT AAG GAC ACA TAT ATG AC	94 °C, 15 s; 52 °C, 15 s; 72 °C, 50 s

^a Allele-specific markers (i.e. primer sets only amplify the allele linked in *cis* to *Nb*)

^b All PCR reactions were started with an initial denaturation step of 2 min at 94 °C

at 37 °C. AFLP fragments were recovered by PCR using the same conditions as the initial amplification. PCR products were cloned into the pGEM-T vector (Promega, Madison, Wis.). To confirm that the cloned DNA was the same size as the original AFLP marker, we carried out an AFLP reaction on the plasmid DNA. The products were fractionated by electrophoresis alongside the original selective amplification products from the bulked segregants. Cloned AFLP markers were then sequenced and converted to PCR-based markers. The sequences and PCR conditions used for derivatives of AFLP markers are shown in Table 1.

Inverse PCR for genome walking

An uncloned library prepared from potato DNA for genome walking by PCR was constructed using the Universal GenomeWalker kit according to the manufacturer's instructions (Clontech, Palo Alto, Calif.). For the construction of each library, 2.5 µg of genomic DNA from the R pool was digested with five different restriction enzymes (*Dra*I, *Eco*RV, *Pvu*II, *Sca*I, *Stu*I). After phenol extraction, the DNA was ethanol-precipitated, resuspended in 20 µl of TE and ligated to genome walker adaptors with 0.5 U of T4 DNA ligase (Boehringer, Mannheim) at 4 °C overnight. This DNA library was used as a template in a primary PCR reaction using the AP1 primer and primers designed based on the ends of the marker GM339. An aliquot of this PCR product was used as template in a second PCR reaction with the AP2 primer and "nested" primers designed based on the ends of the GM339 sequence. The PCR products obtained were cloned directly into the pGEM-T vector and sequenced. AP1 and AP2 primers were provided with the kit.

Sequencing of PCR products and BAC ends

PCR products were cloned into pGEM-T, amplified using M13 universal and reverse primers (Amersham Pharmacia Biotech) and purified using the QIAquick PCR extraction kit (QIAGEN, Valencia, Calif.). The right and left end-sequences of insert DNA from the Cara BAC library were isolated by inverse PCR (IPCR) according to Kanyuka et al. (1999). Sequencing reactions were carried out using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster city, Calif.) according to the manufacturer's instructions. Database searches were performed using the basic local alignment search tool (BLAST; Altschul et al. 1990).

Results

Identification of recombination events in the GP21-SPUD237 interval

De Jong et al. (1997) was previously mapped *Nb* on chromosome V in a 3.3-cM interval delimited by markers GP21/SPUD839 and TG432/SPUD237. We extended the original mapping population to a total of 1,300 S1 progeny to allow the construction of a high-resolution map around the *Nb* locus. This S1 population was screened for recombination events using PCR analysis of DNA samples from individual plants with primers derived from GP21 and SPUD237 markers as described previously (De Jong et al. 1997). The segregation of polymorphism for both markers was 3:1 (971 plants from a total population of 1,300 S1 individuals), which is consistent with *Nb* being in simplex condition in cv. Pentland Ivory. Twenty-six individual plants were identified with recombination events in the interval between

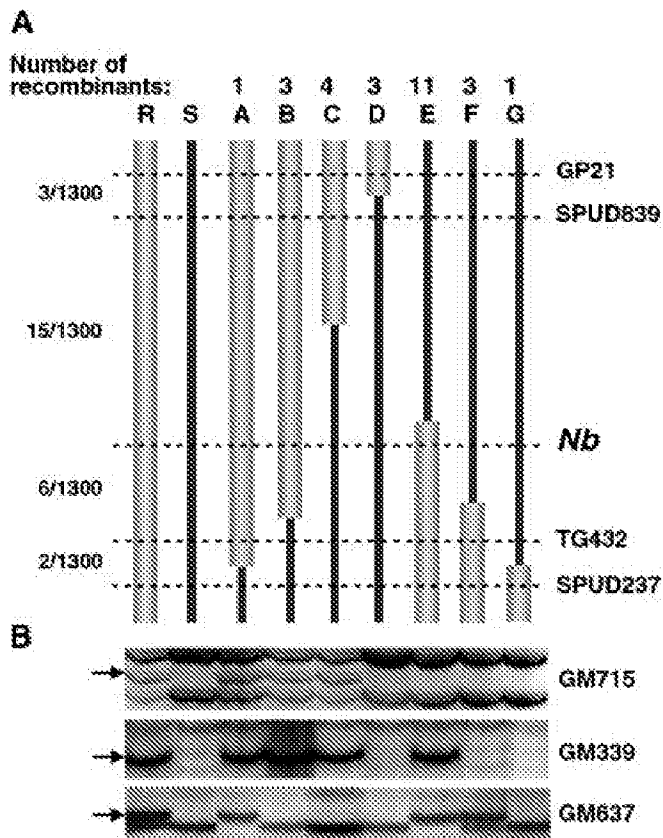


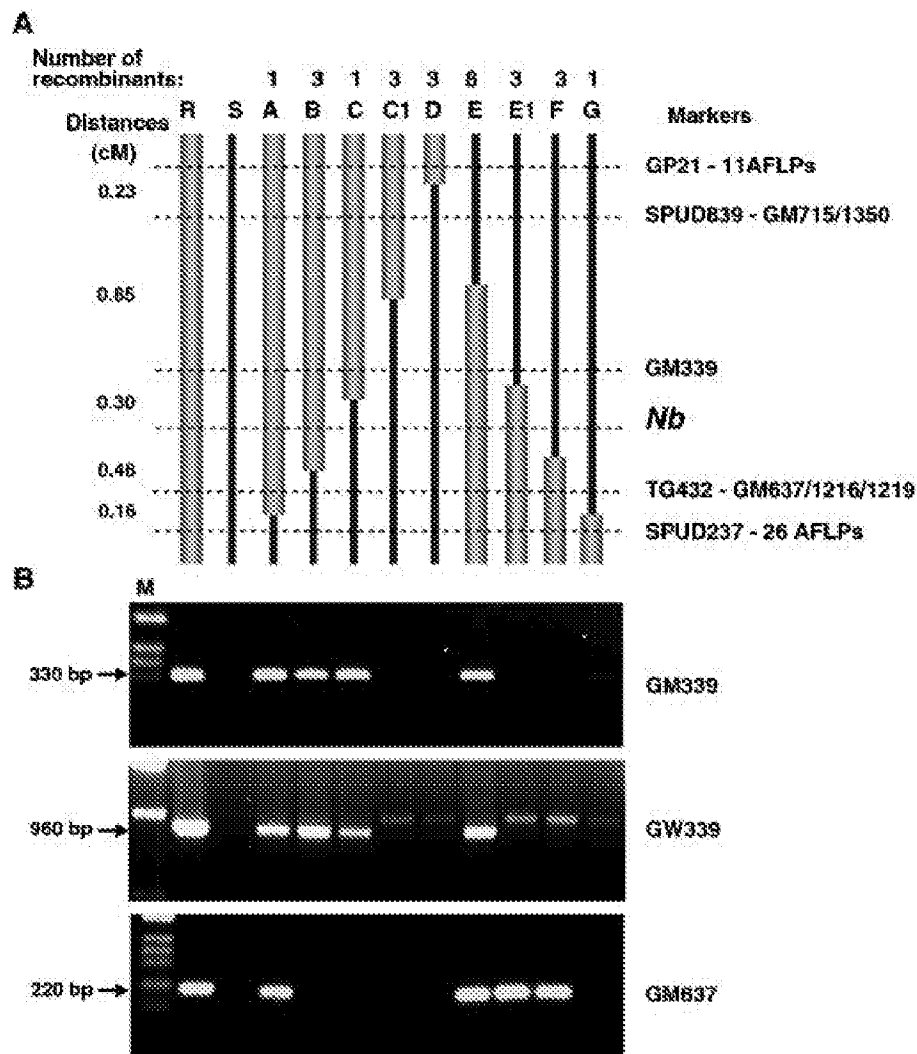
Fig. 1A, B Segregation patterns observed for markers closely linked to *Nb*. **A** Schematic representation of recombinant classes identified around the *Nb* locus (A–G). The resistant (R, solid bar) and susceptible (S, black line) genotypes are shown. Markers defining recombination sites are indicated on the right. The number of recombinants plants (from a total population of 1,300) belonging to a particular class are shown on the left. The number of AFLP markers identified for each interval is also shown. **B** Examples of AFLP markers (one of each class) identified in the intervals indicated in A. Lanes correspond to the recombinant classes shown in A (R, S, A, B, C, D, E, F and G). The name of the AFLP markers is shown on the right. Arrows indicate the linked AFLP fragment to *Nb*.

markers GP21 and SPUD237. The markers SPUD839 and TG432 initially co-segregated with GP21 and SPUD237, respectively (De Jong et al. 1997). After screening of the recombinant plants, these markers were separated from GP21 and SPUD237 by three and two recombination events, respectively (Fig. 1A). Recombinant plants were then tested for resistance to PVX-avirulent strains CP2 or ROTH1 and divided into seven classes according to their resistance/susceptibility phenotype and the molecular pattern defined by each AFLP marker (Fig. 1A). *Nb* was mapped between SPUD839 and TG432 at 1.15 cM and 0.46 cM, respectively (Fig. 1A).

High-resolution genetic map around the *Nb* locus

A high density genetic map of the region carrying *Nb* was required to facilitate the positional cloning of this gene.

Fig. 2A, B High-resolution genetic map of the *Nb* locus. **A** Map position of the *Nb* locus based on the screening of 1,300 segregant plants with AFLP markers linked to the *Nb* locus in the GP21-SPUD237 interval. The genetic distance (centiMorgan, cM) corresponds to the percentage of recombination between molecular markers or molecular markers and *Nb* in a population of 1,300 plants. **B** Shows the analysis of individual plants [resistant to PVX-ROTH1 (*R*) or susceptible (*S*)] with recombination events in the GP21-SPUD237 interval using the GM339 and GM637 PCR-based markers. The *R* pool and *S* pool are indicated as *R* and *S* respectively. Arrows show the positions of the molecular markers



To identify DNA markers in the region delimited by the markers SPUD839 and TG432 (containing *Nb*), AFLP technology (Vos et al. 1995) was employed in conjunction with a bulked segregant approach (Michelmore et al. 1991). The AFLP analysis was carried out on DNA pooled from 20 *R* and 20 *S* plants selected from the S1 progeny of potato cv. Pentland Ivory. A total of 741 randomly selected *Pst*I/*Mse*I primer combinations were analysed. Each AFLP primer combination displayed, on average, 100 DNA fragments so that approximately 74,100 loci were evaluated for polymorphism, from which 69 primer combinations revealed polymorphisms between the *R* and *S* pools. To map these new polymorphic markers relative to SPUD839 and TG432, we repeated the AFLP analysis using the recombinants detected in the GP21 and SPUD237 interval. Thus, DNA samples from recombinant plants of classes A, B, C, D, E, F and G (Fig. 1A) were assayed with the 69 informative primer combinations. Of these, 11 AFLP markers co-segregated with GP21, six AFLP markers (GM715, GM1350, GM339, GM637, GM1216 and GM1219) were mapped in the interval defined by SPUD839 and TG432 (Table 2,

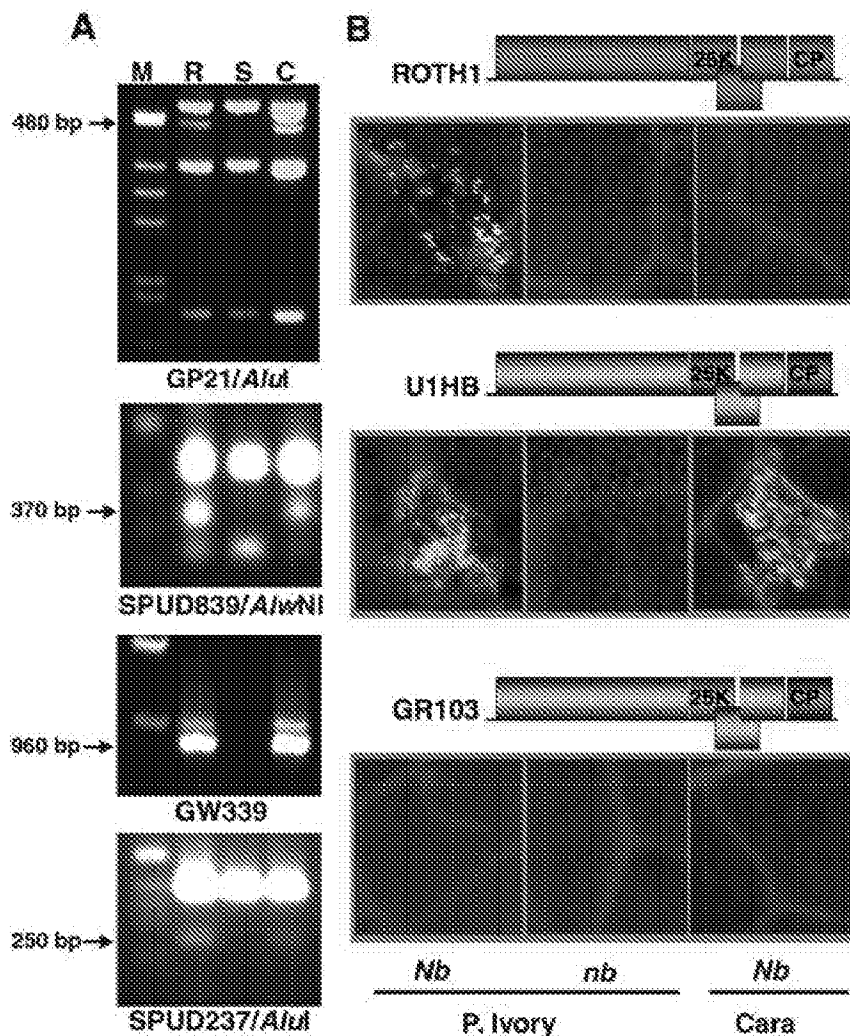
Table 2 Selective bases for amplification of AFLP loci linked to *Nb* in potato

Marker	AFLP primers <i>Pst</i> I+2/ <i>Mse</i> I+3
GM715	CC/GTA
GM1350	TG/TAT
GM339	GG/CTC
GM1216	GT/GTT
GM1219	GT/CTT
GM637	AA/CGA

Figs. 1B, 2A), 26 AFLP markers co-segregated with SPUD237 and the remaining 26 markers were mapped outside the GP21-SPUD237 interval.

In the second step, the AFLP markers GM715, GM1350, GM339, GM637, GM1216 and GM1219 were assigned to more precise chromosomal positions by AFLP analysis of individuals of each class of recombinant plants. GM715 and GM1350 co-segregated with SPUD839. GM339 mapped between SPUD839 and *Nb* at 11 and four recombination events, respectively. The other three markers (GM637, GM1216 and GM1219) co-segregated with TG432 (Fig. 2A). Results from this

Fig. 3A, B Molecular and biological assays for the presence of *Nb* in Cara. **A** Analysis of genomic DNA from potato cv. Pentland Ivory (*R*) and (*S*) pools and potato cv. Cara (*C*) with markers GP21, SPUD839, GW339 and SPUD237. The PCR products were digested with *AluI* for the GP21 and SPUD237 markers and with *AflwNI* for the SPUD839 marker. The DNA fragments linked in *cis* to the *Nb* locus are marked by an arrow. **B** Pentland Ivory resistant (*Nb*) and susceptible (*nb*) leaves and Cara leaves were inoculated by particle bombardment with the PVX avirulent strain ROTH1 and the avirulent (U1HB) or the virulent (RG103) PVX hybrid. Leaves were stained with aniline blue at 4 days post-inoculation to detect callose deposition as an indicator of HR



analysis place *Nb* in an interval of 0.76 cM, flanked by the marker GM339 and the cosegregating markers TG432, GM637, GM715 and GM1219 (Fig. 2A). To develop a fast screening method, the AFLP markers closest to *Nb*, GM339 and GM637, were converted to PCR markers. GM339-derived primers amplified a 330-bp fragment and GM637-derived primers, a 220-bp fragment (Fig. 2B). The GM339 and GM637 primers amplified only the resistant allele and were used to analyse the 26 plants with recombination events between GP21 and SPUD237. The results obtained from this analysis were in accordance with the map presented in Fig. 2A.

The presence of repeated DNA sequences has been shown to cause allele-specific primers to identify BAC clones from the susceptible chromosome or from non-allelic sites. Presumably, this artefact results from the high abundance of primer-binding sites in the cloned DNA (Kanyuka et al. 1999). Therefore, to confirm our results we carried out Southern analysis on the DNA of the R and S pools digested with restriction enzymes *HindIII*, *EcoRI* and *EcoRV* using markers GM339 and GM637. The results showed that each marker was present as a single copy and exclusively in the resistant pool (data not shown).

Resistance test for the presence of *Nb* in potato cv. Cara

Potato cvs. Pentland Ivory and Cara share part of their genetic background (Cockerham 1955; Hawkes 1994). To find out whether this common background includes *Nb*, we carried out PCR analysis with GP21, SPUD839, GM339, TG432, GM637 and SPUD237. In each instance, the amplified fragments from cv. Cara genomic DNA showed the same restriction pattern as the equivalent fragments of cv. Pentland Ivory (Fig. 3A; data not shown). Moreover, the nucleotide sequence of the two allele-specific markers GM339 and GM637 were identical in both cultivars, and it seemed likely that *Nb* could be present in cv. Cara. However, we could not test this hypothesis because *Rx1* protects cv. Cara from most natural strains of PVX. If both *Nb* and *Rx1* resistance genes are present in cv. Cara, the *Nb*-mediated response would be masked by the extreme resistance response conferred by *Rx1* (Cockerham 1970). Therefore, to find out whether cv. Cara carries a functional *Nb* we carried out a series of tests with recombinant PVX isolates that were based on the naturally occurring PVX strains UK3, HB and ROTH1. The design of the recombinant isolates exploited our previous finding that the coat protein in UK3 is an *Rx*

Fig. 4 Sequence analysis of marker 98L. The nucleotide sequence alignment of marker 98L with a sequence corresponding to a cluster of resistance genes on chromosome XII (StRGC, Van der Vossen et al. 2000) was produced with the BLAST programme (Altschul et al. 1990)

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Solanum tuberosum resistance gene cluster (StRGC), complete sequence
Length = 187352
Score = 325 bits (164), Expect = 3e-86
Identities = 393/462 (85%), Gaps = 9/462 (1%)
Strand = Plus / Plus

98L : 163      tcagttcatttgattttctagatacatctcaaatttggtgtatctttgcatacacacaga 222
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
StRGC: 111154 tcagttcaattgtattttctatatatatctcaggcttattgtatccttgatacacacaga 111213

98L : 223      gtttatagcatgttttaaacacatctttctttatattgcactgttttaaaattgccttat 282
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
StRGC: 111214 gtttatagcatgttttaaacagctctt-ctttatatcgctctgttttaaaattgccttat 111272

98L : 283      attgaaatgagtcagtcaggttgagttatggtgagccaggtaagttattcaatttttta- 341
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
StRGC: 111273 attgaaagaagtcagtcaggttgagttgacttgagcaaggtaaaacttttcagtttcttcc 111332

98L : 342      agatttccttctagcttatgttgcttagcatttcaactcacatactcgtaacattcaatgn 401
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
StRGC: 111333 aaatttccttctagccttatgttgcttagtttccagctcgacatactcgtaacattca-tgt 111391

98L : 402      actaatgccagttggcctgcacgtcttattgatgcagatttaggtaaccagaatcagcat 461
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
StRGC: 111392 actgatgccagttggcctgcacgtcttattgatgcagatataggtaaccggatcagcat 111451

98L : 462      ccagcgctcgttgatccagttgagcagttcagagtcagtggtgagcctccttgcatcca 521
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
StRGC: 111452 cctgcacgtcgttgatccagctgagcgctccagagtcagtggtgagcctccttgcatccc 111511

98L : 522      ggaggactctcttatt---tgcttttagnnnttcatttattaaggatggtgtggggtttg 578
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
StRGC: 111512 gaaggactcaattattttgtgtctctagtttttatttatt-aggatggtgagggtctg 111570

98L : 579      tcccaaccatccatctcagttatttttagaggtttatagaca 620
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
StRGC: 111571 tcccaa-catccatctcagta-ttttagaggcttcatagaca 111610

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avirulence determinant (Bendhamane et al. 1995) and that the 25 kDa protein in ROTH1 is an *Nb* avirulence determinant (Malcuit et al. 1999). Thus, we examined the recombinant PVX isolate GR103 (R. Lu, unpublished data) that is based on the UK3 isolate but with a coat protein derived from the *Rx* resistance-breaking strain HB (Fig. 3B). A second recombinant PVX isolate, U1HB, which is based on GR103 but with the *Nb* avirulence-determining 25-kDa protein derived from ROTH1 (Fig. 3B). GR103 is able to overcome resistance specified by both *Rx* and *Nb*, U1HB is able to induce *Nb*-mediated HR but not *Rx1*-mediated extreme resistance and the natural isolate ROTH1 is recognised by plants carrying both resistance genes.

To test the presence of *Nb* in cv. Cara, we carried out particle bombardment of detached leaves (Malcuit et al. 1999) to inoculate PVX, followed by aniline blue staining for callose deposition as an indicator of HR (Fig. 3B). HR lesions developed on cvs. Cara- and Pentland Ivory-resistant (*Nb*) leaves bombarded with U1HB, whereas leaves bombarded with GR103 did not (Fig. 3B). Cultivar Cara leaves bombarded with ROTH1 did not develop HR lesions, whereas cv. Pentland Ivory leaves did (Fig. 3B). These results indicate that *Nb* is present in cv. Cara and that *Rx1* is epistatic to *Nb*.

Identification of new markers linked to *Nb* derived from a cv. Cara BAC clone

To identify more markers in the vicinity of *Nb*, we analysed a potato BAC library prepared from cv. Cara (Kanyuka et al. 1999). The screening of this library with markers tightly linked to *Nb* identified several positive BAC pools with GP21, SPUD839, TG432, GM637 and SPUD237 (data not shown).

One of the positive BAC clones identified (BAC98) was found to carry the TG432 marker and allele-specific marker GM637 within a genomic DNA insert of 120 kb. *RsaI* digestion of the TG432 PCR product revealed that BAC98 carried the TG432 allele linked in *cis* to *Nb* (data not shown). This BAC was selected as a potential source of markers linked to *Nb*.

To determine the end sequence of BAC98, we isolated its left (L) and right (R) ends by IPCR (Kanyuka et al. 1999), determined their nucleotide sequences and produced primers for the PCR-based markers 98L and 98R. The 98L marker amplified one band of 650 bp in BAC98 linked in *cis* to *Nb* (Fig. 4). Sequence analysis of this marker revealed that 98L from cvs. Cara and Pentland Ivory were identical. Analysis of the cv. Pentland Ivory recombinant plants with this marker showed that 98L co-segregated with GM637 and TG432. The 98R marker amplified BAC35 and BAC195, which also contained SPUD237 (data not shown). Further PCR screening of the cv. Cara BAC library with 98L and GM339 failed to

identify a BAC clone that spanned the gap between both markers.

As GM339 is only 330 bp in length, we used a genome walking strategy (see Materials and methods) to clone its flanking sequences. The resulting new marker, GW339, was 960 bp in length (Fig. 2B) and was used to re-screen the cv. Cara BAC library. This improved marker also failed to identify any BAC clone that would close the gap between GM339 and 98L.

Interestingly, the marker 98L shows homology in parts to a sequence in a region of the potato genome corresponding to a cluster of resistance genes from chromosome XII (Van der Vossen et al. 2000) (Fig. 4).

Discussion

In this report we describe new molecular markers tightly linked to the *Nb* locus. As part of the strategy for cloning *Nb*, a large number of segregating S1 progeny (1,300 plants) from a self-pollinated potato cultivar, Pentland Ivory, has been screened, and a high-resolution genetic map was constructed around the *Nb* locus using AFLP technology (Vos et al. 1995) in conjunction with a bulked segregant approach (Michelmore et al. 1991). A total of 69 *Pst*I/*Mse*I primer combinations showed polymorphism between the R and S pools. From these, only six were found in the interval between DNA markers GP21 and SPUD237, spanning a region of 2 cM, whereas 11 and 26 AFLP markers co-segregated with GP21 and SPUD237, respectively, indicating that there is a distortion in the distribution of markers in this region. The lower degree of polymorphism in this interval could be due to the fact that *Pst*I recognises a GC-rich hexanucleotide sequence that could be subjected to methylation (McClelland et al. 1994). Therefore, this methylation-sensitive enzyme would cleave preferentially in transcribed regions that are normally hypo-methylated and where the level of sequence conservation is high (Antequera and Bird 1988). Like most cultivated species from the *S. tuberosum* ssp. *tuberosum* group, Pentland Ivory was derived from *S. tuberosum* ssp. *andigena* that originally carried the *Nb* gene (Jones 1981; Ross 1986). Previous studies have shown that there is variation in the frequency of recombination in areas that have been introgressed from alien species (Kanyuka et al. 1999; Liharska et al. 1996). Therefore, the low level of polymorphism between GP21 and SPUD237 could also be explained by the presence of introgressed DNA from *S. tuberosum* ssp. *andigena* into cv. Pentland Ivory.

In the high-resolution genetical map described above, we positioned the *Nb* locus in an interval of about 0.76 cM between the AFLP markers GM339 and GM637 (Fig. 2A). Given that the average recombination frequency in potato is about 1,000 kb cM⁻¹ (Tanksley et al. 1992), the distance between the closest flanking markers would correspond to approximately 760 kb. However, previous studies in potato have shown that the relationship between genetical and physical distances can vary

considerably. For instance, in the case of potato cv. Cara, which was used to isolate the *Rx1* gene, recombination frequencies were found to vary from 180 kb cM⁻¹ to 2,677 kb cM⁻¹, estimated from the number of recombination events in individual BAC clones (Kanyuka et al. 1999). Therefore, the physical distance in the genetical interval between markers GM339 and GM637 cannot be estimated accurately at this stage. However, the small number of AFLP markers identified near the GP21 and SPUD237 interval and their resistant-allele specificity may indicate that the physical distance in this genetic interval is small.

Here we show that *Nb* is present in cv. Cara using a biological assay with a recombinant virus derived from the *Rx* resistance-breaking strain PVX-HB that carries the avirulent 25-kDa protein that is recognised by *Nb* (Malcuit et al. 1999). Screening of the cv. Cara BAC library identified one clone containing the co-segregating markers TG432 and GM637 from the resistant allele within a genomic insert of 120 kb. A new resistant allele-specific marker, 98L, was generated from the left end of this BAC clone. In addition, we found that *Nb*-flanking markers GM339, GM637 and 98L were identical at the nucleotide level in both cvs Cara and Pentland Ivory. The likely explanation for the presence of *Nb* in both cultivars is that *S. tuberosum* ssp. *andigena* was used in breeding programmes to introgress pathogen resistance into cultivated potato cultivars, including cvs. Cara and Pentland Ivory (Hawkes 1994). At least eight virus resistance genes have been identified in *S. tuberosum* ssp. *andigena*, including PVX resistance genes *Nb* and *Rx1* (Cockerham 1970; Jones 1981). The presence of both *Rx1* and *Nb* in Cara and the fact that *Rx1* is epistatic to *Nb* indicates that there is a lack of selection pressure on the *Nb* gene in this cultivar.

Genetic and molecular analyses have demonstrated that many *R* genes and *R* gene homologues are clustered in the genome of different species (Ellis et al. 1999; Meyers et al. 1998; Parniske et al. 1997). Based on these findings and more detailed analyses of linked *R* genes it has been proposed that the evolution of *R* genes involves intragenic recombination, unequal crossing-over and gene conversion (Ellis et al. 1999; Meyers et al. 1998; Parniske et al. 1997). Resistance to PVX maps to two major clusters of *R* genes in potato. The cluster located on chromosome V contains at least five resistance loci: *R1* conferring resistance to *Phytophthora infestans* (Leonards-Schippers et al. 1992); *Rx2* and *Nb* conferring extreme and hypersensitive resistance to PVX (De Jong et al. 1997; Ritter et al. 1991); *Gpa* and *Gpr1* that confer resistance to the potato cyst nematode species *Globodera pallida* and *Globodera rostochiensis* (Kreike et al. 1994; Rouppe van der Voort et al. 1998). The resistance gene cluster located on chromosome XII contains *Rx1* and *Gpa2*, and these genes confer resistance to PVX and *Globodera pallida*, respectively (Bendahmane et al. 1997; Rouppe van der Voort et al. 1997). *Rx1*, *Rx2* and *Gpa2* were characterised at the molecular level: *Rx2* shows 95% and 88.7% sequence identity to the *Rx1* and

the Gpa2 proteins, respectively. The high homology between sequences from these two clusters indicates that there is some evolutionary relationship between these resistance genes (Bendahmane et al. 2000; Van der Vossen et al. 2000). Interestingly, marker 98L, which is tightly linked to *Nb*, showed significant homology at the nucleotide level to sequences within the R gene cluster on chromosome XII (Van der Vossen et al. 2000) (Fig. 4). The presence of homologous sequences on two different chromosomes in cv. Cara could be explained by unequal crossing-over during recombination of R gene clusters or gene conversion between unlinked loci (Bendahmane et al. 1999; Meyers et al. 1998; Parniske et al. 1997).

The cloning and characterisation of the *Nb* gene might reveal new domains involved in the recognition of pathogen elicitors and could provide some clues to explain the differences between HR type of resistance (*Nb*) and extreme resistance (*Rx*) against PVX, which are elicited by two different virus-encoded proteins (Bendahmane et al. 1995; Malcuit et al. 1999). In addition, *Nb* is particularly important because of its genetic linkage to other loci conferring resistance against fungus, nematodes and other viruses. Consequently, the isolation of *Nb* and the physical analysis of this cluster of R genes will provide a good experimental system to study the evolution of R genes in potato and the mechanisms by which plants generate new recognition specificities.

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W. Marczewski · J. Hennig · C. Gebhardt

The *Potato virus S* resistance gene *Ns* maps to potato chromosome VIII

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Abstract The dominant allele *Ns* confers in potato resistance to *Potato virus S* (PVS). To identify the chromosomal location of *Ns*, we mapped the *Ns*-linked marker SCG17₄₄₈ and the ISSR marker UBC811₆₀₀ to linkage group VIII of the RFLP map of a population that did not segregate for *Ns*. The map position of the *Ns* locus on chromosome VIII was confirmed with the detection of linkage between *Ns* and three RFLP markers, GP126, GP189 and CP16, known to be located in a corresponding region on potato chromosome VIII. PCR-based assays were developed for these RFLP markers. The PCR primers specific for GP126 generated polymorphic products (STS marker). In the case of markers GP189 and CP16, informative polymorphism was revealed in the *Ns* population after digestion with the restriction enzymes *Hae*III and *Hind*III, respectively. The genetic distance between *Ns* and the closest *CP16* locus was 4.2 cM.

Keywords *Potato virus S* · Resistance gene *Ns* · *Solanum tuberosum* · Genetic mapping · PCR markers

Introduction

The dominant *Ns* gene, originating from *Solanum tuberosum* ssp. *andigena* (Baerecke 1967), confers resistance to *Potato virus S* (PVS). Plants expressing *Ns* re-

main symptomless, and no PVS titers develop in enzyme-linked immunosorbent assays (ELISA). In graft inoculation tests, the resistant plants react with a fading of the foliage of shoots developed from the axillary meristems as a result of the hypersensitive reaction of the plants to PVS infection. Depression of tuber formation in such plants is also observed (Marczewski et al. 1998). A cross between diploid potato (*Solanum tuberosum* L.) clones DW 91-1187 and DW 83-3121 was used to identify random amplified polymorphic DNA (RAPD) (Marczewski et al. 1998), sequence-characterized amplified region (SCAR) (Marczewski et al. 2001b) and inter-simple sequence repeat (ISSR) (Marczewski 2001) markers linked to the *Ns* locus. The markers SCG17₃₂₁ (Marczewski et al. 2001b) and UBC811₆₆₀ (Marczewski 2001) are currently being used for indirect selection of the *Ns* resistance gene in diploid breeding programs at the Plant Breeding and Acclimatization Institute at Młochów.

Nineteen single dominant genes (*R* genes) for resistance to various pathogens have been positioned on the molecular maps of potato using DNA markers. Seven genes confer resistance to important potato viruses: *Potato virus Y* (PVY), *Potato virus X* (PVX) and *Potato virus A* (PVA) (reviewed in Gebhardt and Valkonen 2001). A single gene might also confer resistance to *Potato leafroll virus* (PLRV) in potato (Marczewski et al. 2001a).

Here, we report the chromosomal localization of the *Ns* locus based on mapping the *Ns*-linked markers on the restriction fragment length polymorphism (RFLP) map of population K31 (Schäfer-Pregl et al. 1998).

Materials and methods

Plant materials

The parental diploid potato (*Solanum tuberosum* L.) clones, DW 91-1187 (susceptible) and DW 83-3121 (resistant), and 119 F₁ hybrids (*Ns* population) were available to map the *Ns* locus. The resistance test to PVS infection has been described by Marczewski et al. (1998). Sixty and fifty-nine F₁ individuals were determined

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W. Marczewski (✉)
Plant Breeding and Acclimatization Institute,
Platanowa 19, 05831 Młochów, Poland
e-mail: w.marczewski@ihar.edu.pl
Fax: 48-22-7299247

J. Hennig
Institute of Biochemistry and Biophysics,
Polish Academy of Sciences, Pawińskiego 5A,
02106 Warsaw, Poland

C. Gebhardt
Max-Planck Institut für Züchtungsforschung,
Carl von Linne Weg 10, Cologne, 50829 Germany

as being susceptible and resistant to PVS infection, respectively. The 1:1 segregation ratio of resistant versus susceptible F_1 plants indicated that the resistant parent was heterozygous for the single dominant *Ns* gene.

Polymerase chain reaction (PCR) amplification

DNA extraction, PCR amplification with a primer UBC811 and electrophoresis were performed as previously described (Marczewski 2001). The PCR conditions used to amplify the DNA fragment SCG17₄₄₈ and patterns of the products generated after *MunI* digestion were as described by Marczewski et al. (2001b).

PCR analyses of RFLP markers GP126, GP189 and CP16 were performed in 20 mM Tris-HCl pH 8.4, 50 mM KCl, 2.5 mM MgCl₂, 0.1 mM of each deoxynucleotide, 10 ng of each primer, 0.4 U *Tag* DNA polymerase (Gibco BRL) and 20 ng of genomic DNA in a final volume of 20 μ l. The primer sequences, listed in Table 1, were designed according to the Primer Select Program (DNA STAR, Madison, Wis. version for Windows, 3.10) based on DNA sequences of the RFLP markers (C. Gebhardt, unpublished). The PCR parameters were: 94 °C for 60 s, followed by 35 cycles of 94 °C for 15 s, 55 °C for 15 s, 72 °C for 60 s, and a final extension time of 3 min at 72 °C.

Mapping of the *Ns* locus

DNA samples of parental lines P3 and P38 and 87 F_1 plants of the diploid mapping population K31 (Schäfer-Pregl et al. 1998) were used to map the two *Ns*-linked markers SCG17₄₄₈ (Marczewski et al. 2001b) and UBC811₆₆₀ (Marczewski 2001) on the RFLP map covering the 12 potato chromosomes that was constructed in the K31 population. Scoring and linkage analysis of PCR-derived DNA fragments in the K31 population was the same as for RFLP

fragments and has been described by Ritter et al. (1990), and Schäfer-Pregl et al. (1998).

Linkage analysis between PCR markers and *Ns* was performed using LINKAGE-1 software (Suiter et al. 1983). Map distances in centiMorgans (cM) were calculated from recombination frequencies by using Kosambi's mapping function (Kosambi 1944).

Results and discussion

Polymorphism of a single *Ns*-linked PCR fragment of 448 bp, designated SCG17₄₄₈ (Marczewski et al. 2001b), was revealed after *MunI* digestion in the parental lines of the K31 cross (Fig. 1, lanes 5 and 6). The *Ns*-linked ISSR marker UBC811₆₆₀ (Fig. 1, lane 2) was not amplified in the parental DNA samples. Instead, a 600-bp ISSR product was observed in the parent P3 (Fig. 1, lane 3), while a 800-bp ISSR product was amplified in the parent P38 (Fig. 1, lane 4). This indicated the presence of different insertion/deletion alleles of the UBC811₆₆₀ marker in the K31 population and/or the detection of different loci.

Eighty-seven F_1 clones of the K31 population were screened for segregation of both *MunI*-digested SCG17₄₄₈ and the two ISSR fragments. The 800-bp ISSR fragment mapped on linkage group XII of the K31 map, linked by 1 cM to the RFLP locus GP183. The 600-bp ISSR fragment and the marker SCG17₄₄₈ both mapped to linkage group VIII. The two marker fragments were linked in repulsion by 2 cM. The most closely linked RFLP loci (1–5 cM) were GP293(b) and GP171 (Schäfer-Pregl et al. 1998). To confirm its position on chromosome VIII, we mapped the *Ns* locus relative to three extra markers, GP126, GP189 and CP16, that map to the same region on potato chromosome VIII as GP171 (Gebhardt et al. 1991) in the *Ns* population. PCR performed with GP126-specific primers resulted in polymorphic PCR products (STS, sequence tagged site). Two DNA fragments, 750 bp and 700 bp in length, were amplified in the resistant parent DW 83-3121 (Fig. 1, lane 8). The 750-bp fragment was not amplified

Table 1 Primer sequences used for PCR amplification of the RFLP loci GP126, GP189 and CP16

Marker name	Forward and reverse primer sequences (5' to 3')
GP126	TAGCCGTTGCCACCCTACA TTGTTGAAGTCTAAGAAATCTGTT
GP189	AGTTGAGGAGCTGTTTGTGA AGGCTTTAGTATTTCTGTGTATTT
CP16	CTTAAACGCGTCAAGTGAAACT TTAGGGACATACAAACAAACCTCA

Table 2 Summary of linked loci, phenotypic frequencies, χ^2 values for goodness-of-fit to expected segregation ratio (two-point: χ^2 AB) and recombination frequency between loci (SE standard error)

	Locus		Phenotypes ^a				χ^2 AB ^b	Recombination frequency $\pm$ SE
	A	B	AB	Ab	aB	ab		
	GP126	<i>Ns</i>	55	9	4	51	73.2	10.9 $\pm$ 2.9
	GP189	<i>Ns</i>	56	8	3	52	79.7	9.2 $\pm$ 2.7
	CP16	<i>Ns</i>	58	4	1	56	100.1	4.2 $\pm$ 1.9
	UBC811 ₆₆₀	<i>Ns</i>	59	1	0	59	115.1	0.8 $\pm$ 0.8
	SCG17 ₄₄₈	<i>Ns</i>	58	1	1	59	111.1	1.7 $\pm$ 1.2
	GP126	GP189	62	2	2	53	103.5	3.4 $\pm$ 1.7
	GP126	CP16	58	6	4	51	82.4	8.4 $\pm$ 2.5
	GP126	UBC811 ₆₆₀	56	8	4	51	76.1	10.1 $\pm$ 2.8
	GP126	SCG17 ₄₄₈	55	9	4	51	73.2	10.9 $\pm$ 2.9
	GP189	CP16	59	5	3	52	89.2	6.7 $\pm$ 2.3
	GP189	UBC811 ₆₆₀	57	7	3	52	82.7	8.4 $\pm$ 2.5
	GP189	SCG17 ₄₄₈	56	8	3	52	79.7	9.2 $\pm$ 2.7
	CP16	UBC811 ₆₆₀	59	3	1	56	103.7	3.4 $\pm$ 1.7
	CP16	SCG17 ₄₄₈	58	4	1	56	101.1	4.2 $\pm$ 1.8
	UBC811 ₆₆₀	SCG17 ₄₄₈	59	1	0	59	115.1	0.8 $\pm$ 0.8

^a Phenotypes: A or B corresponds to the presence of a marker or to PVS-resistant clones; a or b corresponds to the absence of a marker or to PVS-susceptible clones

^b Significant at the 0.01 probability level

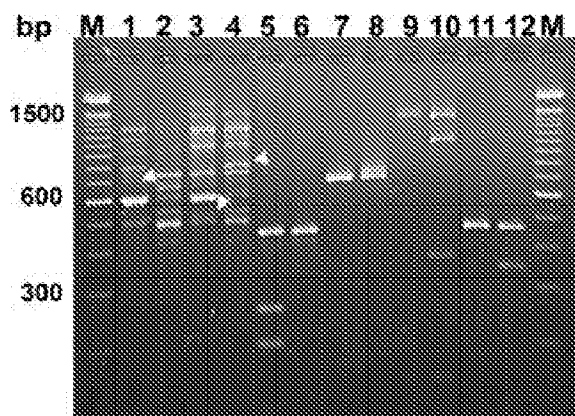
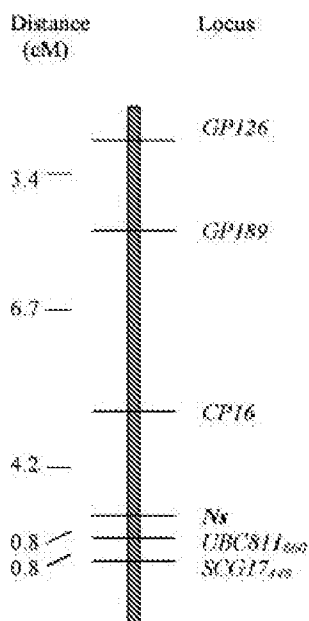


Fig. 1 Electrophoretic patterns of the ISSR, SCAR, STS and CAPS markers in the *Ns* and K31 populations. Lanes: 1, and 2 PCR products amplified with ISSR primer UBC811 in the susceptible (DW 91-1187) and resistant (DW 83-3121) parents of the *Ns* population, respectively; 3, 4 PCR products amplified with UBC811 from the parents P3 and P38 of the K31 population, respectively; 5, 6 SCG17₄₄₈ digested with *Mnl*I in P3 and P38, respectively; 7, 8 STS marker GP126 in DW 91-1187 and DW 83-3121, respectively; 9, 10 CAPS marker GP189 digested with *Hae* III in DW 91-1187 and DW 83-3121, respectively; 11, 12 CAPS marker CP16 digested with *Hind*III in DW 91-1187 and DW 83-3121, respectively; M100-bp ladder. UBC811₆₆₀ (linked with *Ns*), UBC811₆₀₀ and UBC811₈₀₀ are indicated by arrowheads

Fig. 2 Genetic map of the potato chromosome VIII region containing the *Ns* gene for PVS resistance



in the susceptible parent DW 91-1187 (Fig. 1, lane 7). PCR amplification with GP189-specific primers revealed a single band of 1,500 bp in both parents. Among the restriction enzymes tested for fragment length polymorphism of the PCR products, *Hae*III, *Eco*RI and *Alu*I generated informative, segregating DNA fragments (CAPS, cleaved amplified polymorphic sequence). The 1,500-bp PCR fragment amplified in the susceptible parent was not cleaved with *Hae*III (Fig. 1,

lane 9), whereas three bands of 380 bp, 1,100 bp and 1,500 bp were observed after *Hae*III digestion of the PCR product obtained from the resistant parent (Fig. 1, lane 10). The PCR products obtained with primers for the cDNA marker CP16 were 460 bp long. Of the five restriction enzymes tested, an informative polymorphism was observed after *Hind*III digestion (Fig. 1, lanes 11 and 12).

The STS marker GP126 and the CAPS markers GP189 and CP16 were tested for linkage to the *Ns* locus in 119 progeny of the *Ns* population (Table 2). The map position of *Ns* was found to be 4.2 cM (5 recombinants) distal to CP16 on chromosome VIII. The order of the GP126, GP189 and CP16 loci was the same as in the previously published map of a different mapping population (Gebhardt et al. 2001). The SCAR marker SCG17₄₄₈ and the ISSR marker UBC811₆₆₀ were located distal to *Ns* (Fig. 2).

Ns is located in a region of potato chromosome VIII where no other *R* gene has been identified so far (Gebhardt and Valkonen 2001). Among several putative QTL (quantitative trait loci) for leaf resistance to *Erwinia carotovora* ssp. *atroseptica* mapped in yet another population (Zimnoch-Guzowska et al. 2000), QTL *Eca8B* may be located in a similar segment of linkage group VIII as the *Ns* locus. The localization of the *Ns* locus extends the potato function map for pathogen resistance and is a useful starting point for the map-based cloning of the *Ns* gene, which will be the subject for our further studies.

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Development of diagnostic markers for use in breeding potatoes resistant to *Globodera pallida* pathotype Pa2/3 using germplasm derived from *Solanum tuberosum* ssp. *andigena* CPC 2802

Claire Moloney · Denis Griffin · Peter W. Jones ·
Glenn J. Bryan · Karen McLean · John E. Bradshaw ·
Dan Milbourne

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Abstract Quantitative resistance to *Globodera pallida* pathotype Pa2/3, originally derived from *Solanum tuberosum* ssp. *andigena* Commonwealth Potato Collection (CPC) accession 2802, is present in several potato cultivars and advanced breeding lines. One genetic component of this resistance, a large effect quantitative trait locus (QTL) on linkage group IV (which we have renamed *GpaIV^s_{adg}*) has previously been mapped in the tetraploid breeding line 12601ab1. In this study, we show that *GpaIV^s_{adg}* is also present in a breeding line called C1992/31 via genetic mapping in an F₁ population produced by crossing C1992/31 with the *G. pallida* susceptible cultivar Record. C1992/31 is relatively divergent from 12601ab1, confirming that *GpaIV^s_{adg}* is an ideal target for marker-assisted selection in currently available germplasm. To generate markers exhibiting diagnostic potential for *GpaIV^s_{adg}*, three bacterial artificial chromosome clones were isolated from the QTL region, sequenced, and used to develop 15 primer sets generating single-copy amplicons, which were examined

for polymorphisms exhibiting linkage to *GpaIV^s_{adg}* in C1992/31. Eight such polymorphisms were found. Subsequently, one insertion/deletion polymorphism, three single nucleotide polymorphisms and a specific allele of the microsatellite marker STM3016 were shown to exhibit diagnostic potential for the QTL in a panel of 37 potato genotypes, 12 with and 25 without accession CPC2082 in their pedigrees. STM3016 and one of the SNP polymorphisms, C237(119), were assayed in 178 potato genotypes, arising from crosses between C1992/31 and 16 *G. pallida* susceptible genotypes, undergoing selection in a commercial breeding programme. The results suggest that the diagnostic markers would most effectively be employed in MAS-based approaches to pyramid different resistance loci to develop cultivars exhibiting strong, durable resistance to *G. pallida* pathotype Pa2/3.

Introduction

The potato cyst nematode *Globodera pallida* is one of the most significant soilborne pests of potatoes worldwide. In Northern Europe, field populations of *G. pallida* comprising mixtures of pathotypes Pa2 and Pa3 are prevalent, mixed populations sometimes being referred to as pathotype Pa2/3. Developing cultivars expressing high levels of resistance to these heterogeneous populations is a major goal of potato breeders. The availability of DNA-based markers, which are easy to score, cost-effective and diagnostic for resistance to *G. pallida* Pa2/3 would greatly speed up the process of new variety development by allowing a marker-assisted selection (MAS)-based approach to develop lines in which multiple resistance sources were combined, or pyramided, preferably with each individual resistance locus present in high dosage states in the tetraploid potato genome.

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C. Moloney · D. Griffin · D. Milbourne (✉)
Crops Research Centre, Teagasc, Oak Park, Carlow, Ireland
e-mail: dan.milbourne@teagasc.ie

P. W. Jones
Department of Zoology, Ecology and Plant Science,
University College Cork, Butler Building,
Distillery Field, North Mall, Cork, Ireland

G. J. Bryan · K. McLean · J. E. Bradshaw
Genetics Programme, Scottish Crop Research Institute,
Invergowrie, Dundee DD2 5DA, UK

A prerequisite for this MAS-based approach to resistance breeding is the genetic mapping of loci conferring resistance to *G. pallida* pathotypes Pa2 and Pa3 in potato. Several such loci have been mapped, including *Gpa* (Kreike et al. 1994), *Gpa2* (Roupe van der Voort et al. 1997), *Gpa3* and *Gpa4* (Wolters et al. 1999), *Gpa5* and *Gpa6* (Roupe van der Voort et al. 2000), *Grp1* (Roupe van der Voort et al. 1998), *GpaV^{spl}* and *GpaXI^{spl}* (Caromel et al. 2005), *GpaXI^{tar}* (Tan 2008) and several unnamed loci (Bryan et al. 2002; Caromel et al. 2003). These loci mainly originate from diploid wild relatives of cultivated potato, including *Solanum vernei*, *S. sparsipilum*, *S. spegazinii* and *S. tarijense*, or, in the case of *Gpa2*, *S. tuberosum* ssp. *andigena* CPC1673. The loci differ in their effects, some conferring complete or partial resistance to populations of one or other of the pathotypes, and others conferring varying levels of resistance to populations of both pathotypes. However, no single locus confers complete resistance to all populations of both pathotypes of *G. pallida*.

Quantitative resistance to *G. pallida* pathotype Pa2/3, originally derived from *S. tuberosum* ssp. *andigena* Commonwealth Potato Collection (CPC) accession 2802, is present in some cultivars and advanced breeding lines. This resistance source is particularly useful from a breeding perspective due to the fact that *S. tuberosum* ssp. *andigena* is a cultivated tetraploid form of potato closely related to modern *S. tuberosum* ssp. *tuberosum*, obviating the extensive backcrossing to the *Tuberosum* genepool required to eliminate undesirable donor traits when the resistance is introgressed from wild species. A major genetic component of this resistance has previously been genetically mapped in a segregating population resulting from a cross between the *G. pallida*-resistant advanced breeding line 12601ab1 and the cultivar Stirling (Bradshaw et al. 1998; Bryan et al. 2004). A large effect QTL in the duplex configuration was mapped to the distal arm of chromosome IV of 12601ab1. The QTL maps to a position similar to that of the reasonably well characterised R gene locus to which the blight resistance genes *R2*, *R2-like*, *Rpi-blb3* and *Rpi-abpt* have previously been mapped (Park et al. 2005a, b), although insufficient common markers exist between the map of 12601ab1 and the other studies to make accurate positional comparisons. A second, smaller QTL was also detected on LG XI of 12601ab1. The major LGIV QTL was not named in the studies of Bradshaw et al. 1998 or Bryan et al. 2004, and was referred to as *Gpa4* in the review by Gebhardt and Valkonen (2001). Given the increasing number of PCN resistance loci being isolated, we propose that, for clarity, this QTL be referred to in future as *GpaIV^{spl}*, according to the nomenclature described first by Caromel et al (2005) using the scheme [pathogen species, potato linkage group (roman), long/short arm (superscript), source species (subscript)].

Unfortunately, markers found to be linked to QTL in mapping experiments frequently do not satisfy the requirements for use in marker-assisted breeding. Markers such as AFLPs and RFLPs generally require conversion to some form of sequence-characterised marker. Even when sequence-characterised markers are available, they may not be fully diagnostic for the trait in question because the particular allele of the marker found linked to the QTL in the original mapping population is not consistently found associated with the QTL in a broader set of germplasm, due to recombination, or because that particular marker allele is present at a high frequency in the genepool of material used for breeding. For example, Niewohner et al. (1995) developed a PCR assay for detecting RFLP alleles at a marker locus, CP56, which was closely linked in coupling to the nematode resistance gene *Gro1* on potato chromosome VII in a segregating population. However, when tested in 136 diverse tetraploid potato varieties, the marker allele originally found linked to *Gro1* was not correlated with PCN resistance. Conversely, from an AFLP marker linked to a *S. vernei*-derived QTL conferring resistance to *G. pallida* pathotype Pa2/3 resistance located in the major disease resistance locus on potato chromosome V, Bryan et al. (2002) developed a dominant single-locus PCR marker that was consistently associated with PCN resistance in genotypes with *S. vernei* in their pedigree. In a slightly different approach to develop a diagnostic marker to the same quantitative resistance locus, Sattarzadeh et al (2006) sequenced PCR amplicons derived from end sequences of BACs spanning the region containing the *R1* blight resistance gene region to identify SNPs linked to resistance in a segregating population, identified a subset of these SNPs diagnostic for resistance in a broad germplasm set of genotypes with and without *S. vernei* in their background and subsequently used this information to develop a highly diagnostic PCR-based marker for *S. vernei*-derived resistance to *G. pallida* pathotype Pa2/3.

Our objectives in this study were to confirm that *GpaIV^{spl}* is an appropriate target for MAS by showing that it is present in another breeding line called C1992/31, which is divergent from 12601ab1 (Fig. 1), to identify DNA polymorphisms linked to *GpaIV^{spl}* that exhibit diagnostic potential in a broad set of breeding germplasm, and to validate the utility of these markers for MAS in a commercial breeding programme.

Materials and methods

Plant material and DNA isolation

Three sets of germplasm were used in the experiments described in this study.

As part of the potato breeding programme at Oak Park Research Centre, an F_1 population comprising 94 progeny individuals was produced by crossing the advanced tetraploid breeding line C1992/31 (male parent) with the *G. pallida* Pa2/3 susceptible potato cultivar Record (female parent). This is referred to as the T3683 population in this study.

Secondly, a panel of 37 clones comprising breeding material from both Oak Park and the Scottish Crop Research Institute (SCRI) was established. This ‘validation panel’ was composed of 12 breeding lines and cultivars that are known to have *S. tuberosum* ssp *andigena* accession CPC2802 in their pedigrees, and 25 which do not. The genotypes are: with CPC2802—C1992/31, C1992/42, H1H3-6, H1H3-140, 10920ad9, 12601ab1, 12636a2, 12674ab1, Vales Everest, Eden, 97MT200B61, Scarborough.

Without CPC2802—10300-13, 15119ac5, 657/d3*, 8204a4, Cara*, Desiree*, Druid*, Famosa, Habibi*, Lady Balfour, Maris Peer*, Morene*, Navan*, Orla, P55/7, Panda*, Pentland Ivory*, Record, Rooster, Sandra, Saturna*, T2270/50*, T4069/22, Z89/6 (*also in breeding panel, see below).

A third set of germplasm was assembled that comprised almost all of the available progeny with C1992/31 as one parent, which were undergoing selection in the fourth year of the Oak Park Potato Breeding Programme. A total of 178 clones originating from crosses between C1992/31 and 16 other genotypes were used for this study. Twelve parental genotypes are represented in the validation panel, the others were Ausonia, Buchan, Estima and Ulster Glade. None are known to possess significant resistance to *G. pallida*. This germplasm set is referred to as the ‘breeding panel’ in this study.

DNA was isolated from all of the above using either a Qiagen DNAeasy kit or a standard CTAB-based DNA extraction.

Assessment of resistance to *G. pallida* Pa2/3

PCN testing was carried out on the 94 individuals of the T3683 population (including C1992/31) and the 178 individuals in the breeding panel. Tubers from each genotype were used for resistance testing to *G. pallida* pathotype Pa2/3 (Lindley) in a closed container test with four replicates (one tuber per replicate) in a completely randomised design experiment as described by Phillips et al. (1980). Data were collected in the form of the numbers of cysts visible on roots through the transparent walls of the containers 8 weeks after inoculation. The mean cyst count over the four replicates was established for each genotype. Data from the T3683 population were subjected to the variance stabilizing square root transformation for all subsequent

calculations. Data from the breeding panel were not transformed.

Broad-sense heritability of PCN resistance in the T3683 population was calculated in Minitab (Minitab Inc 2006) using the formula $h^2 = V_g/(V_g + V_e/r)$. Components of variance were calculated from the mean squares in ANOVA using Minitab.

Development of sequence-characterised markers in the *GpaIV^s_{adg}* region

To develop sequence-characterised markers specific for the region surrounding the *GpaIV^s_{adg}* locus, a BAC library of the diploid potato RH, the male parent of the ultra high density mapping population of potato (van Os et al. 2006) was screened with the sequenced AFLP marker DPGAM-CAG_155.0 and the microsatellite marker STM3016, which map to the *GpaIV^s_{adg}* region in 12601ab1. This resulted in the isolation of three BACs (GB003E01, GB005C13—isolated using DPGAMCAG_155.0, and GB080G22—isolated using STM3016). One of these (GB005C13) was submitted to a preliminary round of low-coverage shotgun sequencing, which resulted in the availability of a number of sequence contigs. Thirteen of these contigs exhibited homology to Solanaceous ESTs when subjected to BLASTn analysis (results not shown). PCR primers were designed to these sequence contigs, amplified in Record, C1992/31 and ten progeny from the mapping population. To identify polymorphisms, the resulting amplicons were subjected to restriction digest with a range of 4-cutter restriction endonucleases. One primer set, called Contig237, developed by this process, was subsequently used for mapping in the T3683 population.

To develop SNP markers, all three BACs were subsequently sequenced to sixfold coverage (GATC, Germany) using a standard Sanger shotgun approach, and assembled (using the SeqMan module of Lasergene) to phase 1 (unordered, unoriented contigs). Preliminary annotation of the BAC contigs using ab initio gene prediction and homology approaches revealed that none of the BACs contain R-genes (data not shown). BACs GB003E01 and GB080G22 were submitted to GenBank as AC236732 and AC236733, respectively. GB005C13 could not be submitted due to sequence quality issues, but largely overlaps GB003E01. Following the assembly, 11 contigs exceeding 6 kb in size from the three BACs were searched using BLASTn against the entire plant EST database at NCBI. Fourteen primer pairs capable of generating single-copy amplicons in potato, when resolved on a 2% agarose gel, were designed to EST hits within the BAC contig sequences. The 14 primer sets, and the Contig237 primer set, were used to generate amplicons in C1992/31 (resistant parent), Record (susceptible parent) and five of the most resistant

and susceptible progeny from the T3683 population. Direct PCR product sequencing (Agowa, Berlin) was performed on the amplicons using the forward primer. Following the process outlined by Sattarzadeh et al. (2006), SNPs/Indels in the amplicons were identified by manual inspection of the sequence chromatograms, and their probable dosage state estimated by virtue of the relative height/area of overlapping peaks in the sequence chromatogram using the software package DAX version 8.1 (Van Mierloo Software Consultancy) Simplex SNPs/Indels that were consistently present in the resistant parent and resistant samples and absent in the susceptible parent and susceptible samples were considered to be highly likely to be in coupling phase linkage with the *GpaIV^s_{adg}* QTL. The dosage state of diagnostic SNPs in C1992/31 and 12601ab1 was reconfirmed by sequencing the appropriate PCR product using the reverse primer and re-examining the polymorphic nucleotide.

Molecular marker assays

SSR assays were carried out using either radioactively or fluorescently labelled primers. The procedures followed were as described in Febrer et al. (2007). The primer sequences for STM3016 and STM5140 are listed in Bryan et al. 2004.

AFLP assays were performed using the modified protocol of Vos et al. (1995) as described in Bryan et al. (2002, 2004). The rare cutter restriction enzyme *Pst*I (P) and the frequent cutter restriction enzyme *Mse*I (M) were used for all assays, with two and three selective nucleotides, respectively. For bulked segregant analysis (BSA) experiments, 10 µl of the AFLP pre-amp templates for the eight most resistant and eight most susceptible individuals were mixed to make resistant and susceptible bulks. AFLP assays were then performed using 5 µl of each bulk in the selective amplification.

For CAPS marker assays using the Contig237 marker, PCR reactions were performed using the same procedure employed for the radioactively labelled SSR markers, but omitting the labelling step. An annealing temperature of 54°C was used. PCR products were subsequently digested with 5U of *Taq*I restriction enzyme at 65°C for 2 h, before visualisation on a 2% agarose gel. The sequences of the Contig237 primers are

contig237_fwd	GCAGTCCTAATTGCACGTAACA
contig237_rev	CTTACTTGGGCAACCCAGAAT

As described in the previous section, 14 primer sets were designed to sequence contigs derived from BACs known to be from the *GpaIV^s_{adg}* region. The sequences of

the two primer sets subsequently used on the validation panel are listed below

From BAC	Bac8_15_fwd_TCCTCTCTGGTCAGTGTGG
GB005C13	Bac8_15_rev_GAGAATCTCACGGTGGTGGT
From BAC	BAC9_3_fwd_CGCCAACTCTGCTTCATACA
GB080G22	BAC9_3_rev_TGCAGAGGTCAGTTGTTGG

PCRs using these primers were performed as for the Contig237 primer set.

Linkage and QTL analysis in the T3683 population

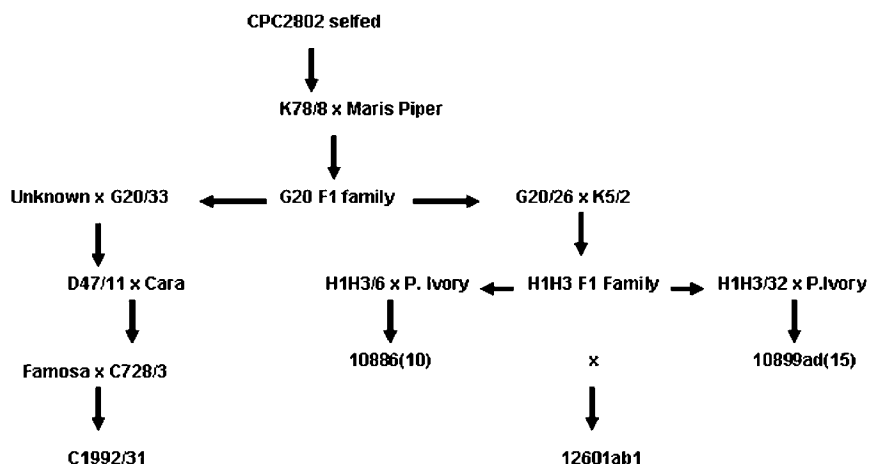
Marker segregation data were analysed using Joinmap 3.0 (Van Ooijen and Voorrips 2001) and TetraploidMap (Hackett et al. 2007). A map was calculated with segregating simplex markers present only in C1992/31 using the default settings of Joinmap 3.0 (LOD score larger than 1.0, pairwise recombination estimates smaller than 0.4, a jump threshold of 5.0, a ripple value of 1.0, Kosambi mapping function). An additional microsatellite marker (STM5140), not conforming to the segregation type above was subsequently added using TetraploidMap (Hackett et al. 2007), which is designed specifically to calculate genetic maps in autotetraploids.

Single marker QTL analysis was performed using a *t* test for two samples in Minitab (2003). The coefficient of determination (r^2) of markers exhibiting significant associations with resistance to *G. pallida* Pa2/3 was calculated by performing a regression analysis on the mean square root transformed cyst scores and the corresponding marker allele scores in Minitab. The marker and trait segregation data were analysed by interval mapping using MapQTL 4.0 (Van Ooijen et al. 2002). A LOD threshold of three was chosen to declare significant QTL effects. The resulting QTL data were represented as an LOD plot against the linkage map.

Analysis of association between diagnostic markers and resistance in the breeding panel

Potentially diagnostic markers were analysed for their ability to select for resistance in the 178 members of the breeding panel by comparing the median (untransformed) cyst counts for genotypes with the appropriate diagnostic allele to those without the allele using a Mann–Whitney *U* test, in Minitab.

Fig. 1 Pedigree of the breeding lines C1992/31 (Oak Park) and 12601ab1 (Scottish Crop Research Institute). Clone K78/8, the G20 F1 family, the clone D47/11 and the H1H3 F1 family were produced at the Plant Breeding Institute, Cambridge, UK in the late 1960s/early 1970s. Subsequent crosses were carried out at Oak Park (left hand side of pedigree) and SCRI (right hand side of figure) to produce C1992/31 and 12601ab1



Results

Segregation of resistance to *G. pallida* Pa2/3 in the T3683 population

The mean cyst count over the four replicates was established for each genotype in the mapping population. This ranged from 0.8 to 44 cysts, while the resistant parent, C1992/31, had a mean cyst count of 14.3. The susceptible control, Maris Piper, had a cyst count of 58. Record was not included in the assay, although historical data indicate that it is susceptible to *G. pallida* Pa2/3. The distribution of square root transformed cyst counts over the population is illustrated in Fig. 2. The broad-sense heritability of the trait was 0.78.

Single marker QTL analysis reveals *GpaIV^{adg}* is present in C1992/31

To investigate whether *GpaIV^{adg}* was present in C1992/31, we first tested the ability of the SSR marker STM3016 to detect a QTL in the T3683 population. STM3016 produced three alleles in C1992/31 exhibiting segregation in the 94 progeny individuals. Using a single marker QTL analysis, only one allele (subsequently called STM3016-122/177), present in C1992/31, but absent in Record, was found to be significantly associated with resistance ($P \leq 0.001$, $t = 8.13$). On the chromatogram output of an ABI3130, STM3016-122/177 is characterised by a main peak at 122 nucleotides that is consistently accompanied by a secondary peak at 177 nucleotides. The allele segregates in a 1:1 ratio in the population (as confirmed by non-significant χ^2 for departures from this ratio: $\chi^2 = 2.084$, $P > 0.05$), indicating that it is present in single-dose in C1992/31. No other allele exhibited any association with resistance. STM3016-122/177 accounts for 45% of the variance in PCN scores in the population. These results indicate that the *GpaIV^{adg}* QTL is present in C1992/31 in the simplex

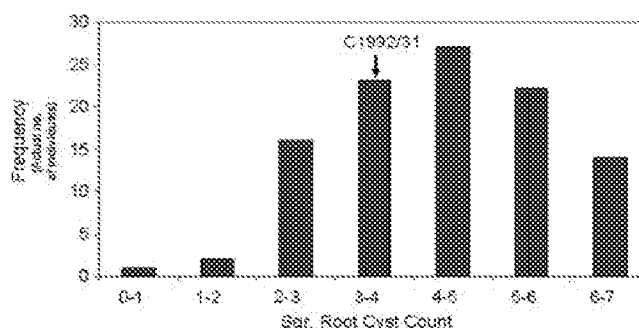


Fig. 2 Distribution of *G. pallida* Pa2/3 cyst counts (square root transformed) over the T3683 population. The resistance value of the resistant parent is indicated approximately, no data were available for the susceptible parent

dosage state (present on only 1 of the 4 homologous chromosomes), and is linked in coupling phase to allele STM3016-122/177.

Development of a linkage map of *GpaIV^{adg}* in C1992/31

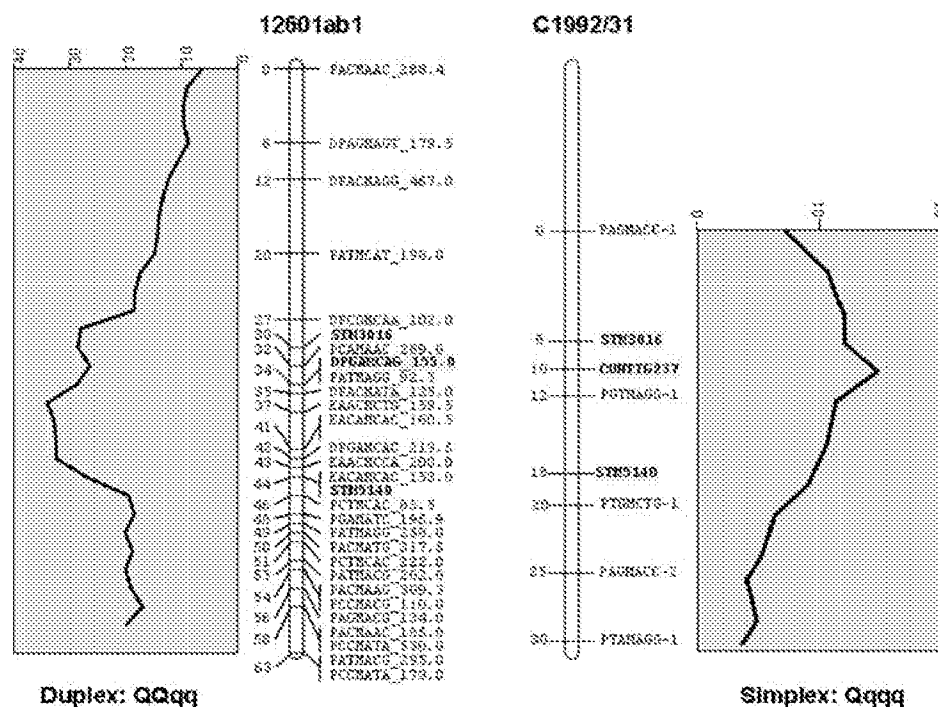
To further characterise the *GpaIV^{adg}* QTL in C1992/31, we generated a linkage map specific to the homologous chromosome carrying both the QTL and STM3016-122/177. Using a BSA-based approach, simplex markers linked in coupling phase to STM3016-122/177 and the QTL were identified. The analysis was carried out on bulks comprising the eight most resistant (R) and susceptible (S) clones from the T3683 population. All of the plants in the resistant bulk possessed the allele STM3016-122/177, while all of the plants in the susceptible bulk lacked the allele. The average PCN scores for the R and S bulks were 3.85 and 40.7, respectively. The two bulks and both parents were subjected to AFLP analysis using 120 *Pst*I/*Mse*I AFLP primer combinations. Primer combinations yielding at least one AFLP band that was present in C1992/31 and the

resistant bulk, but absent in Record and the susceptible bulk were selected for application to the entire population. Five bands from four primer combinations were identified as being potentially linked to *GpaIV^s_{adg}* using this approach (these were designated: PagMacc1, PagMacc2, PgtMagg1, PtgMctg1, PagMata1 and PtaMagg1). On analysis in the T3683 population, all of the markers segregated in a 1:1 ratio (indicating a simplex configuration). An additional marker was developed from the primer set called Contig237 (described in “Materials and methods”). Digestion of the amplicon produced by this primer set with the enzyme *TaqI* produced a segregating allele (referred to as Contig237-a) that exhibited a similar segregation pattern to that of AFLP and SSR markers mapping to the *GpaIV^s_{adg}* region in the T3683 population. Preliminary analysis of STM3016-122/177, the five AFLP markers and the Contig237-a CAPS marker in the T3683 population suggested that they were all linked in coupling phase. In terms of linkage analysis, dominant markers linked in simplex coupling phase in tetraploids can be treated in the same manner as heterozygous coupling markers in diploids, allowing the data to be analysed in a standard mapping package designed for diploids (in this case, JoinMap 3.0). On analysis in Joinmap 3.0, a genetic map of that region on chromosome IV in C1992/31 was created using the seven linked markers (Fig. 3b).

The *GpaIV^s_{adg}* QTL has been extensively characterised by Bryan et al. (2004) in the genotype 12601ab1, using an interval mapping approach developed specifically for autotetraploids (Hackett et al. 2007). This yielded a marker

dense map of the QTL interval comprising mainly AFLP markers, and the potato SSR markers STM3016 and STM5140, which allow the orientation of the LGIV map relative to reference maps (Fig. 3a). In order to orient the C1992/31 map relative to that of 12601ab1, the SSR STM5140 was also analysed on the T3683 population. This marker did not yield any alleles in the configuration that could be included by analysis in JoinMap. However, a single C1992/31-derived allele of STM5140, segregating in a 5:1 ratio, indicating its presence in the duplex dosage state in that genotype, was placed on the map by re-analysing the data in TetraploidMap, which handles both codominant and dominant markers, in all possible configurations in autotetraploids. On the basis of this analysis, the position of STM5140 was approximated on the Joinmap-derived map of C1992/31 (Fig. 3b). Interval mapping of the linkage and phenotypic data identified a QTL in C1992/31 exhibiting a peak at marker Contig237-a (LOD = 15, percentage of variation = 52%), flanked by markers STM3016-122/177 and PgtMagg1. Figure 3 shows a comparison of the LG IV linkage/QTL maps in 12601ab1 and C1992/31, aligned by virtue of three common markers. Although the C1992/31 map is less marker dense than that of 12601ab1, it is apparent that a large effect QTL is present in approximately the same location in the two breeding lines. Given the shared pedigree of the two breeding lines, we propose that the *GpaIV^s_{adg}* QTL is present in both lines, in the simplex dosage state in C1992/31, and the duplex dosage state in 12601ab1.

Fig. 3 Interval maps of the *GpaIV^s_{adg}* locus on linkage group IV in 12601ab1 (**a** left from Bryan et al. 2004) and C1992/31 (**b** right this study). Locus-specific markers common to both studies are highlighted in **bold** on both maps. Marker Contig 237 on the C1992/31 map is equivalent to marker DPGAMCAG_155.0 (the *D* prefix indicates a duplex marker) on the 12601ab1 map. The position of a duplex allele of SSR marker STM5140 was estimated on C1992/31 as described in the “Results”, the marker was not used in the interval mapping



Identifying and testing markers for diagnostic potential in the validation panel

A total of 15 primer sets were designed to BAC-derived sequence from the *GpaIV^s_{adg}* region and tested on the T3683 population. Four of the primer sets (including the Contig237 primers) revealed a total of seven simplex SNPs and one single nucleotide In/Del in the simplex state in apparent coupling phase linkage with *GpaIV^s_{adg}* in C1992/31.

Table 1 Simplex polymorphisms associated with resistance evident from the examination of the sequence of amplicons produced by three primer sets in C1992/31 and Record

Primer	Amplicon size (bp)	Configuration-C1992/31	Configuration-Record	Base pos C1992/31
BAC8-15	635	TTT/A (In/Del)	TTTT	241
BAC9-3	650	CCC/G	CCCC	45
BAC9-3	650	TTT/A	TTTT	87
Contig 237	423	AAA/T	AAAA	119

Total amplicon size, base position of the polymorphism in the sequence and the configurations in the sequence and their dosage SNPs/In/Del nomenclature is according to their primer name and base position in the amplicon (see Electronic Supplementary Material for the sequences of the amplicons)

The four primer sets BAC8_15, BAC9_3, BAC7_16 and Contig 237 were applied to the entire validation panel. Even though they were associated with CPC2802 resistance in C1992/31, four of the SNPs examined apparently represented alleles present at a high frequency in the *S. tuberosum* genepool on the basis of the fact that they were also found in some of the non-CPC2802-derived clones. In contrast, three SNPs, C237 (119) BAC9-3 (45) and BAC 9-3 (87) and the In/Del, BAC8-15 (241), seem to be diagnostic. Table 1 summarises the configuration and position of the polymorphisms in the sequenced amplicons of Record and C1992/31. The actual sequences of the amplicons, with the relevant polymorphic positions highlighted, are provided as electronic supplementary material. The SSR marker STM3016 was also applied to the validation panel, and allele STM3016-122/177, which was linked to the QTL in the T3686 population, was also diagnostic for resistance in this germplasm set. For all of these markers, the target allele is only present in genotypes known to have CPC2802 in their pedigree and is never present in genotypes which do not (Table 2).

On examination of the results of the SNP/InDel markers and STM3016 on the validation panel, it is apparent that 3 out of the 12 CPC2802 derived lines have lost some or all of the potential diagnostic marker alleles described above,

Table 2 Thirty-seven genotypes comprising the validation panel and their scores for marker alleles, exhibiting diagnostic potential for the *GpaIV^s_{adg}* QTL developed during this study

	GB005C13		GB080G22	
	Bac8-15 (241)	C237 (119)	Bac9-3 (45) and (87)	STM3016-122/177
Marker validation panel for CPC2802 resistance				
CPC 2802-derived genotypes				
C1992/31	+	+	+	+
C1992/42	+	+	+	+
H1H3-6	+	+	+	+
H1H3-140	+	+	+	+
10920ad9	+	+	+	+
12601ab1 ^a	+	+	+	+
12636a2	+	+	+	+
12674ab1	+	+	+	+
Vales Everest	+	+	+	+
Eden	+	+	—	—
97MT200B61	—	—	—	—
Scarborough ^b	—	—	—	—
All non-CPC 2802 genotypes ^c	—	—	—	—

+ presence of diagnostic allele, — absence of diagnostic allele

^a The only CPC2802-derived genotype for which the dosage state of the *GpaIV^s_{adg}* QTL is definitively duplex

^b Scarborough is susceptible to *G. pallida* Pa2/3

^c Twenty-five cultivars and breeding lines without CPC2802 in their pedigree are present in the validation panel, these are: 10300-13, 15119ac5 (*S. vernei*-based resistance to *G. pallida* Pa2/3), 657/d3, 8204a4, Cara, Desiree, Druid, Famosa, Habibi, Lady Balfour, Maris Peer, Morene, Navan, Orla, P55/7, Panda, Pentland Ivory, Record, Rooster, Sandra, Saturna, T2270/50, T4069/22, Z89/6

through recombination or loss of the QTL-containing chromosome during meiosis. It is interesting to examine these individuals in terms of their allele score for these SNP/InDel markers and STM3016 to assess which have the greatest relative diagnostic power. For the purposes of this discussion, it is important to remember that markers BAC8-15(241) and C237(119) are derived from BAC GB005C13, while markers STM3016 and BAC9-3(45) and (87) are derived from BAC GB080G22, and indeed, marker alleles from these two clusters had the exact same scores over the entire validation panel. Thus, we are effectively examining only two genetic loci, defined on the map in Fig. 3 by STM3016 and Contig237/DPGAMCAG_155.0. The latter is closest to the peak of the QTL effect on the maps of C1992/31 and 12601ab1, and it might be assumed that it is more closely linked to the gene underlying the *GpaIV^s_{adg}* QTL. Closer examination of the recombinant individuals in the T3683 population suggests that the probable arrangement of these markers relative to the underlying gene/s is STM3016—Contig237—Resistance locus. Given these observations, it would be reasonable to hypothesise that the “Contig237/ DPGAMCAG_155.0-derived” markers would be better predictors of resistance than the “STM3016-derived” markers. Of the genotypes that have lost diagnostic markers, 97MT200B61 and Eden exhibit resistance and Scarborough which is the F₁ progeny of a cross between Eden and the variety Brodick, is susceptible. All three have lost the STM3016-derived markers. Eden, which is resistant, possesses the Contig237/ DPGAMCAG_155.0-derived markers, while its progeny Scarborough, which is susceptible, has lost them. However, 97MT200B61, which is three generations removed from 12601ab1 and 12636a2 and also resistant, has lost the Contig237/DPGAMCAG_155.0-derived markers. Thus, the markers representing locus Contig 237/DPGAMCAG_155.0 successfully diagnose the resistance status of the genotype on 11 out of 12 occasions in comparison to STM3016-derived markers, which successfully diagnose the resistance status on 10 out of 12 occasions.

Testing the utility of SSR marker STM3016 and SNP marker C237(119) for MAS in a breeding programme

To test the predictive abilities of the some of the markers developed in this study in a real commercial breeding programme, two markers, representing the STM3016 and Contig237/DPGAMCAG_155.0 loci (namely the STM3016 SSR marker and the C237(119) SNP marker) were tested in a panel of breeding material that comprised 178 progeny clones with C1992/31 as one parent, which were undergoing conventional selection in the fourth year of the Oak Park Potato Breeding Programme (Table 3).

Table 3 The composition of the breeding panel as described in the “Materials and methods” of the study

Cross	No. of genotypes
C1992/31 × Desiree	21
C1992/31 × 657d3	3
C1992/31 × Estima	1
C1992/31 × Habibi	1
C1992/31 × Ausonia	25
C1992/31 × Cara	6
C1992/31 × Panda	2
Buchan × C1992/31	2
Cara × C1992/31	5
Druid × C1992/31	11
Maris Peer × C1992/31	23
Morene × C1992/31	7
Navan × C1992/31	12
Pentland Ivory × C1992/31	21
Saturna × C1992/31	31
T2270/50 × C1992/31	6
Ulster Glade × C1992/31	1
Total	178

All genotypes are from the third field generation of the Oak Park breeding programme in 2004

In parallel to the genotyping experiment, the resistance of each clone in the breeding panel was assessed as previously described. Due to time constraints, and the large number of samples to be analysed, any clone with more than 100 cysts was given a nominal score of 100. The mean cyst counts ranged from 17 to 100+ cysts. Counting of representative samples from the susceptible clones revealed actual cyst counts of up to 160. The susceptible controls, Desiree and Maris Piper had mean cyst counts of 123 and 112, respectively. The “standard” resistant control Morag had a cyst count of 36. The SCRI cultivar, Vales Everest (which has *GpaIV^s_{adg}*) had a cyst count of 13.75, and the SCRI advanced breeding lines 12601ab1 and 12380 (*S. vernei* derived resistance) had counts of 14.5 and 1.25, respectively. The distributions of untransformed cyst counts for breeding panel members with and without markers STM3016-122/177 and C237(119) are shown in Fig. 4. The medians are 37.75 (with STM3016-122/177) and 75.75 (without), and 33.50 (with C237(119)) and 75.75 (without). Both differences are statistically significant ($P < 0.0001$, Mann–Whitney *U* test). Similar results were obtained when each of the specific crosses was analysed independently. Figure 4 shows the consequences of using the two markers for MAS in a breeding programme. The use of either marker results in the selection of a subpopulation of individuals which exhibits a median corresponding to the resistant end

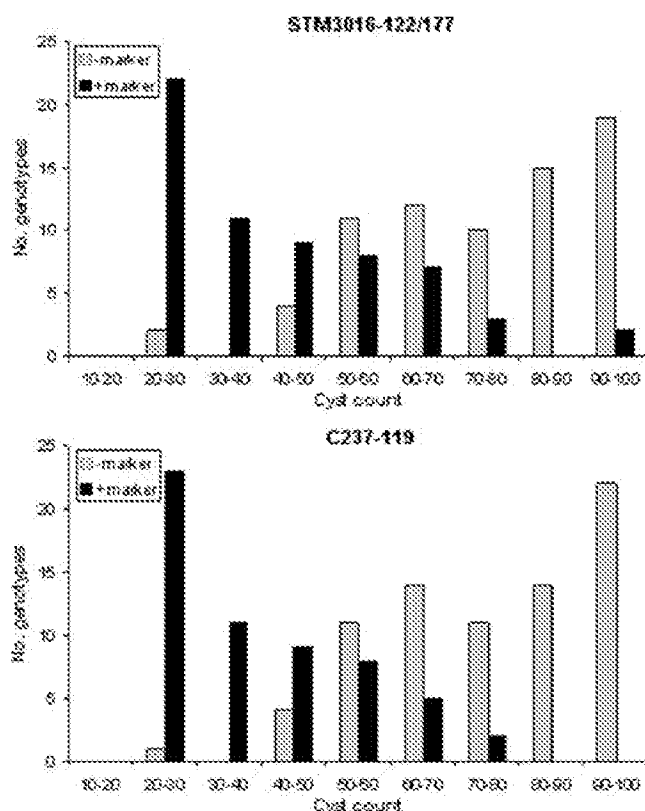


Fig. 4 Histograms of the distribution of cyst counts (untransformed) amongst the 178 individuals of the breeding panel with (black) and without (grey) the *GpaIV^{adg}* diagnostic marker alleles STM3016-122/177 and C237(119)

of the spectrum. The practical implications of these results for MAS are discussed below.

Discussion

By demonstrating the presence of the *GpaIV^{adg}* QTL in relatively divergent material from two independent breeding programmes, we have confirmed that it is a viable target for MAS-based strategies to develop potato varieties exhibiting resistance to *G. pallida* pathotype Pa2/3. Our initial comparisons between 12601ab1 and C1992/31 were based on the anchor marker STM3016, and one allele of this marker (STM3016-122/177) subsequently exhibited diagnostic potential for *GpaIV^{adg}* across a wide range of germplasm. However, STM3016 is several centimorgans from the peak of the QTL in the maps of both 12601ab1 and C1992/31, and the results in the validation panel suggest that the diagnostic allele may occasionally be uncoupled from the resistance gene by recombination. In addition, unless they reveal four distinct alleles at a locus, microsatellite markers give no indication of the dosage state of the associated target gene, a factor which is of great

interest in a tetraploid, since more highly multiplex parents transmit the target gene to the progeny at a greater frequency. The diagnostic SNP/InDel polymorphisms developed during the study will provide a basis for the development of dosage-specific markers for breeding. Examination of diagnostic SNP/InDel polymorphisms between C1992/31 and 12601ab1, the only genotype in the study known to be duplex for *GpaIV^{adg}* generally reveals a shift in peak heights, indicating the expected duplex state in the latter genotype (data not shown). However, as outlined by Sattarzadeh et al. (2006), this method of dosage detection is subject to inconsistent results due to factors such as the influence of the surrounding nucleotides or events such as asymmetric amplification of different alleles. For this reason, and the high per assay cost of Sanger sequencing, dosage detection based on the methods such as pyrosequencing, allele-specific hybridisation or quantitative PCR would be more appropriate for routine application in breeding.

Our results suggest that the gene/s responsible for the QTL effect in C1992/31 might lie between markers Contig237 and PGTMAGG-1. In the longer term, it would be useful to develop another SNP-based marker, or a series of diagnostic markers flanking the QTL, allowing a highly informative marker haplotype, rather than an individual marker genotype to be used to test for the presence of the introgression segment, eliminating the problem of recombination between the marker and the gene. Although it might be possible to convert the aforementioned AFLP marker for this purpose, further BAC sequencing in this region, followed by SNP screening in the same manner as performed in this study, is more likely to achieve the development of such markers.

The experiments on the validation panel offer a retrospective analysis of the diagnostic ability of *GpaIV^{adg}*-linked markers in advanced breeding material. However, our goal was to develop markers for MAS, we felt it was also necessary to assess their utility as a selection tool in a commercial potato breeding programme in a manner similar to the way that they could potentially be employed in that programme. To achieve this, STM3016 and the C237(119) SNP marker were tested in a panel of breeding material that comprised 178 progeny clones with C1992/31 as one parent, from year 4 of the Oak Park Potato Breeding Programme. At this point in the programme, there are generally 2,500 genotypes undergoing field evaluation in plots comprising 10 plants per genotype. Within the programme, PCN testing is generally not carried out until the subsequent year, when 300 genotypes are generally retained, and even then, testing is carried out on a relatively limited amount of material. Deployment of marker-based testing for CPC2802-derived resistance at the year 4 stage of the programme would allow it to be combined with a

reasonable level of traditional selection for generally commercially desirable phenotypes. This could then be followed by confirmation in the selected clones of PCN resistance and other desirable traits. Thus, testing the marker in this material gives a useful measure of the probable utility of the marker in a “real-world” breeding scenario.

As outlined in the results, the use of the diagnostic markers resulted in the selection of a subpopulation of individuals from the breeding panel that exhibits a median corresponding to the resistant end of the spectrum. However, there is still considerable variation around the medians, and this must be due to factors such as recombination breaking the linkage between the markers and *GpaIV^{s_{adg}}*, environmental variation in the PCN tests and genetic variation at other loci. The wild species *S. vernei*, which is another common source of resistance to *G. pallida*, is in the pedigree of several of the non-CPC2802 parents of the breeding panel. Although all of these parents are considered susceptible to varying degrees, we tested them with the HC and SPUD1636 markers (Sattarzadeh et al. 2006; Bryan et al. 2002) which are diagnostic for the large effect chromosome V resistance QTL from this source (results not shown). None were positive for either marker, largely eliminating any contributions from that locus, although it is possible that other, smaller effect loci from *S. vernei* and other sources are present.

The results in the breeding panel suggest that the breeder could confidently use the markers to practise negative selection at this stage in the breeding programme, discarding all genotypes that lack the marker, since there are very few instances in which the most resistant or susceptible genotypes are incorrectly predicted by the marker genotype for either marker. Thus, adopting a negative selection strategy would eliminate the most susceptible individuals from the population. Although useful for raising the median levels of resistance in a breeding programme, this approach may not be the best strategy for breeding cultivars expressing very high levels of resistance to *G. pallida* pathotype Pa2/3. It is clear from this and other studies that no single resistance source gives complete resistance to all populations of *Globodera pallida* pathotypes Pa2 and Pa3. The main reason for this likely to be the relatively complex structure of the PCN populations, leading to heterogeneity of avirulence factors in these populations. Using MAS to combine multiple resistance loci with differing specificities has the potential of broadening the resistance spectrum of varieties, resulting in more durable resistance. In addition, combining different resistance loci can result in apparent additive effects on resistance levels as Rouppe van der Voort et al. (2000) found for *Gpa5* and *Gpa6*. The effect can be quite pronounced, for instance, Caromel et al (2005) found that combining

GpaV^{s_{spl}} and *GpaXI^{s_{spl}}* resulted in the expression of a strong hypersensitive response in the roots of genotypes containing both loci, which was not present when either locus was present individually. Conversely, combining different resistance loci does not always result in an additive effect. For example, Tan (2008) demonstrated that combining root knot nematode resistance QTL from *S. tarijense* and *S. chacoense* did not result in any reduction in infestation beyond that conferred by the more effective of the two loci.

MAS then, is ideal not only for the actual process of pyramiding *G. pallida* pathotype Pa2/3 resistance loci from various sources, but also in helping to identify the most effective combinations of these loci. The availability of the diagnostic markers described in this study will allow the combination of the *GpaIV^{s_{adg}}* QTL with other resistance loci to develop potato varieties, with high levels of resistance to mixed populations of *G. pallida* pathotypes Pa2 and Pa3.

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Gene *Rpi-bt1* from *Solanum bulbocastanum* Confers Resistance to Late Blight in Transgenic Potatoes

T. Oosumi • D. R. Rockhold • M. M. Maccree •
K. L. Deahl • K. F. McCue • W. R. Belknap

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Abstract *Phytophthora infestans*, the causal agent of late blight is the most devastating pathogen of cultivated potato worldwide. Utilizing map based cloning; a genomic region containing a cluster of six nucleotide binding site-leucine-rich repeat resistance gene analogs was isolated from the wild potato species *Solanum bulbocastanum*. Four genes were

pseudogenes, with coding sequences interrupted by either frame shift mutations or premature stop codons. However, neither of the two uninterrupted genes conferred resistance to *P. infestans* when introduced into susceptible potatoes. Specific primers for one of the pseudogenes were used to amplify an uninterrupted cDNA from *P. infestans*-infected *S. bulbocastanum* leaves. A corresponding gDNA was amplified from a late blight-resistant *bulbocastanum-tuberosum* introgression line (*Rpi-bt1*). The *Rpi-bt1* gene under transcriptional control of the constitutive potato *Ubi3* promoter was found to confer resistance to *P. infestans* in several transgenic potato lines in a whole plant greenhouse assay.

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T. Oosumi
Dry Creek Laboratories,
Hughson, CA 95326, USA

D. R. Rockhold • M. M. Maccree • K. F. McCue (✉) •
W. R. Belknap
USDA, Agricultural Research Service,
Western Regional Research Center,
Crop Improvement and Utilization Research Unit,
Albany, CA 94710, USA
e-mail: Kent.McCue@ars.usda.gov

K. L. Deahl
USDA, Agricultural Research Service,
Genetic Improvement of Fruits and Vegetables Laboratory,
Beltsville, MD 20705, USA

Present Address:
T. Oosumi
J.R. Simplot Company, Simplot Plant Sciences,
Boise, ID 83706, USA

Present Address:
M. M. Maccree
USDA, Agricultural Research Service,
Crops Pathology and Genetics Research Unit,
UC Davis Plant Pathology Department,
Davis, CA 95616, USA

Resumen *Phytophthora infestans*, el agente causal del tizón tardío, es el patógeno mas devastador de la papa cultivada en el mundo. Mediante la clonación basada en mapas, se aisló de la especie silvestre de papa *Solanum bulbocastanum* una región genómica que incluía un grupo de repetición con seis análogos de genes de resistencia de sitios de unión de nucleótidos ricos en leucina. Cuatro genes fueron pseudogenes, con secuencias de codificación interrumpidas ya fuera por mutaciones por cambio de marcos o por codones de terminación prematura. No obstante, ninguno de los dos genes sin interrupción confirió resistencia a *P. infestans* cuando se introdujeron a papas susceptibles. Se utilizaron iniciadores específicos para uno de los pseudogenes para amplificar un ADNc ininterrumpido de hojas de *S. bulbocastanum* infectadas con *P. infestans*. También se amplificó un ADNg correspondiente de una línea de introgresión (*Rpi-bt1*) de *bulbocastanum-tuberosum* resistente al tizón tardío. Se encontró que el gen *Rpi-bt1* bajo control transcripcional del promotor de papa *Ubi3* confiere resistencia a *P. infestans* en varias líneas de papa transgénica en un ensayo con planta completa bajo invernadero.

Keywords *Phytophthora infestans* · *Tuberosum* · NBS-LRR · BAC · *Ubi3*

Abbreviations

NBS-LRR	Nucleotide Binding Site-Leucine-Rich Repeat
RACE	Rapid Amplification of cDNA Ends
BAC	Bacterial Artificial Chromosome
-ps	pseudogene

Introduction

Late blight, caused by the oomycete *Phytophthora infestans*, is one of the most destructive plant diseases worldwide. *P. infestans* was the causative agent of the Irish Potato Famine of 1846–1850. Recently, late blight has been effectively controlled by the use of fungicides. However, late blight has become an increasingly important problem to agriculture in the United States and many other countries in the past decade, as more aggressive fungicide-resistant and host-specialized isolates of *P. infestans* have appeared.

Breeding for resistance represents an obvious alternative to fungicide application. Dominant vertical resistance (R) genes from *S. demissum* formed the basis of potato breeding against *P. infestans* from the 1930s to 1950s (Black et al. 1953). While eleven vertical R-genes from *S. demissum* have been introgressed into cultivated potato (Gebhardt and Valkonen 2001; Malcolmson and Black 1966), these race-specific vertical R-genes were found to be non-durable and rapidly overcome when introduced into cultivars (Nelson 1978). In contrast to vertical resistance, horizontal resistance generally involves multiple genes and provides a more general, stable, pathogen resistance in a race-nonspecific manner. Horizontal resistance is not correlated with the hypersensitive response, and instead involves limiting pathogen spread in the host. *S. bulbocastanum*, for example, contains a dominant R gene locus apparently conferring horizontal resistance to *P. infestans* when introgressed into the cultivated potato (Naess et al. 2000; Naess et al. 2001). A gene named *RB* (also known as *Rpi-blb1*) has since been isolated from the *RB* resistance locus of *S. bulbocastanum* and found to confer broad spectrum resistance to *P. infestans* when introduced into potato (Song et al. 2003; van der Vossen et al. 2003).

The *RB* locus consists of a cluster (Meyers et al. 2005) of nucleotide binding site-leucine-rich repeat (NBS-LRR) proteins, similar to most previously identified R genes (Martin et al. 2003). Here we report map-based cloning of the *Rpi-bt1* from the *Rpi-bt* locus of *S. bulbocastanum* isolated from the *bulbocastanum*-*tuberosum* (*bt*) introgression. *Rpi-bt1* is a NBS-LRR R-gene, with high similarity to *RB*, which in transgenic potatoes confers a high level of resistance against *P. infestans*.

Materials and Methods

Plant Material

Solanum bulbocastanum and *S. tuberosum* cvs. Lenape (Akeley et al. 1968) and Atlantic (Webb et al. 1978) were obtained from the Inter-Regional Potato Introduction Station (NRSP-6), Sturgeon Bay, Wisconsin. Plants were maintained in tissue culture and grown in a glasshouse in Albany, CA.

For mapping, the back-cross 2 (BC2) *P. infestans* resistant clone J101k6A22 (Naess et al. 2001) was crossed with the Aberdeen, ID breeding selection A86102-6 (Joseph Pavsek, unpublished) to generate the A9510 back-cross 3 (BC3) population. The BC3 population consisting of 100 progeny segregating for *P. infestans* resistance (scored Resistant, Susceptible, or Resistant/Susceptible) was employed in the present study.

Disease Assay

Detached leaf assays for late blight resistance employed unblemished, fully expanded sub terminal leaflets from 6 to 8 week old greenhouse-grown test plants. Inocula were obtained from two-week-old cultures of *P. infestans* (strain US8, Florida isolate) grown on rye agar. Inoculations were made by placing a 10 μ L droplet of a sporangial suspension (4×10^4 mL⁻¹) that had been incubated at 8 C for 2.5 h (to release zoospores) on both sides of the midrib of the abaxial leaf surface. The inoculated leaflets were placed in Petri dishes containing moistened filter paper to maintain 100% relative humidity. Inoculated material was incubated for 1 d at 15 C in darkness, then for 4 d at 15 C with 6 h/d photoperiod ($400 \text{ Em}^{-2} \text{ s}^{-1}$). A computer-driven image analysis system (Bioquant IV, R and M Biometrics, Nashville, TN) was used to obtain measurements of lesions. The lesion diameter was determined by projecting the whole leaves onto a grid lining the Bioquant Digitizing Pad. The digitizing pad was coupled with an IBM PC and measurements were generated using Bioquant Systems software.

A whole-plant glasshouse test (Stewart et al. 1983) was used to determine which of the transgenic lines containing the genomic *Rpi-bt1* transgene (described below) were resistant to *P. infestans*. Tubers were planted in 15-cm pots and grown in a 16 h light and 8 h dark photoperiod using high-pressure sodium lamps as supplemental lighting. Inocula were obtained from cultures of *P. infestans* (strain MD-02-pet-1 A2, US-8 genotype) grown on lima bean agar medium in the dark at room temperature. After two weeks of incubation, the plates were flooded two times with sterile water and scraped lightly using an L-shaped glass or plastic rod to collect sporangia. The liquid from the plates were filtered into a 1 L glass beaker using two

layers of cheesecloth. The total volume was roughly estimated and sporangia concentration was determined using a hemacytometer. Using sterile water, the volume of the inoculum was adjusted to give a final concentration of 5×10^3 sporangia mL^{-1} . The inoculum was transferred into a sprayer (approximately 2 mL sec^{-1}) after incubation at 4 °C for 1 h followed by room temperature incubation for 30 min. Plants of each clone (in flower bud stage) were inoculated with *P. infestans*. Each plant was scored daily using Malcolmson's scoring scale (1–9) for increasing resistance (Cruickshank et al. 1982), starting 7 days after inoculation, and plants of each clone were compared.

AFLP and RFLP Mapping

The BC3 population segregating for *P. infestans* resistance was mapped using a combination of AFLP (Vos et al. 1995) and RFLP markers. AFLP mapping employed the commercially available primer/enzyme sets (Applied Biosystems, Inc.). The late blight resistance locus has been mapped to chromosome 8 (Naess et al. 2001). RFLP mapping employed tomato markers kindly provided by S. Tanksley (CT64, CT214, TG385, TG478, TG505, CD60, and TG282). Markers were amplified using M13 forward and reverse primers and random primed (GE Healthcare) [^{32}P]-labeled sequences were used to probe genomic blots of the BC3 parents. After the initial parental screening to identify polymorphisms, selected RFLP markers known to be located on potato chromosome 8 were also mapped in the BC3 population.

BAC Library Screening

The RFLP markers CT64, TG282, TG505, and CD60 were used to probe a *S. bulbocastanum* BAC library (Song et al. 2000). The library was probed with the random primed (GE Healthcare) [^{32}P]-labeled sequences. BAC clones corresponding to the RFLP markers were isolated and end-sequenced with M13 forward and reverse primers. These and subsequent BAC end-sequences were used to generate specific primer pairs (see Table 1 for the CD60 contig primers) for screening of pooled BAC clones by PCR to assemble contigs of overlapping BAC clones (Cai et al. 1995). To assess linkage of BACs to the resistance phenotype, BAC end sequence primer pairs were also employed in PCR screening of genomic DNA from the original population segregating for late blight resistance.

Isolation of Candidate Genes

Using BAC end and synthetic primer sequencing the *Rpi-bt* locus was localized to BAC clone C29F2F3 of the CD60

contig. Portions of the *Rpi-bt* locus were sequenced by digestion of BAC C29F2F3 and C29F2F2R2 and synthetic primer sequencing of the subcloned fragments. Three contigs covering approximately 75 kbp of the *Rpi-bt* locus were assembled. Genomic fragments containing each of the 6 complete NBS LRR coding sequences were obtained by restriction enzyme digestion of BACs C29F2F3 and C29F2F2R2. The *Rpi-bt1-ps* was isolated as an 11 kbp SphI-PacI fragment; and *Rpi-bt2* was isolated as a 10 kbp SphI-SacII fragment from C29F2F2R2. *Rpi-bt3* was isolated as an 18 kbp SacII-BamHI fragment; *Rpi-bt4-ps* and *Rpi-bt5-ps* were isolated on a 34 kbp SacII-PmeI fragment; and *Rpi-bt6-ps* was isolated as a 24 kbp PacI-PmeI fragment from C29F2F3. A genomic DNA for *Rpi-bt1* was obtained by PCR amplification using the DNA of a late blight-resistant BC3 line as a template and *Rpi-bt1-ps* specific primers (forward CCAACATCTTACTTCATTTC and reverse GATACCGTTAGCTATAAGAC).

Sequences were compared to the GenBank non-redundant database using the BLAST Network Service of the National Center for Biotechnology Information (Altschul et al. 1990). Pustell matrix analysis (Pustell and Kafatos 1982) was used to compare DNA sequences and generate alignment presented in the figures (MacVector 8.0).

Identification of Transcripts from the *Rpi-bt* Locus

To identify potential mRNAs from *Rpi-bt* genes, RACE (Rapid Amplification of cDNA Ends)-PCR was employed using polyA⁺ RNA prepared from *P. infestans*-infected *S. bulbocastanum* leaves. Transcript cDNAs were generated using the Marathon cDNA Amplification Kit (Clontech) followed by PCR amplification with gene specific primers for *Rpi-bt2* (5'RACE GGCATACAAGTCAATCCATGG CAACG and 3' RACE ACAGAGAGACAAGTTGCTA CACGCC) and *Rpi-bt3* (5'RACE GAATGCTACTGCCA CGCAAGTTCAAC and 3' RACE TTCCCTGAGGCT GAGTTATCATCACC). A messenger RNA transcript corresponding to the pseudogene (-ps) *Rpi-bt1-ps* was amplified with *Rpi-bt1-ps* specific primers (5'RACE AGT GATTCATCCAGTCTGGGAGACG and 3' RACE CCAAGCTGTGCTAGAAGATGCTCAG) from a late blight resistant BC3 line (*Rpi-bt1*).

Confirmation of the *Rpi-bt1* Gene Source

To confirm the source of the *Rpi-bt1* gene PCR was employed using genomic DNA from the backcross 1 and 2 parents ('Katahdin' and 'Atlantic') and *S. bulbocastanum*. PCR fragments were generated with forward primer (CCAAGCTGTGCTAGAAGATGCTCAG) in the first exon and reverse primer (GGCTTTATCCTGTTCATTTA GACTTGGGG) in the first intron. PCR fragments were

Table 1 BACs and primers used for mapping the NBS-LRR locus shown in Fig. 2. Probes were amplified from 5'/3' primer pairs for BAC end sequence derived from M13 forward or reverse primers

BAC	F/R ^a	Primer Sequence	T _m (°C) ^b	Product (bp)
C29	F1 5'	CCCAAACGCCAAACCATA	53.7	
	F2 3'	AAGCTTAAGTTGTTCAAGTTTTTC	53.5	201
	R1 5'	GAAACCCCTAAAAAACTTGATCC	54.7	
	R2 3'	GACGCGACTTAAAGTCGT	53.7	396
C29F2	F1 5'	CTCAAAAAGTAGCATTAAGCTAG	54.7	
	F2 3'	TGTGATCATTTAGCATTGACC	54.0	154
	R1 5'	AGCTTGACTTGATGCAGG	53.7	
	R2 3'	TCAAATCACCCCACTCTG	53.7	141
C29F2F2	F1 5'	GAATCCATCAATACGACAAC	53.2	
	F2 3'	CTTTGCTCTAATATAGGGAG	53.2	203
	R1 5'	AGCTTTGACATGTTCCCC	53.7	
	R2 3'	GCCAAATGACATGTGCAG	53.7	273
C29F2F2R1	F1 5'	CTGATAACAGAAGAAATGCTG	54.0	
	F2 3'	CTCAATAACTGAATACTCCTC	54.0	165
	R1 5'	GGAGAATAAGAAGGCACC	53.7	
	R2 3'	GGATGGCACATGGTTTTTC	53.7	274
C29F2F2R1F3	F1 5'	GCTTGATGAAAAGAGGTCG	53.7	
	F2 3'	TGGTGTACTCCCAACAAGT	53.7	210
	R1 5'	CTTTCACCGCCAAGTTTG	53.7	
	R2 3'	CTGGAAGTAGTCTCTAGC	53.7	351

^a Location of probe and primer orientation based on M13 forward and reverse primer BAC end sequencing^b Primer T_m calculation (69.3+0.41*%GC-650/#bp)

subjected to EcoRI restriction enzyme digestion to confirm the presence of the unique EcoRI site in the *S. bulbocastanum* BAC DNA and the BC3 gDNA isolates.

Screening of Candidate Genes for *Phytophthora* Resistance

Experiments to determine the efficacy of either *Rpi-bt2* or *Rpi-bt3* in conferring late blight resistance were based on mobilization of these genes plus at least 3 kbp of 5' and 3' flanking sequence into susceptible potatoes by *Agrobacterium*-mediated transformation. The 10 kbp *Rpi-bt2* fragment and the 18 kbp *Rpi-bt3* fragment were mobilized into the binary transformation vector pCGN1547 (McBride and Summerfelt 1990). These binary vector constructs were used to introduce the *Rpi-bt2* or *Rpi-bt3* genes into Lenape (Akeley et al. 1968) and Atlantic (Webb et al. 1978) by a standard transformation/selection protocol (Snyder and Belknap 1993). Transgenic and control lines were screened for *P. infestans*-resistance by the detached leaf assay (described above).

In order to express *Rpi-bt1* in transgenic plants a chimeric transgene was constructed. Transcription of the genomic copy *Rpi-bt1* gene was placed under the control of the potato *Ubi3* promoter for constitutive moderate-level expression (Garbarino and Belknap 1994a; Garbarino and

Belknap 1994b). The *Ubi3* polyadenylation signal was fused to the 3' end. The *Rpi-bt1* transgene was mobilized into the binary transformation vector pBINPLUS/ARS (Belknap et al. 2008; McCue et al. 2006). This binary vector construct was used to introduce the *Ubi3::Rpi-bt1* transgene into Atlantic. Transgenic and control lines were screened for *P. infestans*-resistance in the detached leaf and whole plant assays (described above).

Results and Discussion

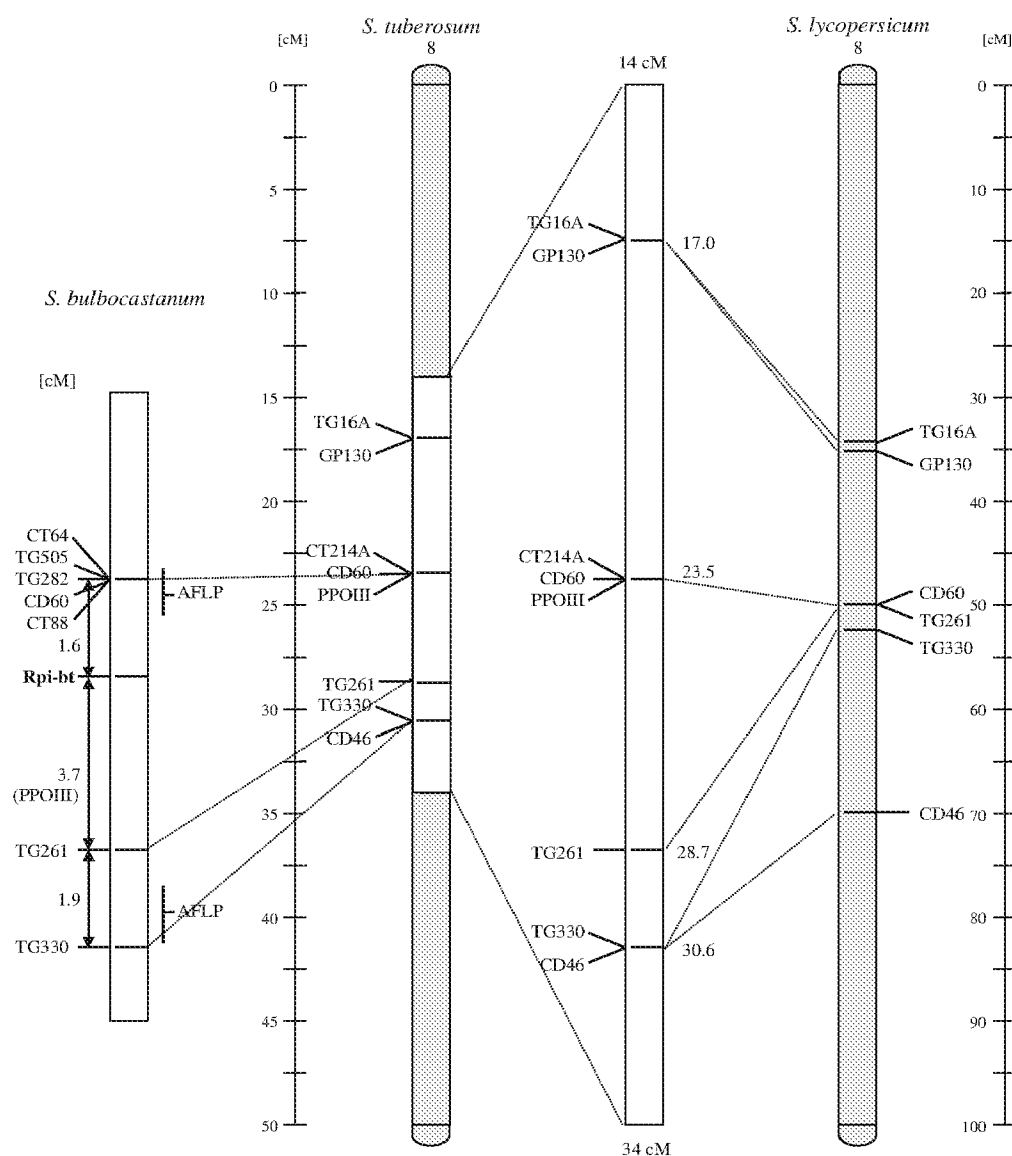
Mapping of the *P. infestans* Resistance Locus

Because *S. bulbocastanum* cannot be crossed with potato, DNA from this diploid species was originally introgressed into *S. tuberosum* cv. Atlantic (Webb et al. 1978) by somatic fusion at the University of Wisconsin resulting in the somatic hybrid J101 (Naess et al. 2001). This somatic fusion was then backcrossed to 'Katahdin' resulting in the back-cross 1 (BC1) population. The resistant individual J101K6 from this BC1 population was then again crossed with Atlantic, resulting in the BC2 population containing the resistant clone J101k6A22 (Naess et al. 2001). This

BC2 clone was crossed with the 'Aberdeen', ID breeding selection A86102-6 (Joseph Pavék, unpublished) to generate the A9510 BC3 population employed in the mapping studies. This BC3 population consisted of 100 progeny segregating for *P. infestans* resistance. A combination of AFLP (Vos et al. 1995) and RFLP markers was employed to map the late blight resistance gene locus. The late blight resistance locus was previously mapped to chromosome 8 (Naess et al. 2001). The segregating population was subjected to AFLP mapping. Exhaustion of the commercially available AFLP primer/enzyme sets resulted in identification of over 400 polymorphic bands. RFLP mapping of the segregating population identified two markers, CT64 and TG505, that produced polymorphic bands that link with the R phenotype. The relative positions

of the AFLP and RFLP markers closest to the *Rpi-bt* locus are shown in Fig. 1. AFLP analysis failed to generate multiple markers in the proximity of the RFLP probes, potentially due to the limited size of the BC3 population. A *Phytophthora resistance locus* was previously mapped between TG261 and CT88 (Naess et al. 2001). In tomato three genes, CD60, TG282, and TG505 are tightly linked with TG261 and the four genes map on the same locus (Tanksley et al. 1992). In potato CD60 lies in a region within 5.3 cM of TG261 (Tanksley et al. 1992). The clustering of these markers, together with the absence of multiple AFLP markers in the proximity of clustered RFLP probes suggested that additional mapping was unnecessary, and the RFLP markers were selected to probe a *S. bulbocastanum* BAC library (Song et al. 2000).

Fig. 1 The genetic map of the *S. bulbocastanum* late blight resistance gene locus compared to genetic maps of *S. tuberosum* and *S. lycopersicum* chromosome 8. The approximate position of the locus is indicated by *Rpi-bt*. The positions of RFLP markers relative to this locus are indicated. The relative positions of AFLP markers flanking the R gene are indicated. Data are from (Naess et al. 2001; Tanksley et al. 1992) adapted from the SOL Genomics Network (<http://www.sgn.cornell.edu>)



Identification of Candidate Resistance Genes

BAC C29 was originally identified by hybridization of library filters to the labeled CD60 RFLP marker. BAC end-sequence data from the M13 forward and M13 reverse primers (F and R) allowed design of specific primer pairs for both ends of each insert (Table 1). To obtain adjacent overlapping BACs (chromosome walking) subsets of the BAC library were pooled and screened by PCR using the end-specific primer sets (Cai et al. 1995). After six rounds, an approximately 700 kbp contig was assembled proximal to the CD60 RFLP marker on *S. bulbocastanum* chromosome 8 (Fig. 2). Using BAC end-sequence primer sets, BACs C29F2 and C29F5 were found to be linked to the resistance phenotype by PCR screening with BAC end sequence primer sets on genomic DNA from the original population segregating for late blight resistance. Computational (BLAST) alignment of the R end sequence of BAC isolate C29F2F2R2 with the GenBank non-redundant database revealed the position of a member (*Rpi-bt3*) of a NBS-LRR protein similar to many previously identified R genes (Martin et al. 2003).

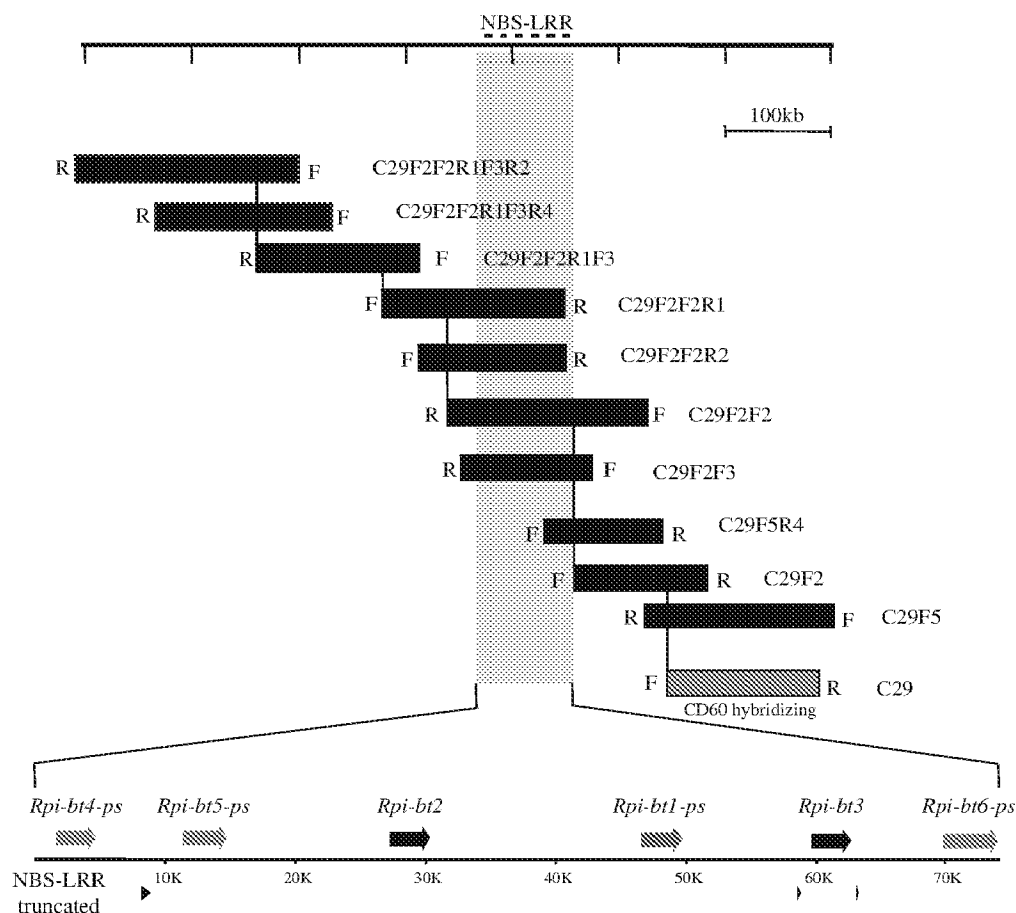
The NBS-LRR regions of the *Rpi-bt* locus were sequenced in six contigs totaling 52 kbp covering a region

of approximately 75 kbp. This region contains a cluster (Meyers et al. 2005) of six complete NBS-LRR genes (*Rpi-bt1-6*) as well as three truncated genes (Fig. 2). As shown in Fig. 2, four of the six complete genes were found to be pseudogenes, with coding sequences interrupted by either frame shift mutations or premature stop codons. These data suggested that late blight resistance at this locus was associated with *Rpi-bt2* and/or *Rpi-bt3* expression.

Identification of *Rpi-bt* Late Blight Resistance

Initial experiments focused on the two *Rpi-bt* genes with complete NBS-LRR coding sequences. To determine the efficacy of either *Rpi-bt2* or *Rpi-bt3* in conferring late blight resistance, these genes plus at least 3 kbp of 5' and 3' flanking sequence were mobilized into susceptible potatoes by *Agrobacterium*-mediated transformation. Transgenic potato plants containing either the *Rpi-bt2* or *Rpi-bt3* transgenic construct were screened for resistance to late blight by detached leaf assay (Tognitz et al. 1995). Both *Rpi-bt2* and *Rpi-bt3* genes failed to confer resistance to *P. infestans* in the detached leaf assay (data not shown). To examine the efficacy of *Rpi-bt2* and *Rpi-bt3* to confer resistance to late blight in vivo, transgenic potato plants

Fig. 2 Physical map of the *Rpi-bt* NBS-LRR resistance locus. The assembly of the 75 kbp contig on *S. bulbocastanum* chromosome 8 was anchored by a BAC clone hybridizing to the RFLP marker CD60. Primer pairs from BAC end-sequence used to identify overlapping BACS are indicated by the vertical lines. BAC sequence analysis revealed the *Rpi-bt* locus on BAC C29F2F3. Sub-cloning and sequencing of this BAC revealed six complete and three truncated NBS-LRR coding sequences. Two of the six complete genes, *Rpi-bt2* and *Rpi-bt3*, were found to encode uninterrupted open reading frames (dark arrows). The remaining four complete genes labeled as pseudogenes (light arrows) are interrupted by frame shift mutations (*Rpi-bt1-ps* and *Rpi-bt4-ps*) or premature stop codons (*Rpi-bt5-ps* and *Rpi-bt6-ps*)



were grown in the glass house and scored for disease after inoculation with *P. infestans* US-8 genotype (data not shown). None of the transgenic lines exhibited levels of resistance different than controls in two separate time course experiments.

The similarity of the NBS-LRR proteins of the *Rpi-bt* locus (Fig. 2) to the proteins encoded by known disease resistance genes is significant. In addition, the PCR screening with the BAC-end sequence primers (C29F2, and C29F5) from the *Rpi-bt* locus indicated linkage to resistance in the segregating population. Since transgenic lines containing the gDNA for *Rpi-bt2* and *Rpi-bt3* did not confer resistance in the detached leaf assays, expression profiles for these two genes were examined in *S. bulbocastanum*. Specific primers were prepared to the *Rpi-bt2* and *Rpi-bt3* genes and RACE-PCR was employed to amplify potential mRNAs from polyA⁺ RNA isolated from *P. infestans*-infected *S. bulbocastanum* leaves. Messenger RNA products corresponding to *Rpi-bt2* and *Rpi-bt3* were not amplified. Instead transcripts similar to *Rpi-bt2* and

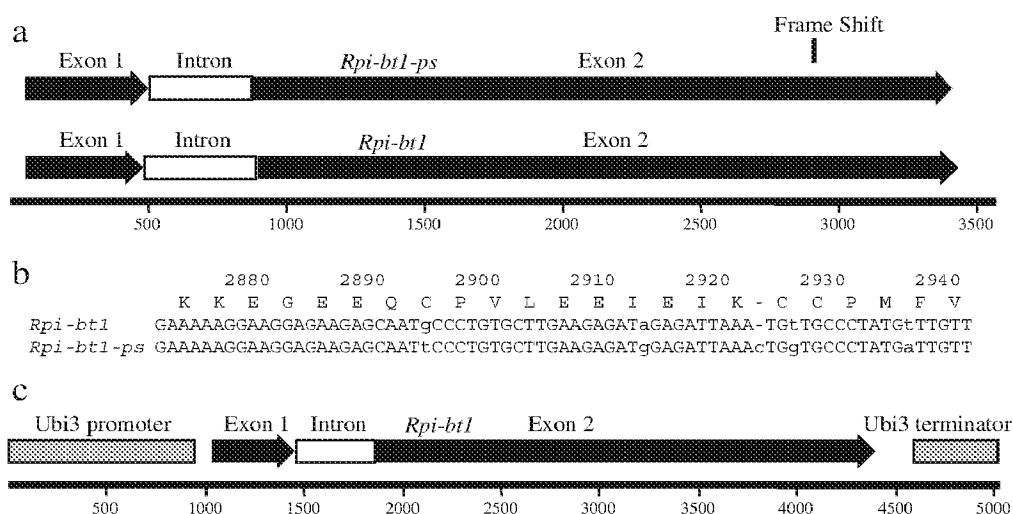
Rpi-bt1 were isolated. It was therefore possible that one or more of the four pseudogenes present in the *Rpi-bt* locus represented an inactive allele of a gene active on the other chromosome of this diploid species (Song et al. 2003).

A BLAST search of the GenBank non-redundant database (Altschul et al. 1990) using the deduced amino acid sequence of *Rpi-bt1-ps* corrected for the insertional frame shift returned resistance gene analog (RGA) proteins at highest identity (75%). Since the *Rpi-bt2* and *Rpi-bt3* genes were not identified in the RACE-PCR of infected *S. bulbocastanum* leaves, this suggested that the *Rpi-bt* locus might be heterozygous in *S. bulbocastanum*, with one allele active and others such as *Rpi-bt1-ps* interrupted by frame shift or stop mutations (Helgeson et al. 1988). A messenger RNA transcript was amplified with *Rpi-bt1-ps* specific primers from a late blight resistant BC3 line that contained a complete coding sequence lacking the *Rpi-bt1-ps* frame shift and was designated *Rpi-bt1*. The *Rpi-bt1* deduced amino acid sequence is 94% identical to the predicted amino acid sequence of *Rpi-bt1-ps* corrected for the frame

Fig. 3 Alignment of the deduced amino acid sequences of *Rpi-bt1* (GenBank Accession FJ188415) with RGA2 (GenBank Accession Q7XA41). Only RGA2 residues that differ from RPI1 are shown, relative gaps are indicated by dashes. Shaded regions indicate NBS (*Rpi-bt1* residues 178–415) and LRR (*Rpi-bt1* residues 515–957) domains

RPI1	MAEAFLOVLLDNLTCFIQGEGLGLILGPKDEFKQLSTFTTIQAVLEDAQKKQLKDKAIENWLQKLNAAAY	70
RGA2	I S LK V LF Q QR S M S E NN PL T	70
RPI1	EADDILDECKTEAPIRQKKNKYCYHPNVITFRHKIGKRMKKIMEKLDVIAAERIKFHLDERTIERQVAT	140
RGA2	V Y K -T FSGSE R K P V DQV K KA E EN H KIV AVR	139
RPI1	RQTGFVLNEPQVYGRDKEKDEIVKILINNVSNAQTLPVLPILCMGHLGKTTLAQMVNDQPVIEHFPKXI	210
RGA2	E S T D H S T S	209
RPI1	NICVSEDFNEKRIKIEVESIEEESL-GGDLAPLQKKLRDLNGEKYLLVLDDVNNEDQDKWAKLRQVL	279
RGA2	D A GRP L E QE R Q N A	279
RPI1	KVGASCASVLTTRLEKVGSIIMTLPVLEENLSQEDCWLLFMQAPFCHQEEINLNLVAICKEIVKRCGC	349
RGA2	P S	349
RPI1	VPLAATLGGILKPKREERQWEHVRDSEIWKLPQESSILPALSLSYHMLPLDLRQCPTYCAVFPKDTM	419
RGA2	C A P N D Q K A AK	419
RPI1	EKGNLISLWMAHGFIISKGNLELENVGNEVWNELYLRSFFQIEIEVKSGQTYFKMHDLIHDLATSLFSAST	489
RGA2	EK L M D D K D K N	489
RPI1	SSSNIREIIVENYIHMMSIGFTKVVSSYLSHLQKVFSLRVNLNLSDIKRLQPLSSIGDLVHLRYLNLSN	559
RGA2	NKHS T AE FF T PP E I G STFNK Y -	558
RPI1	TSIRSLPWLCKLQNLQTLIDLGCHSLCCLPKETSRLGSLRNLLLDGCYGLTCMPPRIGSLTCLTLSEF	629
RGA2	SGM K QY TK SQS GQ	628
RPI1	VVGIGQKXSQGLLENLNLGYSEITHLERVIONDAKEANLSARENLSLSMKWDDDERPRIYESEKVE	699
RGA2	-R GY G K S K G S -NNFG E E K	696
RPI1	VLEALKPHSNLTCLTIRGFRGIRLPDWNHSLKQNVSTIETISCKNCACLPFPGLPCLSLSLWDCSAR	769
RGA2	S K Y H E I L SNFR D E HW D	766
RPI1	VEYVD-----SGFPTERRPPLRLKLNIREFDNLKGLLXKPGSEECQPVLEEISIKCCPMFVPIPTLSSVE	832
RGA2	EEVDIDVH I D WD GS F MI HE FL--- NL	833
RPI1	KLVVSGDKSDAIGFSSISINMALTSLQIRYNKEDASLPEEMFKSLANLKYLNISFYFNLKELPTSLASLN	902
RGA2	R----- R C VAT F N T RCN	883
RPI1	ALKHLEINSCYALSLPEEGVGLISLTQLSITYCEMLQCLPEGLQHLTALTNLSEVFCPTLAKRCEKGI	972
RGA2	S K QL C LE S E FVEH N K T S KIRG Q I	953
RPI1	GEDWYKIAHIPRVFIY-*	
RGA2	H S N N I	

Fig. 4 Structures of *Rpi-bt1-ps* and *Rpi-bt1* genes and the chimeric transgene. **a** Comparison of structures of the *Rpi-bt1-ps* pseudogene from BAC C29F2F3 and the transcriptionally active *Rpi-bt1* genomic copy isolated by PCR. The position of the frame shift mutation in the pseudogene is indicated. **b** Alignment of *Rpi-bt1* genomic sequences in the region of the frame shift mutation in the *Rpi-bt1-ps* pseudogene. Position numbers and translation derived from the active *Rpi-bt1* gene. **c** Structure of the genomic DNA *Rpi-bt1* chimeric transgene employed in complementation analysis



shift. PCR amplification of *Rpi-bt1* from genomic DNA isolated from a late blight-resistant BC3 line generated an amplicon encoding an ORF 100% identical to the *Rpi-bt1* cDNA with a 412 bp intron. Amplification of *Rpi-bt1-ps* and *Rpi-bt2* was only successful from the BAC library and *S. bulbocastanum* gDNA and was not present in the BC3 population (data not shown) indicating the *Rpi-bt* allele represented in the BAC library was not the allele present in the BC3 population segregating for resistance.

The source of the *Rpi-bt1* gene was confirmed by PCR analysis and restriction enzyme digestion. The primers used to isolate *Rpi-bt1* cDNA and gDNA were designed from the *Rpi-bt1-ps* of the *S. bulbocastanum* BAC sequence. The *Rpi-bt1* genomic copy was isolated from the BC3 line and was not present in the BAC library. The *S. bulbocastanum* *Rpi-bt1* cDNA is 100% identical with the BC3 gDNA (minus intron). The gDNA of *Rpi-bt1* is 95.6 % identical to *Rpi-bt1-ps* from the *S. bulbocastanum* BAC, which is within the variation expected for alleles and highly suggestive that *Rpi-bt1* is the active allele of the *Rpi-bt* locus isolated from the *S. bulbocastanum* BAC library. Both the BAC and BC3 gDNA sequences encode a unique *EcoRI* restriction site in the intron. Amplification of gDNA from *S. bulbocastanum* and the *Rpi-bt1* gDNA produced the predicted 554 bp PCR fragment which was subsequently cleaved by restriction endonuclease *EcoRI* into two fragments of 373 and 181 bp. Fragments amplified from the BC1 and BC2 parents 'Katahdin' and 'Atlantic' were not cut by *EcoRI* (result not shown). The *Rpi-bt* locus was also absent from the susceptible BC3 parent A86102-6 confirming *S. bulbocastanum* as the source of *Rpi-bt1*.

The predicted amino acid sequence from the *Rpi-bt1* gDNA is 91% identical to the predicted amino acid sequence of *Rpi-bt1-ps* (insertion removed). Subsequent to the isolation of *Rpi-bt1*, Song et al. (2003) reported isolation of the RB locus from *S. bulbocastanum*. A current BLAST

search of the GenBank non-redundant database (Altschul et al. 1990) using the deduced amino acid sequence of *Rpi-bt1* returns the proteins RGA1, RGA2 and RGA3 from the RB resistance locus (Song et al. 2003; van der Vossen et al. 2003) as the most significant alignments at 76–78% identity. The deduced amino acid sequence for RPI-BT1 predicted from the gDNA of *Rpi-bt1* is most similar to the deduced amino acid sequence for RGA2 (the functional RB gene that confers late blight resistance) at 78% identity and 86% positive homology. The amino acid sequence alignment of RPI-BT1 and RGA2 is shown in Fig. 3.

In order to express *Rpi-bt1* in transgenic plants a chimeric transgene was constructed. Transcription of the *Rpi-bt1* gene is directed from the potato *Ubi3* promoter, which will result in constitutive moderate-level expression (Garbarino and Belknap 1994a; Garbarino and Belknap 1994b). The *Ubi3* polyadenylation signal was fused to the 3' end of the

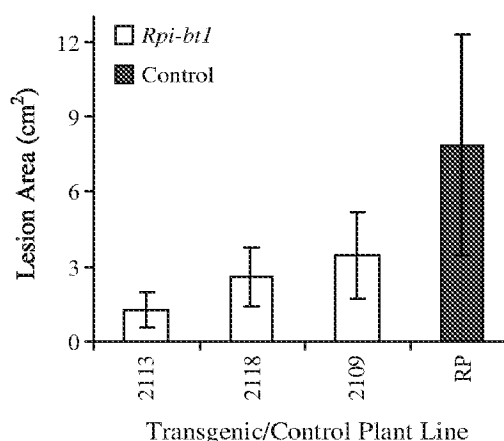
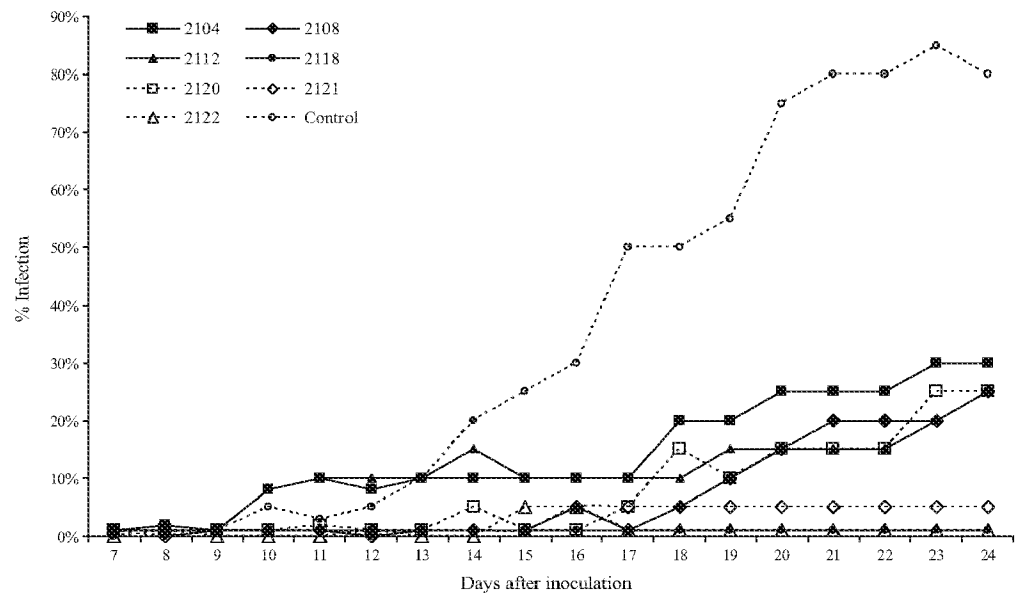


Fig. 5 Analysis of the *Rpi-bt1* resistance gene in detached leaf assays. Lesion area in transgenic potato lines expressing the chimeric *Rpi-bt1* transgene compared to the control cv. Red Pontiac (RP). Data represent the mean and standard deviation of 7 to 9 leaves (average 8.5) per plant line assessed 4 days after inoculation

Fig. 6 Complementation analysis of the *Rpi-bt1* gene in whole plant assays. Time course and extent of infection in selected glass-house grown *Rpi-bt1* transgenic and control cv. Atlantic potatoes. Data represents a single pot per plant line from a representative inoculation and time course



sequence (Fig. 4). The transgene was mobilized into the binary transformation vector pBINPLUS/ARS (Belknap et al. 2008; McCue et al. 2006). This binary vector construct was employed to introduce the transgene into the susceptible potato variety Atlantic. Transgenic potato plants were screened for resistance to late blight by detached leaf assay (Trognitz et al. 1995). Lesion size in the transgenic lines was reduced compared to the Red Pontiac control (Fig. 5). To examine the efficacy of *Rpi-bt1* to confer resistance to late blight in vivo, transgenic potato plants were grown in the glass house and scored for disease after inoculation with *P. infestans* US-8 genotype (Fig. 6). In contrast to the experiments with *Rpi-bt2* and *Rpi-bt3*, six of the transgenic lines had intermediate levels of resistance, and two of the transgenic lines (2,118 and 2,122) exhibited no infection 24 days after inoculation.

As shown in Figs. 5 and 6, the *Rpi-bt1* transgene constructed from the genomic cDNA conferred resistance to *P. infestans* in transgenic potatoes. While the *Rpi-bt2* and *Rpi-bt3* genes do not, individually, confer a resistant phenotype, this does not preclude a role for these genes or additional alleles in enhancing *Rpi-bt1*-mediated resistance originating from this locus.

Conclusion

Infection by *P. infestans* remains a threat to potato growers worldwide. Identifying sources of horizontal and vertical resistance to the various races of *P. infestans* remains a challenge as the genetics of the oomycete will evolve in parallel with the identification and deployment of resistance genes and strategies. Establishing durable resistance to the commonly encoun-

tered races of *P. infestans* will have an important but potentially limited life. The wild potato relative *S. bulbocastanum* is a good source of strong horizontal resistance to infection by a wide number of *P. infestans* races. Two separate loci *RB/Rpi-blb1* (Song et al. 2003; van der Vossen et al. 2003) and *Rpi-blb2* (van der Vossen et al. 2005) have now been described, each containing multiple RGAs. Additional functional genes from these loci may also confer resistance to other pathogens and diseases in the future. However, neither introgressed loci nor single genes have proven to confer the level of resistance found in the wild parent. Similarly the *Rpi-bt1* gene described here is not sufficient to confer the level of field resistance observed in the wild parent. This may be due in part to its expression via the constitutive *Ubi3* promoter. We have subsequently isolated a native promoter fragment for *Rpi-bt1* which is available for future studies. It is also possible that genes from both loci are required for the wild type levels of resistance seen in *S. bulbocastanum*. As a first step experiments are underway to combine the *RB* and *Rpi-bt1* genes in a single line using a transgenic approach. The Primers used to identify the *S. bulbocastanum* as the source of the *Rpi-bt1* gene may be useful as markers for breeders in combination with the markers for *RB* (Colton et al. 2006) to combine both of these loci. Hopefully the *Rpi-bt1* gene in concert with additional R genes from *S. bulbocastanum* or other potato relatives a strong durable resistance to infection by *P. infestans* will be achieved.

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Molecular cloning of the potato *Gro1-4* gene conferring resistance to pathotype Ro1 of the root cyst nematode *Globodera rostochiensis*, based on a candidate gene approach

Jürgen Paal^{1,†}, Heike Henselewski¹, Jost Muth^{1,‡}, Khalid Meksem², Cristina M. Menéndez^{1,§}, Francesco Salamini¹, Agim Ballvora¹ and Christiane Gebhardt^{1,*}

¹MPI für Züchtungsforschung, Carl von Linné Weg 10, 50829 Köln, Germany, and

²Department of Plant, Soil and General Agriculture, Southern Illinois University at Carbondale, Carbondale, IL 62901-4415, USA

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*For correspondence (fax +49 221 5062 413; e-mail gebhardt@mpiz-koeln.mpg.de).

†Current address: ALTANA Pharma AG, Byk-Gulden-Str.2, 78467 Konstanz, Germany.

‡Current address: Fraunhofer IME, Worringerweg 1, 52074 Aachen, Germany.

§Current address: Universidad de la Rioja, Departamento de Agricultura y Alimentación, C/ Madre de Dios 51, 26006 Logroño, Spain.

Summary

The endoparasitic root cyst nematode *Globodera rostochiensis* causes considerable damage in potato cultivation. In the past, major genes for nematode resistance have been introgressed from related potato species into cultivars. Elucidating the molecular basis of resistance will contribute to the understanding of nematode–plant interactions and assist in breeding nematode-resistant cultivars. The *Gro1* resistance locus to *G. rostochiensis* on potato chromosome VII co-localized with a resistance-gene-like (RGL) DNA marker. This marker was used to isolate from genomic libraries 15 members of a closely related candidate gene family. Analysis of inheritance, linkage mapping, and sequencing reduced the number of candidate genes to three. Complementation analysis by stable potato transformation showed that the gene *Gro1-4* conferred resistance to *G. rostochiensis* pathotype Ro1. *Gro1-4* encodes a protein of 1136 amino acids that contains Toll-interleukin 1 receptor (TIR), nucleotide-binding (NB), leucine-rich repeat (LRR) homology domains and a C-terminal domain with unknown function. The deduced *Gro1-4* protein differed by 29 amino acid changes from susceptible members of the *Gro1* gene family. Sequence characterization of 13 members of the *Gro1* gene family revealed putative regulatory elements and a variable microsatellite in the promoter region, insertion of a retrotransposon-like element in the first intron, and a stop codon in the NB coding region of some genes. Sequence analysis of RT-PCR products showed that *Gro1-4* is expressed, among other members of the family including putative pseudogenes, in non-infected roots of nematode-resistant plants. RT-PCR also demonstrated that members of the *Gro1* gene family are expressed in most potato tissues.

Keywords: *Solanum tuberosum*, *Globodera rostochiensis*, nematodes, resistance gene, candidate gene family.

Introduction

About 60 parasitic nematode species feed on potato plants (Jensen *et al.*, 1979). Species causing yield reduction are cyst nematodes of genus *Globodera*, root lesion nematodes of genus *Pratylenchus*, and root knot nematodes of genus *Meloidogyne* (Brodie, 1999). *Globodera rostochiensis*, the golden cyst nematode, and *G. pallida*, the white cyst nematode, are considered the most damaging for potato production (Brodie, 1984; Evans and Trudgill, 1992). Both

Globodera species are distributed worldwide and occur either intermixed or individually in potato production areas (Mai, 1977). They presumably originated in the Andean mountains of South America where they co-evolved with their hosts (Evans and Brodie, 1980).

Globodera root cyst nematodes have as hosts Solanaceae species, among those important crops such as potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum*),

and eggplant (*S. melongena*; Southey, 1965). In contrast to other plant parasitic nematodes (e.g. *Heterodera schachtii*), propagation of *Globodera* on the model plant *Arabidopsis thaliana* is not possible (Sijmons et al., 1991). *Globodera* larvae rest in the soil in eggs encapsulated in a small (diameter < 1 mm), spherical cyst. In spring and early summer, excretions of potato roots stimulate the hatching of the larvae and act as an attractant. Mechanically supported by their stylet and enzymatically by oesophageal secretions, the nematodes invade the potato roots. In the inner cortex, only the females induce the formation of a large syncytium feeding site, whereas the males die after mating. The syncytium expands until it is in close proximity to the xylem. The female produces 200–300 eggs, and its spherically enlarged body penetrates the root. The animal's body passes through a white and yellow (only *G. rostochiensis*) colored stage before it develops into a brown cyst containing infectious larvae and dies. The cysts are remarkably resistant to unfavorable environmental conditions and nematicides and can persist more than 10 years in the soil, which makes control difficult (reviewed in Sijmons, 1993; Williamson and Hussey, 1996).

Genes for nematode resistance have been introgressed in potato cultivars from other tuber bearing *Solanum* species such as *S. andigena*, *S. vernei*, and *S. spegazzinii* (Ross, 1962, 1986). Several of these genes have been mapped, including genes for resistance to *G. rostochiensis*, *G. pallida*, and *Meloidogyne chitwoodi* (reviewed in Gebhardt and Valkonen, 2001). *Gro1*, a major, dominant locus conferring resistance to all pathotypes of *G. rostochiensis* has been localized on potato chromosome VII (Barone et al., 1990), and high-resolution mapping assigned *Gro1* to an interval of 1.4 cM (Ballvora et al., 1995). Short sequence signatures conserved between the tobacco *N* gene for resistance to tobacco mosaic virus (Whitham et al., 1994) and *RPS2* of *A. thaliana* conferring resistance to the bacterium *Pseudomonas syringae* (Bent et al., 1994; Mindrinis et al., 1994) were used to amplify from the potato genome several different resistance-gene-like (RGL) DNA fragments. The RGL fragments *St332* and *St334* co-localized with *Gro1* and identified a clustered family of closely related genes at the *Gro1* locus (Leister et al., 1996). *St332* and *St334* were 95% identical at the nucleotide level and shared 72% sequence similarity with the nucleotide-binding (NB) domain of the *N* gene from tobacco.

The cloning of four nematode resistance (*R*) genes from plants has been reported. *Hs1^{pro-1}* from sugar beet confers resistance to the beet cyst nematode *H. schachtii* (Cai et al., 1997). The tomato gene *Mi-1.2* on chromosome 6 mediates resistance to three different root knot nematode species of the genus *Meloidogyne* and to the potato aphid *Macrosiphum euphorbiae* (Milligan et al., 1998). The *Gpa2* gene on potato chromosome XII controls pathotype-specific resistance to *G. pallida* (Van der Vossen et al., 2000). Recently,

the tomato gene *Hero* on chromosome 4 was cloned, which confers resistance to various pathotypes of *G. rostochiensis* and *G. pallida* (Ernst et al., 2002). Five major classes of plant genes for resistance to different types of pathogens are currently described (reviewed in Dangl and Jones, 2001). All nematode *R* genes, except *Hs1^{pro-1}*, are structurally related and fall into the class of *R* genes that share a leucine zipper (LZ) motif, an NB, and a leucine-rich repeat (LRR) domain. *Hs1^{pro-1}* codes for an unusual protein with an LRR domain, which does not fit a common consensus motive for plant disease *R* genes.

Here, we report the cloning and characterization of the *Gro1* gene family on potato chromosome VII. We show by complementation analysis that *Gro1-4*, one member of the family, encodes a functional nematode *R* gene. Our cloning strategy relied on the hypothesis that at least one member of the candidate gene family detected by RGL fragments *St332* and *St334* at the *Gro1* locus was indeed a gene for resistance to *G. rostochiensis*.

Results

Identification, cloning, and characterization of *Gro1* candidate genes

Six potato genotypes were subjected to Southern gel blot analysis using *TaqI* as restriction enzyme and the NB-homologous sequence *St332* as probe (Figure 1). The diploid clones P40 and P18 were the resistant (*Gro1/gro1*) and susceptible (*gro1/gro1*) parent, respectively, of population F1840 used for high-resolution mapping of *Gro1* and *St332* (Ballvora et al., 1995; Leister et al., 1996). Plant R458 was resistant to *G. rostochiensis* pathotype Ro1 (*Gro1/gro1*) and was one of 121 recombinant plants selected from 1100 plants of population F1840 for having a crossing-over between two markers that flanked the *Gro1* locus (Ballvora et al., 1995). The diploid genotype P6/210 was a progeny of the cross between the diploid clones P41 and P40 and was selected for having both the *Gro1* nematode *R* gene and the *R1* gene for resistance to late blight (genotype *Gro1/gro1*, *R1/r1*; Ballvora et al., 2002). P6/210 was the source of the lambda and BAC genomic libraries. The nematode-susceptible, tetraploid variety Désirée was used for molecular complementation analysis. Three accessions of the diploid, wild potato species *S. spegazzinii*, the historical source of the *Gro1* resistance (Barone et al., 1990; Ross, 1986), were included in the Southern gel blot. The *St332* probe detected, for each genotype, a distinct *TaqI* fingerprint with 7–16 restriction fragments between 500 and 2000 bp in length (Figure 1). Twelve *TaqI* fragments were shared between the *S. spegazzinii* accessions and resistant as well as susceptible *S. tuberosum* genotypes, indicating similarity between the two species for this particular chromosome

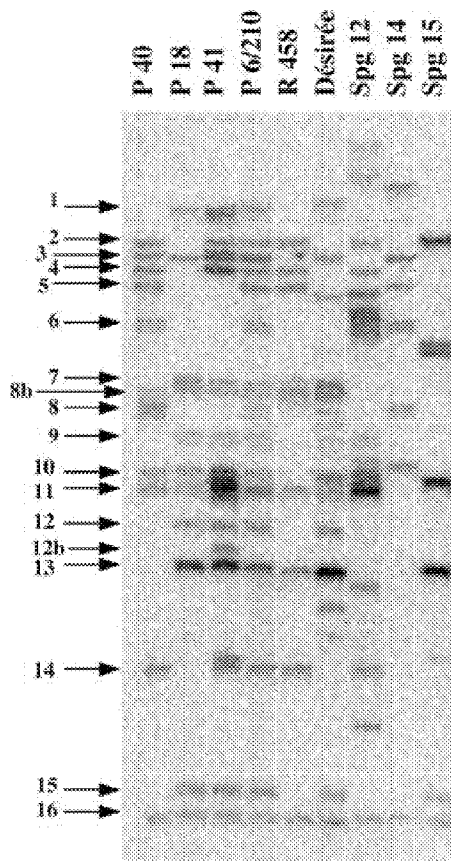


Figure 1. *TaqI* RFLPs of the *Gro1* candidate gene family.

Genomic DNA of different potato genotypes was restricted with *TaqI*, separated on denaturing 4% polyacrylamide gels, blotted, and hybridized to the NB domain homologous marker *St332* (Accession U60080). Diploid clones P40 and P18 were the resistant (*Gro1/gro1*) and susceptible (*gro1/gro1*) parents, respectively, of the F1840 mapping population. Diploid P6/210 was a nematode-resistant (*Gro1/gro1*) F₁ hybrid from the cross P41 × P40, and was used for genomic library construction. R458 was a nematode-resistant (*Gro1/gro1*) plant of the F1840 mapping population and was the only recombinant within the cluster of *Gro1* candidate genes. The susceptible, tetraploid variety Désirée (*gro1/gro1/gro1/gro1*) was used for plant transformation and functional complementation analysis. Spg12, spg14, and spg15, having unknown resistance phenotype, were different accessions of *S. spegazzinii*, the wild, diploid potato species, from which the *Gro1* resistance gene should have originated. *TaqI* fragments and the corresponding candidate genes were numbered as shown on the left.

fragment, but not pointing to any specific *Gro1* candidate gene. The *TaqI* fragments present in the parental genotypes P40, P18, and P41 and inherited by progeny P6/210 and/or R458 were numbered from 1 to 16 (Figure 1). The members of the *St332* gene family having these *TaqI* fragments were named, accordingly, from gene 1 to 16. Genes 1, 7, 9, 12, 12b, 13, and 15 were absent in P40, the source of the *Gro1* nematode resistance, and were therefore excluded as *Gro1* candidates. Genes 2–6, 8b, 8, 10, 11, 14, and 16 were present in P40. The nematode-resistant plant R458 lacked, however, genes 3, 6, 8, and 10 (Figure 1), which restricted the *Gro1* candidates to genes 2, 4, 5, 8b, 11, 14, and 16. These seven

members of the *St332* gene family were present in P40 and inherited by R458 (Figure 1). Genes 2, 4, 5, and 14 co-segregated with nematode resistance in the F1840 population (Leister *et al.*, 1996), whereas gene 8b was linked in coupling phase to the susceptible allele *gro1* of P40 (not shown), excluding this gene as a candidate. The presence of gene 8b in the nematode-resistant plant R458 indicated, however, that the recombination break point in plant R458 was located within the *St332* gene cluster linked to *Gro1*. Gene 11 mapped to the *Gro1* locus but was also present in the susceptible parent P18, whereas gene 16 did not segregate in population F1840 (Leister *et al.*, 1996).

Lambda and bacterial artificial chromosome (BAC) genomic libraries were screened using *St332* as probe. Positive clones were isolated and subjected to fingerprint analysis with *TaqI* restriction enzyme (not shown). Based on presence of individual *TaqI* restriction fragments, BAC and/or lambda clones for all members of the *St332* gene family of genotype P6/210 were identified, with the exception of gene 9 (Figure 1; Table 1). Five BACs contained two, and a sixth BAC contained three of the *Gro1* candidate genes. This showed that genes 4 and 14, and 2, 5, and 11 were physically tightly linked. The combination of genes 2, 5, and 11 present in three BACs was used to deduce their linear order 5-2-11 on BAC clone BA98P9 (Table 1). Whether the genomic clones originated from either P40 or P41 could be inferred from the presence of the corresponding *TaqI* fragments in either P40 or P41 (Table 1; Figure 1), with the

Table 1 BAC and lambda P6/210 genomic clones containing members of the *St332* gene family and their parental origin

BAC clones	Lambda clones	Origin	<i>TaqI</i> fragment number
BA107K18	79, 94	P41	1
–	74, 48	P40	2
BA260K12, BA60B11	–	P40	5 + 2
BA261J14, BA78o5	–	P40	2 + 11
BA98P9	–	P40	5 + 2 + 11
BA10N22, BA70K20	10	P40	3
BA34L19, BA81L19	110	P41	3
BA60L16, BA73N5	–	P40	4
BA26N18	–	P40	4 + 14
–	65, 113	P40	5
BA115B4, BA49J20	–	P40	6
BA34L19, BA53o21	–	P41	7
BA62C17, BA71o17, BA78M21, BA119F19	36	P40	8
BA107K18	107	P41	10
–	75	P40	11
–	101	P41	11
–	33	P41	12
BA42o6, BA87D4, BA254K3	50	P41	13
BA25J5	98	P40	14
BA22K21, BA248H17	16	P41	15
BA106C14	–	P40	16

Table 2 PCR-based markers for mapping gene *16* and for assignment of genomic clones either to parent P40 or P41

Genomic clone used as marker source	Gene no.	Position of primer pairs	Type of polymorphism	PCR product size (bp)	Primer sequences (5' → 3')
BA106C14	16	At T3 end	CAPS (<i>A/lul</i>)	629	GACAAAAGTAGAATAATTTAGTAGCTCTAC CGCTACTTAACATACTATGTATC
BA106C14	16	At T7 end	Specific PCR for P40 allele	1713	CTCTCAGTTCCTCCTCCGCGTGCAA GTACATTGTTAGCTGCTGAATCATGTTACC
BA25J5	14	At T3 end	SNP	1684	GTTAAGAGAGTCTTTAAAGCTTTTAAAGAGTCGT CCAAGTGTATATTACTAACAAAATTG
Lambda 10	3	At T3 end	Specific PCR for P40 allele	2089	GGCTACATGGTCTTATGGA GCAGTTGTATACCGTCAACAAA
Lambda 110	3	At T7 end	Specific PCR for P41 allele	487	GGATACTACTGTGGAAAACC TGCAACTAAGGACAGGCTC
Lambda 107	10	In gene 10	Specific PCR for P41 allele	465	ACAAAAATGTGATGAACCTGTAAAC AACACGTTCTAAATCTGTGCC
Subclone b32b of BA98P9	2	In gene 2	Specific PCR for P40 allele	481	AGTATAACAGGGATATATGCTC TAGGCATCCATTTGTGAAAAAG
Lambda 75	11	In gene 11	Specific PCR for P40 allele	449	ATGTTTGAAAAACCTTGTTGGT AGATGTGCATTCATTAGCAAATAT
Lambda 101	11	In gene 11	Specific PCR for P41 allele	572	ACCTGACTAATGAAGAAGAAGC TCAAAGCAGTAGCACCCAAAC

exception of genes 2–4, 10, 11, 14, and 16. The *TaqI* fragments characteristic for these genes were, in fact, present in both parents of P6/210 (Figure 1). PCR-based markers were developed, therefore, for mapping gene *16* and for identifying genomic clones having the P40 allele of these genes (Table 2). The PCR-based markers for both ends of BAC BA106C14 including gene *16* mapped in population F1840 to the short arm of chromosome IV, 5 cM proximal to RFLP marker GP180 (not shown), therefore excluding gene *16* as candidate for *Gro1*, which is located on chromosome VII. Allele-specific markers for genes 2, 3, 10, 11, and 14 identified BAC and lambda genomic clones originating from P40 or P41. The parental origin of genomic clones having gene 4 was deduced from its physical linkage to gene 14 (Table 1). Based on the assignment of P6/210 *TaqI* fragments and genomic clones to either the P40 or P41 allele, the *St322* gene cluster of the P40 allele linked to *Gro1* consisted of at least eight genes (2–6, 8, 11, and 14), whereas the P41 allele linked to *gro1* consisted of at least nine genes (1, 3, 7, 9–13, and 15).

Members of the *St322* gene family were sequenced from lambda or BAC genomic clones by primer walking, starting with outward-directed primers binding to the *St322* homologous region (Accessions AY196151–AY196163). The organization in four exons and three introns of eight genes of the P40 allele linked to *Gro1* was determined by sequence comparison to 5' and 3' rapid amplification of cDNA ends (RACE) products (see below) and is shown in Figure 2. Database homology searches revealed that the *St322* gene family was homologous to the Toll-interleukin 1 receptor (TIR)/NB/LRR class of plant genes for pathogen resistance. The three domains were encoded by the first three exons.

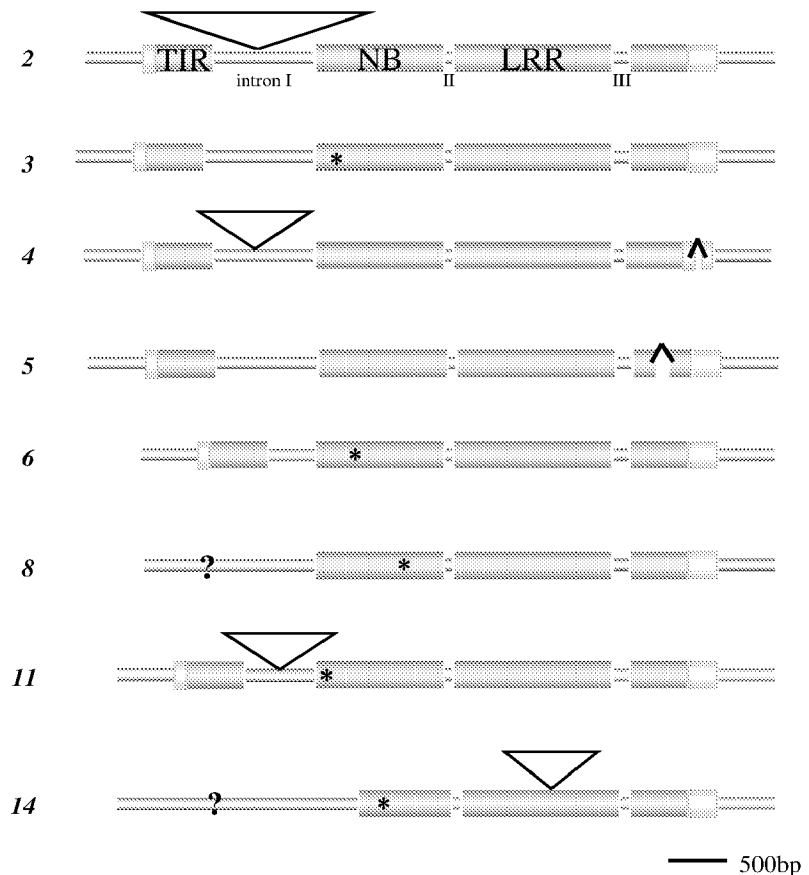
The TIR coding exon I was identified in six of the eight genes and was separated from the NB coding exon II by an intron of variable size because of the insertion of retroelements in intron I of genes 2, 4, and 11. The retroelement insertion in intron I of gene 2 was itself interrupted by another insertion (not shown in Figure 2), resulting in an intron size of 11.2 kbp. In the NB coding region, stop codons at different positions were identified in genes 3, 6, 8, 11, and 14 and were confirmed by sequencing both DNA strands. In addition, the NB coding exon of gene 14 was truncated at the 5' end. In all eight genes, the NB coding exon was separated from the LRR coding exon III by a small intron having similar size in the different genes. The LRR coding exon of gene 14 contained an inverted repeat element. Exon IV did not show homology to other genes with known function. Based on sequence analysis, genes 2, 4, and 5 encoded putative functional TIR/NB/LRR-type proteins whereas genes 3, 6, 8, 11, and 14 were considered non-functional because of internal stop codons. Genes 11 and 14 were excluded therefore as candidates, leaving genes 2, 4, and 5, all linked to the *Gro1* allele of P40, as the three remaining candidates for the *Gro1* nematode *R* gene.

Subcloning and complementation analysis

The candidate genes 2, 4, and 5 were subcloned into the binary plant transformation vector pCLD04541 and transferred to the susceptible potato cv. Désirée via *Agrobacterium tumefaciens*-mediated transformation. The 22-kbp subclone b32b containing gene 2 (Accession AY196153) was obtained from BAC clone BA98P9 (Table 1). Gene 4 (subclone b07c with 14.5 kbp, Accession AY196151)

Figure 2. Structural alignment of eight members of the *Gro1* family linked in coupling phase to the resistant *Gro1* allele of P40 (Accession numbers AY196151–AY196163).

Asterisk (*) indicates stop codon mutations. Triangles indicate retroelement insertions in intron I of genes 2, 4, and 11, and an inverted repeat element insertion in the LRR domain of gene 14. Question mark indicates that no TIR coding exon I was found in the sequenced genomic region. A deletion in genes 4 and 5 is indicated with a 'roof' symbol. TIR, Toll/Interleukin-1 receptor homology region; NB, nucleotide-binding domain; LRR, leucine-rich repeat domain. Exons are shown as dark gray boxes. Boxes in light gray are 5' and 3' untranslated regions upstream of exon I and downstream of exon IV, respectively.



and gene 5 (subclone b16j with 10.5 kbp, Accession AY196152) were subcloned from BACs BA26N18 and BA60B11, respectively. Subclones b32b, b07c, and b16j included 2.3-, 4.0-, and 2.7-kbp sequence upstream of the putative transcription start site and 3.4-, 1.1- and 3.0-kbp sequence, respectively, downstream of the putative stop site. Between 30 and 37 independent transgenic lines were regenerated per construct and tested for resistance to *G. rostochiensis* pathotype Ro1 (Table 3). All plants transformed with gene 2 or 5 constructs were susceptible. Of 30 lines transformed with the gene 4 construct, 14 lines were resistant and 16 lines were susceptible (Table 3). Candidate gene 4 was therefore able to confer resistance to *G. rostochiensis* pathotype Ro1 and was named *Gro1-4*. Using the b07c construct, the frequency of

nematode-resistant transgenic plants was 47%. Five resistant and five susceptible *Gro1-4* transgenic lines were analyzed for the presence of *TaqI* fragment 4 that was absent in untransformed cv. Désirée (Figures 1 and 3). The *TaqI* fragment 4 was present, as expected, not only in the five resistant plants but also in the five susceptible plants tested (Figure 3). It was concluded that the susceptible phenotype of these five plants may be the consequence of position effects, gene silencing, or rearrangements of the transgene.

Structure of the *Gro1-4* nematode resistance gene

The 14.5-kbp sequence of subclone b07c carrying the nematode *R* gene *Gro1-4* was determined (Accession AY196151). No other open-reading frames besides the TIR/NB/LRR gene were found. Intron I included a retroelement and was 5465-bp long. Introns II and III were 76 and 115 bp long, respectively. The putative promoter region contained the ethylene-responsive element 'AWTTCAAA' (Montgomery *et al.*, 1993), four CAAT boxes, of which two were in reverse orientation, and the motif 'CAANN-NATC' that is conserved in 5'-upstream regions of circadian clock-controlled light harvesting complex (Lhc) genes (Piechulla *et al.*, 1998). The putative TATA box was part

Table 3 Complementation test with *G. rostochiensis* pathotype Ro1

Construct	No. of transgenic lines tested	No. of susceptible lines	No. of resistant lines
b32b/gene 2	37	37	0
b07c/gene 4	30	16	14
b16j/gene 5	35	35	0

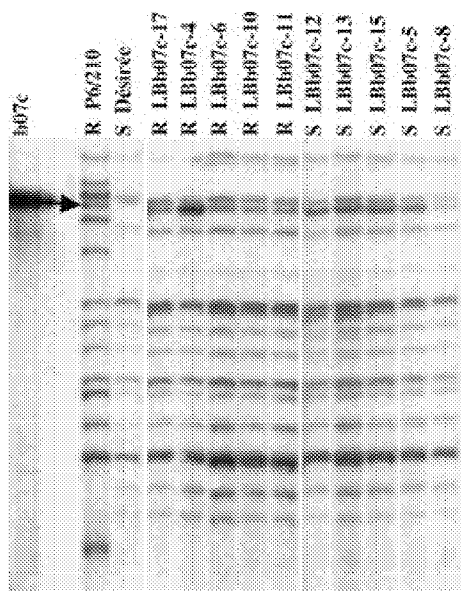


Figure 3. Southern gel blot analysis of nematode-resistant (R) and susceptible (S) transgenic Désirée plants.

From left to right: 1st lane: Subclone b07c containing gene 4 used for *Agrobacterium*-mediated transformation. 3rd lane: P6/210, the source of cloned candidate genes 1–16. 4th lane: Untransformed Désirée. 5th to 15th lane: R and S Désirée plants transformed with the b07c construct. DNA was restricted with *TaqI*, separated on denaturing 4% polyacrylamide gels, blotted, and hybridized to the *St332* probe. The *TaqI* fragment 4 indicative for *Gro1-4* is indicated by the arrow.

of a TA microsatellite with variable length in different members of the gene family (Figure 4a). The microsatellite in genes *gro1-5* and *gro1-6* was shorter when compared to genes *gro1-2* and *Gro1-4*. The microsatellite, and thus a putative TATA box, was missing in genes *gro1-3* and *gro1-11*. Two ATG translation start codons were present in the *R* gene *Gro1-4*. Translation from the first ATG results in the formation of a signal peptide (Figure 4b), as predicted by SIGNALP. Based on a purine present at position –3 and +4 (Kozak, 1991), translation more likely starts at the second ATG codon. The deduced amino acid sequence (1136 amino acids, Figure 4c) comprised a TIR homology domain as predicted by PROFILESCAN, an NB domain including the kinase 1a (P-loop), kinase 2, and kinase 3a motifs (Saraste *et al.*, 1990; Walker *et al.*, 1982), the GLPLAL amino acid signature, and an LRR domain with 11 LRR modules (predicted by PROFILESCAN). The consensus motif of the

LRR modules XLXXLXX(C/N/T)X(X)LXXXP indicated that the *Gro1-4* protein may be located in the cytoplasm (Jones and Jones, 1997). No function could be predicted for the C-terminal part of the resistance protein encoded by exon IV. At the amino acid level, *Gro1-4* and *gro1-2* were 95% identical to each other and both genes were 90% identical to *gro1-5*. The deduced *Gro1-4* protein differed by 29 amino acids from *gro1-2* and *gro1-5* (Figure 4c). Sixteen amino acid changes were non-conservative. When compared with functionally characterized plant *R* genes in the database, *Gro1-4* was most closely related to the tobacco N protein for resistance to tobacco mosaic virus (Whitham *et al.*, 1994) and to the RPP1-WsA protein for resistance to *Peronospora parasitica* from *A. thaliana* (Botella *et al.*, 1998) sharing 38 and 35% sequence identity, respectively. Homology with the functionally most similar tomato nematode resistance protein Hero (Ernst *et al.*, 2002) was low (24% identity). At the nucleotide level, the NB region of *Gro1-4* shared 93 and 92% sequence identity with the tomato NB-LRR *R* gene homologs Q2 and Q112, which map to syntenic positions on tomato chromosome 7 (Pan *et al.*, 2000).

Expression of the *Gro1* gene family

Total RNA was isolated from roots of the nematode-resistant line P6/210 20 days after infection with *G. rostochiensis* pathotype Ro1. No expression of the *Gro1* gene family was detectable on Northern gel blots probed with *St322*. On a virtual Northern blot of the same tissue, using as template PCR-amplified whole cDNA instead of RNA, the *St332* probe detected three cDNA bands of 3.6, 2.3, and 1.8 kbp (not shown). The size of the largest 3.6-kbp product corresponded to transcripts predicted for the genes *gro1-2*, *Gro1-4*, and/or *gro1-5*. The smaller cDNA fragments can be products of truncated or alternatively spliced transcripts or of transcripts from unknown members of the *Gro1* gene family.

RACE PCR was performed on poly(A)⁺ RNA from roots of P6/210 20 days after infection with the nematode. 5' and 3' RACE products of variable length were cloned and sequenced. The *in silico* assembly of the sequence of the longest 5' and 3' RACE products resulted in a putative transcript of 3.6 kbp, the same size as detected on the virtual Northern blot (not shown). The deduced amino acid sequence of the 3.6-kbp assembled RACE transcript was

Figure 4. Promoter region, 5' untranslated nucleotide sequence, and deduced amino acid sequence of the *Gro1-4* nematode resistance gene.

(a) Promoter and 5' untranslated region of *Gro1-4* (Accession AY196151) aligned with corresponding regions of *gro1-2* (AY196153), *gro1-3* (AY196154), *gro1-5* (AY196152), *gro1-6* (AY196155), and *gro1-11* (AY196157), all inherited from P40. Putative CAAT boxes and the microsatellite region are underlined. Putative regulatory elements (ethylene-responsive element 'AATTCAAA', circadian expression motif 'CAATGGAATC', and TATA box) are shaded gray. The first putative ATG translation start codons are printed bold. The second ATG is present in all genes (not shown). The arrow indicates the putative transcription start based on the 5' end of the longest RACE product.

(b) Putative signal peptide of *Gro1-4*.

(c) Deduced amino acid sequence of *Gro1-4*. Conserved structural motifs are indicated. The 29 amino acids that distinguish *Gro1-4* from *gro1-2* and *gro1-5* are shown as large, bold letters. The 16 non-conservative amino acid changes are indicated by black arrowheads.

[illegible]

(b)

MQVAVNIITNFLFRFLVLLSRRPVIK

putative signalpeptide

(c)

1 MNCESSLLPSPDITRWSYDVFLSFRGEDVRKTTVDHLYLALEQKCINTFFKDDERLEKGFISPELVSSIE
71 ESRIALIIIFSEKNYANSTWCLDELTRIMECKNVEGQIVVPVFYIVDVPSTVVRKQKRSIFGEAFSKHEARFQED
TIR (Toll/Interleukin-1 receptor homology) - region
141 RVQKWRAALEEAANTISGWDLFNTANGHEARVMEKIAEDIMARIGSQRHASNARNLVGMESHMHVEYKMLG
211 IGSQGVHFLGIIIGMSGVGKTTTLARVIYDNIRSQFQACFLHEVRDRSAKQGLERLQKILLSEILVVEKLR
Kinase 1a, P-loop motif
281 INDSFEGANMCKQRLQYKEVLLVLDDVDHIDQLNALAGEREWFGDGSRIIITTEKDKHLVVKYETEKIYRM
Kinase 2 Kinase 3a
351 KTLNNYESLQLFKQNAFRKNRPTRKEFEDLSAQVINHTDGLPLALKVLCSFLYGRGLDENISEVERLKKQIP
421 ENEILKKLEQSEFTGLHNTQKIFLDIACFFSGKKKDSVTRILESFHFQCPVIGIKVLMEKCLITILQGRIT
491 IHQLIQDMGWHIVRREATDDPRMCSRMMKKREDICPVLERNLGTOKNEGMSLHITNEEEVNFQGGKAFMQMT
561 BLRFLKFNNAYVCOGPEFLDELRLWLDWNGYPSKSLFNSFKGDQVLGLELKKSRITQLWKTSEDGLGKLY
631 MNLSSHQKLIRTPDFSVTPNLERLVLEECSLVEINFSIENLGKLVLLNLKNCNNKLTLPKRIKLEKLEI
LRR (leucine-rich repeat) - region
701 IVLTCGSKLRTTFPEIERKMNKCLAEYLGLGATSLSELSPASVENLSGVGVINLSYCKHLESPLSSIFALEKLE
771 TLDVSGCSKLEKLPDDLGLLVGLEELHCHTHTAIQTIPSSMSLLKHLKHLSSGCCNALESQVSSSSSHCKKS
841 MGVNFQNLGSLCSLIMLDLSDCNISDGGILNHLGFLSSLLEILLNENNFSNIPAAASISRFTELRRLKLN
910 CGGRLESPLPELPPSINGIFANECTSLMSIDQLTKYPMLSDATPRNCRQLVENKQHTSMVDLSLLKQMLEAL
980 YMNVRFCLYVPGMEIPEWFTYESWGTQSMVALPTNWFPTPTFRGFTVCEVILGRKMLFILGRNTHEVYGL
1050 ENMIWLNKRYDGLRQKISTSFQPIGSEKPGGLDITLITHIAFERSWKLENDLDYYRNNAFOLEFSACDH
1120 YCKDVVNGELGVRLVYEN*

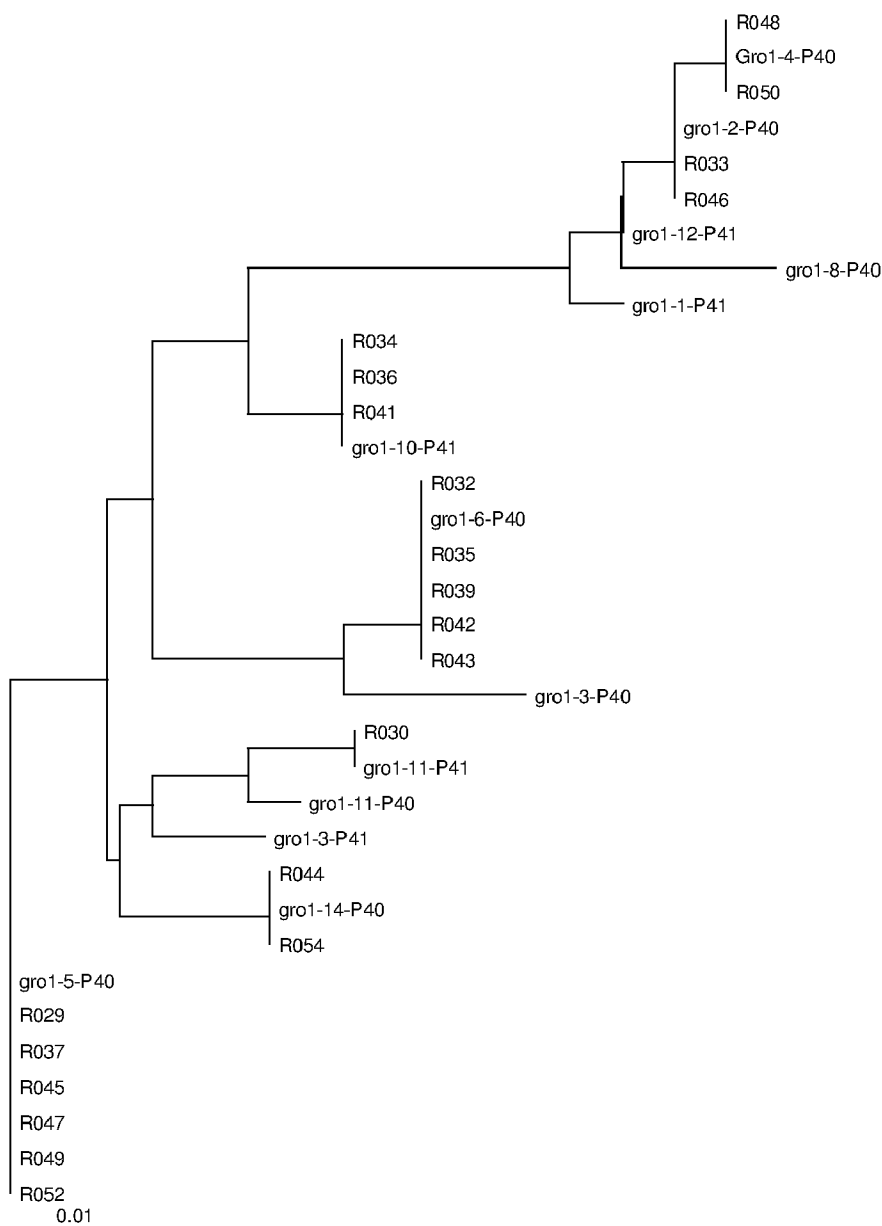


Figure 5. Sequence similarity of cloned RT-PCR products from uninfected roots of P6/210 to members of the *Gro1* gene family. RT-PCR products (sequences R029–R054) were generated with primers NBS3RACE1 addressed to exon II and LRR5RACE1 addressed to exon III, which did not discriminate between 13 different members of the *Gro1* gene family from both P40 and P41 alleles. RT-PCR products were cloned and sequenced. A phenetic tree was constructed from the comparison of 250-bp sequence flanking intron II of 13 members of the *Gro1* gene family with 21 RT-PCR products that matched one of the sequenced genes. Sequence comparison and tree drawing were performed using the programs CLUSTALW and TREEVIEW. Scale: 0.01 = one nucleotide exchange per 100 bp. RT-PCR products R048 and R050 matched the sequence of the *Gro1-4* resistance gene.

most closely related to the *Gro1-4* deduced nematode resistance protein, differing by eight amino acids (not shown).

To dissect the expression of individual members of the *Gro1* gene family, RT-PCR experiments were performed with two primers spanning intron II (Figure 2), which bind to absolutely conserved regions in all sequenced members of the *Gro1* family. A 500-bp cDNA product was generated from total RNA of uninfected roots of P6/210 and cloned. The nucleotide sequences of 21 individual clones were compared to 13 highly homologous *Gro1* genomic sequences originating from P41 or P40 alleles, including *Gro1-4*. Between one and six cDNA products were identified that corresponded to 7 of 13 *Gro1* genomic sequences

and included both P40 and P41 alleles (Figure 5). Among those were two products that corresponded to the *Gro1-4* gene (R048 and R050 in Figure 5). This confirmed that *Gro1-4* was expressed in uninfected roots. Transcripts of members of the *Gro1* gene family having a stop codon within the NB domain were also found (*gro1-6*, *gro1-11*, and *gro1-14*). Based on the number of identical RT-PCR products, genes *gro1-5* and *gro1-6* of the P40 allele had the highest expression level in uninfected roots.

RT-PCR products of the expected size were also generated from stem, leaf, flower, tuber, and stolon cDNA preparations (Figure 6). This showed that members of the *Gro1* gene family are expressed in all tested potato tissues.

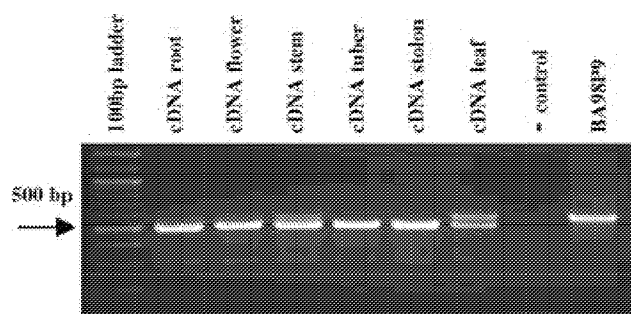


Figure 6. Expression of the *Gro1* gene family in different potato tissues. RT-PCR products were generated with primers NBS3RACE1 and LRR5RACE1 flanking intron II. The RT-PCR product from the genomic control, BAC BA98P9, included intron II and was therefore c. 80 bp larger when compared to the products from cDNA.

Discussion

The candidate gene approach for cloning Gro1-4

Positional cloning of a plant gene of unknown sequence requires the construction of a physical map covering the genetic interval between two markers flanking the target gene. This is followed by complementation or mutation analysis of all genes present within the physical map that co-segregate with the targeted gene's function. This approach is unbiased but usually very laborious and time-consuming, depending on the physical size of the target region, the number of genes to be considered, and the time required for the complementation test. The candidate gene approach has the potential to reduce the number of genes to be considered, but it is biased because of the hypothesis that it makes concerning the identity of the target gene. As the structural similarity was recognized between genes for resistance to different types of pathogens isolated from unrelated plants (Mindrinos *et al.*, 1994), a number of *R* genes have been cloned by a combination of both approaches, among others the first *R* genes of potato (Ballvora *et al.*, 2002; Bendahmane *et al.*, 1999; Van der Vossen *et al.*, 2000). In those cases, the sequence analysis of the available physical map revealed candidate genes with structural similarity to other plant *R* genes, which were then selected and tested for complementation. As the complementation test for resistance to nematodes by stable transformation requires at least 6 months in potato, to limit the number of genes to be tested, a candidate gene approach for cloning the *Gro1* gene was adopted, which did not include prior construction of a physical map. The RGL marker *St322* co-localized with *Gro1* based on a high-resolution genetic map (Ballvora *et al.*, 1995; Leister *et al.*, 1996). We hypothesized that the marker *St322* was, in fact, derived from a gene identical or highly homologous to the nematode *R* gene. The marker *St322* identified a complex family of highly homologous candidate genes, which clus-

tered, except for gene *gro1-16*, at the *Gro1* locus. With the exception of gene *gro1-9*, all candidate genes were subsequently isolated from lambda and BAC genomic libraries. Analysis of inheritance and linkage mapping reduced the number of candidates to five. Sequence analysis of 13 family members revealed putative pseudogenes and further reduced the number of candidates to three genes. The complementation test using these three genes under the control of their endogenous promoter identified the gene *Gro1-4*, which was able to confer resistance to *G. rostochiensis* pathotype Ro1 to the susceptible cv. Désirée.

When testing for overlapping ends the BACs having candidate genes derived from the resistant allele of genotype P40, BACs having genes 3 or 8 did overlap, whereas BACs having genes 5, 2, and 11, or 4 and 14 did not overlap with each other or with BACs having gene 6 (unpublished results). Based on BAC insertion sizes between 70 and 100 kbp, and between one and three genes of the *Gro1* family in each BAC (Table 1), we estimate that the eight genes are spread over at least 400 kbp. This indicates a genome organization at the *Gro1* locus reminiscent of the *Mi* locus for resistance to the root knot nematode *M. incognita* on tomato chromosome 6, with six to eight homologous genes present within 650 kbp (Milligan *et al.*, 1998). This is different from the *Hero* locus for resistance to potato cyst nematodes on tomato chromosome 4, where 14 homologous genes clustered within 118 kbp (Ernst *et al.*, 2002).

Among 1100 plants tested for recombination events in the *Gro1* region (Ballvora *et al.*, 1995), only plant R458 was recombinant within the *Gro1* gene cluster. Starting with the BACs having candidate genes, the construction of partial BAC contigs did not result in the genetic delimitation of the physical map of the *Gro1* region by placement of crossing-over events flanking *Gro1* (unpublished data). The physical size of the locus was therefore much larger than previously estimated (Ballvora *et al.*, 1995). The candidate gene approach, although risky, proved to be a useful and effective short cut for cloning *Gro1-4*.

Structure and function of the Gro1 gene family

The functional *Gro1-4 R* gene and the other fully sequenced *gro1* genes are all members of the TIR/NB/LRR class of plant genes for pathogen resistance. *Gro1-4* is the first plant gene for nematode resistance of this type and the first potato gene for resistance to *G. rostochiensis*. The tomato genes *Mi-1.2* for resistance to *M. incognita* (Milligan *et al.*, 1998) and *Hero* for resistance to *G. rostochiensis* (Ernst *et al.*, 2002), and the potato gene *Gpa2* for resistance to *G. pallida* (Bendahmane *et al.*, 1999) are all members of the coiled coil (CC)/NB/LRR class of plant *R* genes. *Gro1-4* and *Hero* are functionally most closely related, as both genes confer resistance to pathotype Ro1 of *G. rostochiensis*. The two genes occupy, in fact, different positions in the highly

syntenic potato and tomato genomes (Tanksley *et al.*, 1992) and share very little sequence similarity. This suggests that they interact with different nematode avirulence factors and may also trigger different signaling pathways. However, *gro1-16*, the only member of the *Gro1* family not included in the gene cluster on chromosome VII, maps to a similar position on potato chromosome IV as does *Hero* on the syntenic tomato chromosome 4. This can be inferred from similar genetic distances (5–7 cM) of *gro1-16* and the tomato marker CT229, most tightly linked to *Hero* (Ernst *et al.*, 2002), from potato anchor marker GP180 that has been mapped relative to CT229 on the tomato RFLP map (Tanksley *et al.*, 1992). The *gro1-16* gene has not been characterized further by sequencing. It may be part of a *R* gene cluster including different types on the short arm of potato/tomato chromosome 4. In potato, major quantitative trait locus (QTL) for resistance to *Phytophthora infestans* (Leonards-Schippers *et al.*, 1994) and *G. pallida* (Bradshaw *et al.*, 1998) have been mapped to this genomic region.

At the nucleotide level, *Gro1-4* was more than 90% identical with two NB-LRR-type *R* gene homologs of tomato, which map to syntenic positions on tomato chromosome 7 and are tightly linked to the tomato *I3* gene for resistance to *Fusarium oxysporum* (Bournival *et al.*, 1989; Pan *et al.*, 2000). The *I1* gene for resistance to a different race of the same fungus also maps to this region (Sarfatti *et al.*, 1991). The potato *Gro1* and tomato *I3/I1* loci may be orthologous, encoding genes that are highly sequence related but confer resistance to different pathogens such as nematodes and fungi.

The deduced *Gro1-4* resistance protein differs from the *gro1-2* and *gro1-5* susceptibility proteins by 16 non-conservative amino acid changes. One is located between the TIR and NB domains, six are in the LRR domain, and nine in exon IV with unknown functional significance (Figure 4c). One or more of these 16 amino acids may be responsible for the functional difference between *Gro1-4* versus *gro1-2* and *gro1-5*. Alternatively, sequence polymorphisms in the putative signal peptide region and further upstream in the promoter region may alter transcription, translation, and/or transport of *gro1* polypeptides, rendering them non-effective. One such candidate for functionally relevant polymorphisms is a microsatellite sequence of variable length that is located within 300 bp upstream from the second translation start codon of *gro1* genes.

Preliminary data indicate that *Gro1-4*, unlike *Hero* (Ernst *et al.*, 2002), does not confer resistance to all pathotypes of *G. rostochiensis*. In contrast, P40, the source of the *Gro1-4* gene, carries broad-spectrum resistance to *G. rostochiensis* (Barone *et al.*, 1990). The possibility exists that other members of the *Gro1* gene family are also functional nematode *R* genes and confer resistance to pathotypes other than Ro1. The broad-spectrum resistance phenotype

of P40 could then result from the concerted action of several family members. This is subject to further studies.

Expression of the *Gro1* gene family

Despite the fact that *Gro1-4* contains a large retroelement in intron I, *Gro1-4* is expressed at low level in uninfected roots of nematode-resistant plants, together with several other members of the gene family, including putative pseudogenes. The expression of genes that contain retroelements has been reported. The gene encoding starch branching enzyme (SBEIIb) of barley contains a retrotransposon-like element in intron II (Sun *et al.*, 1998). The intragenic insertion of a retrotransposon can result in alternative RNA processing, as has been shown for the maize *waxy* gene (Marillonnet and Wessler, 1997). Transcripts smaller than expected for genes *Gro1-4*, *gro1-2*, and *gro1-5* were detected on a virtual Northern blot using amplified root cDNA and among sequenced RACE products. This indicates occurrence of truncated or alternatively spliced transcripts of members of the *Gro1* gene family. Alternative splicing has also been observed for the *N* gene conferring resistance to tobacco mosaic virus (Whitham *et al.*, 1994).

Members of the *Gro1* gene family were expressed in all potato tissues tested. The distribution of gene-specific transcripts was not analyzed in tissues other than roots. Biological functions other than nematode resistance may therefore be encoded by the *Gro1* gene family.

Experimental procedures

Plant material

P40, P18, R458, P41, and P6/210 are diploid potato genotypes. P40 (H80.696/4) is an interspecific hybrid between *S. tuberosum* and *S. spengazzinii*. P40 is heterozygous for the *Gro1* *R* gene and resistant to all five known pathotypes (Ro1–Ro5) of *G. rostochiensis* (Barone *et al.*, 1990). P18 (H82.337/49) is the susceptible parent of the cross P18 × P40 used to map the *Gro1* resistance locus (Ballvora *et al.*, 1995; Barone *et al.*, 1990). R458 is one of 121 F₁ hybrids of the cross P18 × P40 that were selected from 1100 gametes of P40 for being recombinant between two markers flanking the *Gro1* locus (Ballvora *et al.*, 1995). R458 is heterozygous resistant to *G. rostochiensis* pathotype Ro1 (*Gro1/gro1*). P41 (H79.1506/1) and P40 are the parents of line P6/210 used to construct the lambda and BAC genomic libraries. P6/210 was selected for carrying both the *Gro1* gene for nematode resistance and the *R1* gene for late blight resistance in the heterozygous state (Ballvora *et al.*, 2002). The tetraploid cv. Désirée, which is susceptible to all pathotypes of *G. rostochiensis* was used for complementation assays. Three accessions of the wild tuber-bearing species *S. spengazzinii* are represented by plants spg12, spg14, and spg15 and were kindly provided by J. R. Hoekstra (Bundesforschungsanstalt für Landwirtschaft (FAL) at Braunschweig, Germany). Resistance of the spg plants to *G. rostochiensis* has not been tested.

Genomic libraries and library screening

A potato BAC library constructed as described by Meksem *et al.* (2000) from high-molecular weight DNA of line P6/210 was kindly supplied by LION Bioscience AG (Heidelberg, Germany). The library consisted of c. 100 000 clones with an average insert size of 70-kbp potato genomic DNA, cloned into the *Hind*III site of the pCDL04541 vector (Jones *et al.*, 1992). The library covers the diploid potato genome approximately seven times. The BAC library was screened by hybridization of high-density filters to the ³²P-labeled probe *St*332 (GenBank Accession no. U60080, Leister *et al.*, 1996), as described by Ballvora *et al.* (2002). A potato lambda library was constructed from genomic DNA of line P6/210 that was partially restricted with *Mbo*I and size-fractionated by sucrose density gradient ultracentrifugation using standard procedures (Sambrook *et al.*, 1989). The 15–20-kbp DNA size fraction was ligated into the *Bam*HI pre-digested lambda DASH-II vector, packaged using Gigapack II XL packaging extracts, and amplified in *Escherichia coli* strain XL1-Blue MRA (P2) following the supplier's instructions (Stratagene, La Jolla, CA, USA). Plaque lifts and filter hybridization to the *St*332 probe were performed using standard procedures (Sambrook *et al.*, 1989).

DNA isolation

Lambda plate lysate stocks and large-scale bacteriophage preparations were generated using standard protocols (Sambrook *et al.*, 1989). Lambda DNA was prepared using Qiagen Lambda Maxi Kit (Qiagen, Hilden, Germany). BAC DNA was isolated as described by Ballvora *et al.* (2002) by using the Qiafilter Plasmid Purification Kit (Qiagen). Potato genomic DNA was extracted and purified as described by Gebhardt *et al.* (1989) and Oberhagemann *et al.* (1999).

Southern gel blot analysis

*Taq*I enzyme restriction digest, electrophoresis on denaturing polyacrylamide gels (4% w/v), electroblotting onto a Biotodyne B nylon membrane (Pall, East Hills, NY, USA), and hybridization to the ³²P-labeled *St*332 probe were carried out as described by Gebhardt *et al.* (1989). Four to five micrograms of potato genomic DNA and 10 ng of BAC or lambda DNA were used per sample. Exposed phosphor imager screens were scanned using a STORM 860 Phosphor Imager (Amersham Pharmacia, Rockville, MD, USA).

Gene-specific PCR markers

Gene-specific primers were designed, either on the basis of anonymous end sequences from BAC or lambda clone genomic inserts containing the corresponding *Taq*I fragment (Table 1) or on the basis of sequences specific for the internal *St*332 homologous gene. Gene-specific sequence variation was identified by sequence alignment and comparison of all members of the *St*332 gene family located at the *Gro1* resistance locus of P40. PCRs were carried out in 25 µl volume on a PTC-225 Tetrad thermocycler (MJ Research, Waltham, MA, USA) using the following conditions: 4 min at 94°C followed by 40 cycles of 1 min at 92°C, 1 min at 58°C and 1 min kbp⁻¹ at 72°C, and finally 10 min at 72°C. PCRs consisted of buffer (Invitrogen, Carlsbad, CA, USA), 10 µM primers A and B (Table 2), 0.5 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), 1× BSA, 2% DMSO, 0.2 mM dNTPs, 1.4 mM MgCl₂, and 50 ng genomic, 5 ng BAC, or 1 ng lambda DNA as template.

Linkage mapping

Ninety-two F₁ hybrids of the cross P18 × P40 (mapping population F1840, Leister *et al.*, 1996) were scored for presence or absence of polymorphic PCR products. Linkage analysis relative to mapped RFLP markers was performed as described by Gebhardt *et al.* (1989) using the MAPRF software package (E. Ritter, NEIKER, Vitoria, Spain).

RACE and RT-PCR

Total RNA was isolated from different potato tissues using the RNeasy Plant Mini Kit (Qiagen) and the RNase-Free DNase Set (Qiagen) following the manufacturer's protocols. Tissues with high starch content (tubers and stolons) were treated with Concert Cytoplasmic RNA Reagent (Invitrogen) after powderization under liquid nitrogen. For the virtual Northern blot, total root RNA was isolated from P6/210 20 days after infection with *G. rostochiensis* pathotype Ro1. Double-stranded cDNA was synthesized with the SMART library construction kit (Clontech, Palo Alto, CA, USA) according to the supplier's instructions. Ten micrograms of cDNA were separated on 1% agarose gel in 1× 40 mM Tris-acetate 1 mM EDTA (ethylene-diamin-tetra-acetate) (TAE) buffer, blotted, and probed with the ³²P-labeled *St*332 sequence.

For RT-PCR experiments, double-stranded cDNA was generated from total RNA of different tissues of uninfected P6/210 plants and amplified using the SMART RACE cDNA Amplification Kit (Clontech). Primers for RT-PCR had the following sequences: NBS3RACE1, 5'-GGA AGA AGA AAG ATT CAG TGA CCA GAA TAC TTG A-3'; LRR5RACE1, 5'-CCT TTG AGG TTT TCC AAA GTT GTA TGA TGC G-3'. RACE was carried out using the Marathon cDNA amplification kit (Clontech). RACE products were generated using the following *St*332 internal primers: For 3' RACE, we used primers gsp1, 5'-GAC GGA AGT AGA ATC ATC ATA ACA ACC-3' and gsp2, 5'-TGA ATG GTT TGG YGA CGG AAG TAG AAT CAT C-3'; for 5' RACE, we used primers gspas1, 5'-GGT TGT TAT GAT GAT TCT ACT TCC GTC-3' and gspas2, 5'-CCT GTT TTG CTG AAC GGT CTC TGA CTT CGT G-3'. PCR products were cloned using the pGEM-T vector system (Promega, Madison, WI, USA).

Generation of genomic subclones

Five hundred nanograms of DNA from BAC clones BA26N18 and BA60B11 were partially digested with the restriction enzyme *Tsp*509I. Five hundred nanograms of BAC BA98P9 were completely digested with the restriction enzyme *Bam*HI. DNA fragments were separated on a 1% low-melting-temperature agarose gel (Sea Plaque GTG Agarose, Bioproducts, Rockland, ME, USA) using the CHEF-DR III Puls Field Gel Electrophoresis System (Bio-Rad, Hercules, CA, USA). DNA fragments were recovered using the GELase system (Epicentre Technologies, Madison, WI, USA) and ligated into the pCDL04541 plant transformation vector (Jones *et al.*, 1992), which had been linearized by *Eco*RI or *Bam*HI and dephosphorylated with shrimp alkaline phosphatase (Roche, Mannheim, Germany). Constructs were transformed into DH10B cells (Invitrogen). Positive clones were identified by colony hybridization with ³²P-labeled *St*332 sequence using standard protocols (Sambrook *et al.*, 1989).

Generation of transgenic plants

Competent LBA4404 *Agrobacterium* cells were prepared and transformed via electroporation with pCDL04541 constructs as

described by McCormac *et al.* (1998) using a Gene Pulser (Bio-Rad). Transformation in MS medium (Murashige and Skoog, 1962) of leaves of cv. Désirée by *Agrobacterium* strains and regeneration of transgenic calli on MS medium supplemented with 50 mg l⁻¹ kanamycin and 250 mg l⁻¹ claforan were carried out as described by Rocha-Sosa *et al.* (1989). At least 20 independent transgenic lines for each transgene were regenerated.

Test for resistance to *G. rostochiensis*

Cysts of *G. rostochiensis* pathotype Ro1 were kindly provided by Dr H. J. Rumpfenhorst (Biologische Bundesanstalt, Münster, Germany) and Dr M. Arndt (Bayerische Landesanstalt für Bodenkultur und Pflanzenbau, Freising, Germany). Three to four shoot cuttings of each transgenic line were subcultured for 3–4 weeks on MS medium (Murashige and Skoog, 1962) until small roots were formed. Plantlets were then transferred into pots (diameter 6–8 cm) filled with a mixture of 50% soil (Einheitserde) and 50% sand. Each pot was inoculated with 20 cysts. Plants were grown in the greenhouse under normal daylight conditions or in a phytocabinet (BBC-York, Germany), 16 h at 22°C in the light and 8 h at 18°C in the dark. The roots were visually scored after 6–7 weeks for the presence of newly formed cysts. Transgenic lines were scored resistant when the roots of all infected plants showed no or very few cysts (up to five) and susceptible when more than five new cysts were visible on the roots. Untransformed Désirée plants were used as susceptible control and P40 plants as resistant control.

Sequence analysis

DNA sequences were determined by Automatische DNS Isolierung und Sequenzierung (ADIS), the Max-Planck-Institut für Züchtungsforschung (MPIZ) DNA core facility, on Abi Prism 377 or 3700 automated sequencers (Applied Biosystems, Foster City, CA, USA) using BigDye-terminator chemistry. Pre-mixed reagents were supplied by Applied Biosystems. Oligonucleotides were purchased from Metabion (Martinsried, Germany). Sequences were analyzed using the GCG-software package for UNIX, version 10 (Genetics Computer Group, WI, USA) and the LASERGENE software version 5 (DNASTAR, WI, USA). Sequence homology searches were performed using European Molecular Biology Laboratory (EMBL), National Centre for Biotechnology Information (NCBI), and DNA Data Bank of Japan (DDBJ) database platforms. The SIGNALP program at <http://www.cbs.dtu.dk/services/SignalP> and the PROFILESCAN server at <http://hits.isb-sib.ch/cgi-bin/PFSCAN> were used for functional prediction. Putative promoter sequences were analyzed with PLACE SIGNAL SCAN Search (Higo *et al.*, 1999; <http://www.dna.affrc.go.jp/htdocs/PLACE/>). CLUSTALV at <http://www.ebi.ac.uk/clustalw> was used for multiple sequence alignment, and TREEVIEW was used for tree drawing (Roderick D.M. Page, download from <http://taxonomy.zoology.gla.ac.uk/rod/rod.html>).

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T. H. Park · V. G. A. A. Vleeshouwers · D. J. Huigen
E. A. G. van der Vossen · H. J. van Eck
R. G. F. Visser

Characterization and high-resolution mapping of a late blight resistance locus similar to *R2* in potato

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Abstract Identification of resistance (*R*) genes to *Phytophthora infestans* is an essential step in molecular breeding of potato. We identified three specific *R* genes segregating in a diploid mapping population. One of the *R* genes is located on chromosome 4 and proved phenotypically indistinguishable from the *Solanum demissum*-derived *R2*, although *S. demissum* is not directly involved in the pedigree of the population. By bulked segregant analysis combined with a resistance assay, a genetic linkage map of the *R2*-like locus was constructed with 30 coupling and 23 repulsion phase AFLP markers. Two markers flanking the *R2*-like locus were applied to screen an extended population of 1,586 offspring. About 103 recombinants were selected, and an accurate high-resolution map was constructed. The *R2*-like resistance was localized in a 0.4 cM interval and was found co-segregating with four AFLP markers, which can be used to isolate the *R2*-like gene by map-based gene cloning. By analyzing race-specificity and *R* gene-specific molecular markers, we also found that an *R1*-like gene and an additional unknown *R* gene are segregating in the population.

Introduction

Phytophthora infestans is the causal agent of late blight and one of the most important pathogens in cultivated potato (*Solanum tuberosum* L.). The economic value of the loss of global annual production and the cost of crop protection are estimated to be US \$5 billion annually (Duncan 1999). In early breeding programmes for late blight resistance, 11 resistance (*R*) genes originating from the hexaploid wild species *S. demissum* were introduced into *S. tuberosum* (Black et al. 1953; Malcolmson and Black 1966). These *R* genes confer race-specific resistance, and they are controlled by major single dominant factors (Mastenbroek 1953; Malcolmson and Black 1966). The potato-*P. infestans* interaction is following the gene-for-gene model as proposed by Flor (1971). Although the *R* genes do not give durable resistance, the isolation of these *R* genes is of importance to understand the mechanism of the specific plant defence. Several *R* genes have been localized, e.g. *R1* on chromosome 5 (Leonards-Schippers et al. 1992) and *R3*, *R6* and *R7* clustered on chromosome 11 (El-Kharbotly et al. 1994, 1996). *R1* and *R3* have been isolated (Ballvora et al. 2002; Huang 2005). The *R2* locus has been localized on chromosome 4 using a tetraploid mapping population EJ96-4061 comprising 86 offspring (Li et al. 1998). Eleven AFLP markers linked to the *R2* locus have been identified using bulked segregant analysis [(BSA) Michelmore et al. 1991]), and the *R2* linkage group has been assigned using the reference map of AM3778-16. Three AFLP markers that are tightly linked to *R2* are present in the diploid mapping population RHAM026, which is used in this study.

Recently many different haplotypes of *R3*-like genes were discovered at the *R3* locus in *S. demissum* and ample variation for functional *R* genes to late blight appears to exist in *Solanum* species (Huang et al. 2004, Huang 2005). In this research we identified resistances by assaying race specificities and focused on a locus that is indistinguishable from *R2* in the diploid mapping

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T. H. Park · V. G. A. A. Vleeshouwers · D. J. Huigen
H. J. van Eck · R. G. F. Visser (✉)
Department of Plant Sciences, Laboratory of Plant Breeding,
Graduate School Experimental Plant Sciences,
Wageningen University, Binnenhaven 5, 6709 PD,
Wageningen, The Netherlands
E-mail: Richard.Visser@wur.nl
Tel.: +31-317-482857
Fax: +31-317-483457

E. A. G. van der Vossen
Plant Research International BV, P.O. Box 16, 6700 AA,
Wageningen, The Netherlands

population RHAM026. Because the resistance was introgressed from different wild species, we designate the gene as *R2-like*. From the *R2-like* locus, a high-resolution map was constructed.

Materials and methods

Plant materials

The diploid mapping population RHAM026 was obtained from a cross between AM3778-16 (AM) and RH89-039-16 (RH). The dihaploid female parent AM is derived from the tetraploid breeding clone AM78-3778, which has various wild *Solanum* species in its pedigree, including *S. tuberosum* spp. *andigena*, *S. vernei*, *S. vernei* spp. *ballsii*, *S. oplocense* and *S. edinense*. The detailed pedigree of the mapping population is shown in Fig. 1.

The mapping population was generated from seeds that were sown in vitro after they were sterilized. When the seeds germinated and the seedlings reached approximately 3–5 cm, they were duplicated. One was used for maintenance and another for DNA isolation.

Inoculum preparation and resistance assay

Four different isolates were used to identify race-specific resistance (Table 1). For experiments, a plug of mycelium was transferred to a fresh plate with rye sucrose agar medium (Caten and Jinks 1968). One to 2 weeks later, ice-cold water was added to the mycelium that covered the agar plate. The sporangiospore suspension was pipetted into a tube and incubated at 4°C for 3 h.

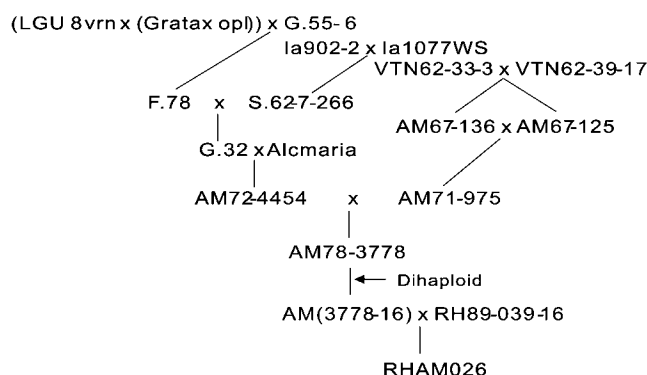


Fig. 1 Pedigree of the diploid mapping population RHAM026 (RH) used in this study

After the release of zoospores, the concentration of inoculum was adjusted to 5×10^4 spores/ml.

A resistance assay was performed by a detached leaf assay. Fully expanded and healthy leaves were collected from greenhouse plants and inoculated on wet paper in humid trays. Droplets of 10 μ l inoculum were applied to the leaves, which were subsequently incubated at 15°C (Vleeshouwers et al. 1999). The symptoms on the leaves were evaluated 3 days and 5 days after inoculation.

DNA isolation

A high-throughput DNA isolation procedure was followed using a Retsch machine (Retsch, Haan, Germany) and 96-deep-well COSTER microtiter plates (Corning, Corning, N.Y., USA). Fresh leaf tissue was obtained by harvesting in vitro plants and grinding with two steel balls in the presence of nuclear lysis buffer (0.2 M Tris-HCl, 0.05 M EDTA, 2 M NaCl, 2% CTAB, with an end pH of 7.5), DNA extraction buffer (100 mM Tris-HCl, 5 mM EDTA, 0.35 M Sorbitol, 20 mM sodium bisulfite, with an end pH of 7.5) and 5% sarcosyl, followed by incubation at 65°C in a water bath for 1 h. Ice-cold chloroform isoamyl alcohol (24:1) was added, and the samples were mixed and centrifuged. The supernatant was transferred to new tubes and an equal volume of isopropanol was added. Another centrifugation step allowed the precipitation of DNA. The DNA pellet was dried and dissolved in $T_{0.1}$ E-buffer (+0.5 μ g RNase).

AFLP analysis

The AFLP analysis was essentially carried out as described by Vos et al. (1995). Primary template DNA was prepared using *Eco*RI and *Mse*I and adaptors fitting to the restriction enzyme sites. Template DNA was diluted ten times prior to the selective pre-amplification with single-nucleotide extended primers, which decreases marker density. For the selective amplification, AFLP reactions with three nucleotides extended primers (*Eco*+3/*Mse*+3 primers) were performed to find markers linked to the resistance locus. The AFLP bands were separated on a 6% polyacrylamide gel in a Li-cor sequencer (Li-cor, Lincoln, Neb., USA).

The AFLP markers were named with the enzyme, the primer combination and the mobility of the fragment as described in reference autoradiograms created by Keygene NV, Wageningen, The Netherlands.

Table 1 Isolates of *Phytophthora infestans* and virulence factors

Isolate	Race specificity	Source
IPO-0	0	W. Flier, Plant Research International, The Netherlands
99018	1, 4	F. Govers, Wageningen University, The Netherlands
90128	1, 3, 4, 7, 8, 11	F. Govers, Wageningen University, The Netherlands
USA618	1, 2, 3, 6, 7, 11	W.E. Fry, Cornell University, USA

Genetic analysis of resistance

The AFLP markers linked to a resistance locus were identified by BSA (Michelmore et al. 1991). The data were analyzed using JoinMap software (Stam 1993). Marker order was determined by Record (van Os et al. 2000), and the map distance was calculated based on the frequency of the recombination between markers. A marker-saturated genetic linkage map was produced by MapChart (Voorrips 2002).

To identify race-specificity of the resistances, *R1*-specific marker 76_2s (forward primer: 5'-CAC-TCGTGACATATCCTCACTA-3' and reverse primer: 5'-CAACCCTGGCATGCCACG-3'; Ballvora et al. 2002) and *R2*-linked AFLP markers EATC/MCGA_186 and EACT/MCAC_189 (Li et al. 1998) were used.

Results

Characterization of resistance

A subset of the population of 28 genotypes and both parents AM and RH were evaluated for resistance to four different isolates, which differ in race specificity (Table 1). All 28 genotypes were susceptible to the isolate USA618, but segregated for resistance to the isolates IPO-0, 99018 and 90128 (Fig. 2a). The differential segregation for resistance to the three isolates suggests that three specific *R* genes are segregating in the population.

To examine whether known or unknown *R* genes segregate in RHAM026, specific molecular markers were designed. The presence of *R1* gene-specific sequences was tested by applying the 76_2s marker (Ballvora et al. 2002) on parents and progenies, and a clear segregation was found (Fig. 2b). The *R2*-linked AFLP markers EATC/MCGA_186 and EACT/MCAC_189 (Li et al. 1998) were also tested. Both markers showed an identical segregation pattern in the progenies (Fig. 2b).

By comparing the genotypic results with the phenotypic results, we divided the 28 genotypes into five groups, which could have different candidate *R* genes

(Fig. 2c). Presence of the 76_2s marker matched with the *R1*-specific resistance to *P. infestans* isolate IPO-0 (groups I and IV), and the *R2*-linked AFLP markers EATC/MCGA_186 and EACT/MCAC_189 perfectly matched with the *R2*-specific resistance to isolate 90128 (groups I and II). Some genotypes that do not contain the *R1* and *R2* markers were susceptible to isolate 90128, but resistant to isolates IPO-0 or 99018 (group III). This might be caused by another resistance gene that we designated *R_{unknown}*. In conclusion, the combination between race-specificity and molecular marker data suggests that *R_{unknown}*, *R1*-like and *R2*-like genes are segregating in the population.

Marker-saturated genetic linkage map

To determine the location of the *R2*-like locus, a genetic linkage study was initiated with the RHAM026 population comprising 78 genotypes. A detached leaf assay with *P. infestans* isolate 90128 revealed 24 resistant and 45 susceptible genotypes, which fits to a 1–2 segregation according to the χ^2 test. This segregation is skewed to susceptible, which might be caused by the relatively small population size or by postzygotic selection against unfavourable allelic combinations (Gebhardt et al. 1991; El-Kharbotly et al. 1994). Nine genotypes were not assigned due to unclear observation. A resistant and a susceptible bulk were composed of eight genotypes each. Two hundred fifty-six *Eco* + 3/*Mse* + 3 primer combinations were examined in the resistant parent, the susceptible parent, the resistant bulk and the susceptible bulk by BSA, and 33 coupling and 31 repulsion-phase candidate markers were revealed. After selecting the candidate markers, the 16 genotypes used to compose the resistant and susceptible bulks were separately checked for linkage to the resistance locus. Finally 30 coupling phase markers and 23 repulsion-phase markers were identified to be linked to the *R2*-like locus (Fig. 3). Seven AFLP markers co-segregate with the resistance locus. The linkage group was determined with two AFLP markers, EATC/MCGA_186 and EACT/MCAC_189, which were bridged to the genetic map of chromosome 4 where the *R2* locus is located (Li et al. 1998).

High-resolution map

To construct a high-resolution map, we selected two flanking AFLP markers (Fig. 3) to screen an extended population for selecting recombinants around the resis-

Fig. 2 Graphical genotyping of 28 offspring and parents AM and RH. **a** Phenotypic results with the *Phytophthora infestans* isolates IPO-0, 99018, 90128 and USA618. R, S and nd indicate resistant, susceptible and undetermined, respectively. **b** Genotypic results obtained with *R1*-specific marker 76_2s and the AFLP markers EATC/MCGA_186 and EACT/MCAC_189 linked to *R2*. aa and ab indicate absence and presence of the marker, respectively. **c** Classes distinguished based on the phenotypic and genotypic data

		AM	RH	28 progenies																									
A	IPO-0	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
	99018	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
	90128	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
	USA618	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
B	76_2s	ab	aa	ab	ab	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa
	EATC/MCGA_186	ab	aa	ab	ab	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa
	EACT/MCAC_189	ab	aa	ab	ab	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa
C Group				I				II				III				IV				V									

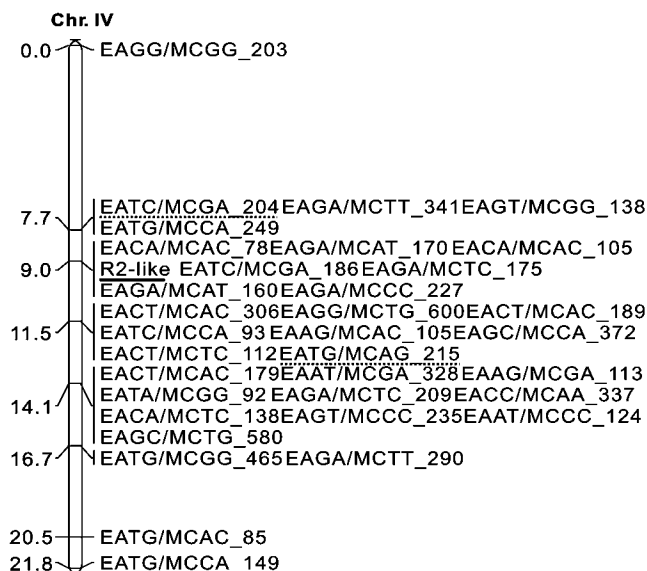


Fig. 3 Genetic linkage map of the *R2-like* locus. The resistance locus is underlined. Two flanking markers used for screening the extended population are dot-underlined. All 30 coupling phase markers, but only four repulsion phase markers and the resistance locus, are included in the map

tance locus. One is EATC/MCGA_204, which was mapped 1.3 cM telomeric from the resistance locus. Another is EATG/MCAG_215, which was mapped 2.5 cM centromeric from the resistance locus in the 78 offspring (Fig. 4). By screening the expanded population of 1,582 genotypes, 103 recombinants were selected resulting in a larger interval of a 6.4-cM genetic distance between these two AFLP markers. We screened the 103 recombinants with 14 AFLP markers located between EATC/MCGA_204 and EATG/MCAG_215 (Fig. 3) and tested them for their resistance to *P. infestans* isolate 90128. Subsequently a high-resolution genetic map of

the 6.4-cM interval containing the resistance locus was constructed (Fig. 5), in which the AFLP markers are precisely separated and mapped. Closest markers on both sides of the resistance locus are EACA/MCAC_105 (three recombinants in 0.2 cM) and a group containing five markers (three recombinants in 0.2 cM). Four AFLP markers are still co-segregating with the resistance locus.

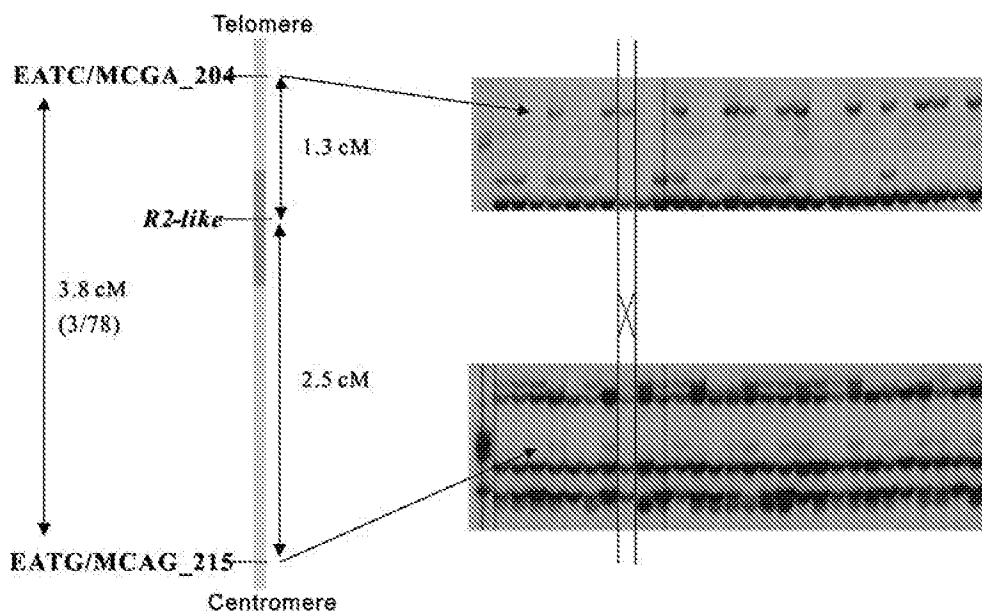
Comparative genetics

The *R2* and *R2-like* loci derived from two different mapping populations were compared. The bridged markers EATC/MCGA_186 and EACT/MCAC_189 are 0.2 cM apart in the *R2-like* diploid population (three recombinants in 1,582 genotypes) and 1.2 cM in the *R2* tetraploid population (one recombinant in 86 genotypes) (Li et al. 1998). One AFLP marker, EATC/MCGA_186, co-segregates with the *R2-like* locus, but is 1.2 cM separated from the *R2* locus. Another AFLP marker EACT/MCAC_189 is 0.2 cM separated from the *R2-like* locus, but co-segregates with the *R2* locus. The position of flanking markers that connect the two genetic linkage maps confirmed that the *R2* and *R2-like* loci are located at the same region of chromosome 4.

Discussion

In this research, we identified three resistance loci in the diploid segregating population RHAM026. One locus was localized on chromosome 4, where the *R2* locus from *S. demissum* was previously mapped using a tetraploid mapping population EJ96-4601 (Li et al. 1998). Accurate specificity studies with a panel of four different *P. infestans* isolates revealed that *R2-like* resistance of

Fig. 4 Schematic representation for recombinant screening towards high-resolution mapping. The AFLP gel images of the two flanking AFLP markers EATC/MCGA_204 and EATG/MCAG_215, which were used to select recombinants in 1,582 genotypes. One example of a recombinant is indicated by X



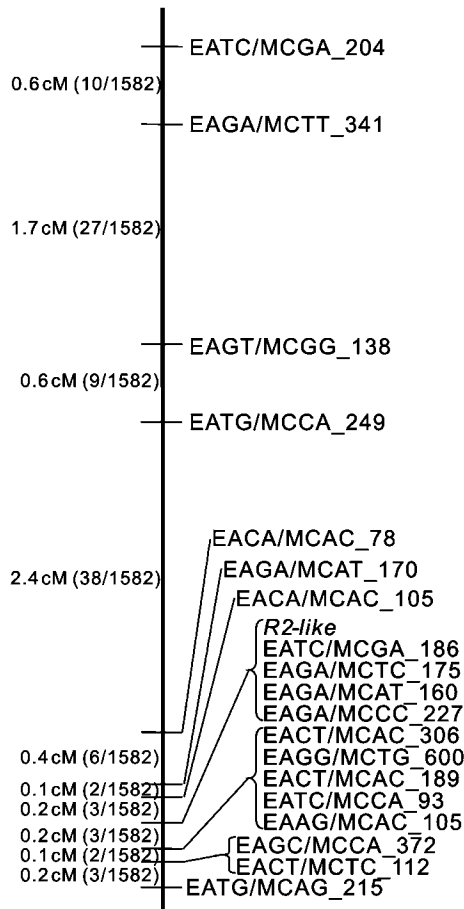


Fig. 5 High-resolution map of the *R2-like* locus. The 1,582 genotypes were screened with two AFLP markers, EATC/MCGA_204 and EATG/MCAG_215, which are dot-underlined, and 103 recombinants were selected. Four markers are co-segregating with the *R2-like* locus

this population is phenotypically indistinguishable from *R2*.

The origin of the AFLP markers EACT/MCAC_189 and EATC/MCGA_186 was previously thought to be *S. demissum*-specific, because the AFLP markers were identified in the fingerprints of three accessions of *S. demissum* (Li et al. 1998). However, we observed that EACT/MCAC_189 is also present in *S. acaule* accessions and EATC/MCGA_186 in *S. bulbocastanum* accessions (data not shown) and therefore conclude that these markers are present in a broader range of *Solanum* germplasm. Although *S. demissum* is not directly present in the ancestors of the *R2-like* diploid population, the introgression fragment could be of wild species origin, e.g. *S. edinense*, which is a natural hybrid between *S. tuberosum* and *S. demissum* (Hawkes 1990). It remains unknown what the origin of the *R2-like* locus is. Even if it is derived from *S. demissum*, definite identity with *R2* (Li et al. 1998) can only be determined after cloning both genes.

To construct a marker saturation and high-resolution map at the *R2-like* locus, we used a diploid population. Compared to the tetraploid level, mapping studies at the diploid level can avoid the complexities of tetrasomic

inheritance and makes the study of potato genetics more feasible (Jacobs et al. 1995; Li et al. 1998). The number of markers is at least fourfold higher in a diploid population than in a tetraploid population, because marker alleles with linkage in repulsion can be used at the diploid level (Wu et al. 1992). We identified 69 AFLP markers with linkage to *R2-like*, using 256 primer combinations. A similar high efficiency was achieved in another study in which 29 AFLP markers with linkage to *R1*, using 108 primer combinations were identified at the diploid level (Meksem et al. 1995). At the tetraploid level, much lower efficiency was achieved. Li et al. (1998) detected only 11 AFLP loci with linkage to *R2*, using 205 primer combinations.

Many R genes and resistance gene homologues are clustered in the genome (Meyers et al. 1998; Michelmore and Meyers 1998). In the potato genome, for instance, at least five R genes against diverse pathogens are located at the gene cluster on chromosome 5 (Leister et al. 1996; Marano et al. 2002), i.e. *Gpa* conferring resistance to the potato cyst nematode *Globodera pallida* (Kreike et al. 1994), *Grp1* conferring resistance to the potato cyst nematode *Globodera rostochiensis* (Rouppé van der Voort et al. 1998), *Nb* conferring resistance to potato virus X (de Jong et al. 1997), *Rx2* (Ritter et al. 1991) conferring resistance to potato virus X and *R1* conferring resistance to *P. infestans* (Leonards-Schippers et al. 1992). Also near the *R2-like* locus on chromosome 4, various known R genes are located. The *Hero* gene, which confers broad-spectrum resistance against *G. rostochiensis* in tomato, is located between CT229 and TG370 (Ganal et al. 1995), *Gpa4* conferring resistance to *G. pallida* (Bradshaw et al. 1998) and *R2* (Li et al. 1998), *Rpi-abpt* (Park et al. 2004) and *Rpi-blb3* (Park et al. 2004) conferring resistance to *P. infestans* are mapped at the same region on chromosome 4.

One of the most challenging methods for gene cloning in a plant species with a large genome is map-based cloning, which can be considered routine in model species such as *Arabidopsis* with a small genome (Lukowitz et al. 2000). Accurate mapping of the target gene and identification of recombinants in the population are essential steps for map-based cloning, and here we report a reliable high-resolution map of the *R2-like* locus within a 0.4-cM interval on chromosome 4 in the potato breeding line RHAM026. The initial research for tightly linked AFLP markers was facilitated using AFLP technology (Vos et al. 1995) in combination with BSA (Michelmore et al. 1991). The *R2-like* locus is flanked by one AFLP marker on the telomeric side, a group of five AFLP markers on the centromeric side and co-segregating with four AFLP markers (Fig. 5). The flanking and co-segregating markers will facilitate isolation of the *R2-like* gene by BAC walking. A 0.2-cM (4/2,109 recombinants) interval was covered by one BAC clone of 100 kb in the cloning of the potato late blight resistance gene *Rpi-blb1* (van der Vossen et al. 2003) and in the cloning of *R1*, a 0.2-cM (2/1049 recombinants) interval was estimated at 250–300 kb (Ballvora et al. 2002). We estimate that the 0.4-cM

interval spanning the *R2-like* gene could be covered by a single BAC clone or a few BAC-walking steps.

Currently a BAC library of diploid genotype AM is being constructed to establish a physical contig with the co-segregating markers and the flanking markers and isolation of *R2-like* gene in near future is quite feasible. After that, physical analysis of the gene cluster containing the *R2-like* gene might accelerate isolation of the homologous genes conferring resistance to *P. infestans* in different genetic backgrounds.

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High-resolution mapping and analysis of the resistance locus *Rpi-abpt* against *Phytophthora infestans* in potato

Tae-Ho Park¹, Vivianne G.A.A. Vleeshouwers¹, Ronald C.B. Hutten¹, Herman J. van Eck^{1,2}, Edwin van der Vossen^{1,2}, Evert Jacobsen¹ and Richard G.F. Visser^{1,*}

¹Graduate School Experimental Plant Sciences, Laboratory of Plant Breeding, Department of Plant Sciences, Wageningen University, Binnenhaven 5, 6709 PD, Wageningen, The Netherlands; ²Plant Research International BV, P.O. Box 16, 6700 AA, Wageningen, The Netherlands; *Author for correspondence (e-mail: Richard.Visser@wur.nl; phone: +31-317-482857; fax: +31-317-483457)

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Abstract

Introduction of more durable resistance against *Phytophthora infestans* causing late blight into the cultivated potato is of importance for sustainable agriculture. We identified a new monogenically inherited resistance locus that is localized on chromosome 4. The resistance is derived from an ABPT clone, which is originally a complex quadruple hybrid in which *Solanum acaule*, *S. bulbocastanum*, *S. phureja* and *S. tuberosum* were involved. Resistance data of the original resistant accessions of the wild species and analysis of mobility of AFLP markers linked to the resistance locus suggest that the resistance locus is originating from *S. bulbocastanum*. A population of 1383 genotypes was screened with two AFLP markers flanking the *Rpi-abpt* locus and 98 recombinants were identified. An accurate high-resolution map was constructed and the *Rpi-abpt* locus was localized in a 0.5 cM interval. One AFLP marker was found to co-segregate with the *Rpi-abpt* locus. Its DNA sequence was highly similar with sequences found on a tomato BAC containing several resistance gene analogues on chromosome 4 and its translated protein sequence appeared to be homologous to several disease resistance related proteins. The results indicated that the *Rpi-abpt* gene is a member of an *R* gene cluster.

Introduction

Late blight caused by *Phytophthora infestans* is one of the most serious threats to potato cultivation. Controlling late blight usually involves the application of a lot of chemicals. Nevertheless, restrictions on chemical control, environmental safety and the development of more virulent forms of the pathogen that are resistant to these chemicals indicate that the most promising approach to

achieve late blight resistance is the introduction of resistance genes from wild *Solanum* species into potato cultivars (Kato et al. 1997).

In the last century, 11 resistance genes against *Phytophthora infestans* were introgressed from the wild *Solanum* species *S. demissum* into potato (Black et al. 1953; Malcolmson and Black 1966). However, these resistance genes confer race-specific resistance controlled by single resistance factors and these genes are not durable (Mastenbroek 1953;

Malcolmson and Black 1966). In the meantime, not only asexual but also sexual forms (A1 and A2) of *P. infestans* have been introduced into Europe, enabling the pathogen to overcome chemicals and resistance genes. Therefore, new sources are required for the creation of durable and race-non-specific resistance. Instead of the genes from *S. demissum*, resistance loci and QTLs originating from wild species such as *S. berthaultii*, *S. pinnatisectum* and *S. microdontum* have been localized on chromosomes 10, 7 and 4, respectively, as new sources of resistance (Ewing et al. 2000; Kuhl et al. 2001; Sandbrink et al. 2000). *S. bulbocastanum* has also been considered as another new source of resistance to *P. infestans* (Niederhausen and Mills 1953). *S. bulbocastanum* is a self-incompatible diploid wild species from Mexico which cannot be crossed to the common potato *S. tuberosum* (Hermesen and De Bore 1971). Recently two resistance genes *Rpi-blb1/RB* and *Rpi-blb2* derived from *S. bulbocastanum* were cloned (Song et al. 2003; van der Vossen et al. 2003, 2004). The *RB* gene was localized on chromosome 8 (Naess et al. 2000). Because of the inability of direct sexual crosses between *S. tuberosum* and *S. bulbocastanum*, BC₂ populations derived from somatic hybrids were used (Helgeson et al. 1998). Due to the same reason, the *Rpi-blb1* gene was cloned using an intraspecific hybrid between resistant and susceptible *S. bulbocastanum* genotypes (van der Vossen et al. 2003). To clone *Rpi-blb2*, which is located on chromosome 6, both an intraspecific hybrid and a complex interspecific hybrid, which was designated ABPT, were used (van der Vossen et al. 2004). ABPT is a quadruple species hybrid created through bridge crosses using *S. acaule* (A), *S. bulbocastanum* (B), *S. phureja* (P) and *S. tuberosum* (T) (Figure 1a; Hermesen and Ramanna 1973).

Map-based cloning is one of the most challenging methods for gene cloning in plant species with a relatively large genome (Lukowitz et al. 2000). In addition to *Rpi-blb1/RB* and *Rpi-blb2*, the *S. demissum* derived *R1* (Ballvora et al. 2002) and *R3a* (Huang 2005) have also been isolated using the map-based cloning approach. The first essential step for map-based gene cloning is to determine the accurate position of the target gene.

In this paper, we identify another new resistance gene named *Rpi-abpt* in ABPT material, which differs from that used by van der Vossen et al. (2004). We constructed a high-resolution map of

the *Rpi-abpt* locus and analyzed this region by investigating the sequence of closely linked markers. We also unraveled the predicted wild species origin of the resistance gene.

Materials and methods

Plant materials

The pedigree of the mapping population RH4X-103 is shown in Figure 1b. The tetraploid resistant female parent 707TG11-1 was derived from a quadruple hybrid ABPT clone (Hermesen and Ramanna 1973). The diploid susceptible male parent RH89-039-16, producing unreduced 2*n* pollen, is one of the advanced clones widely used for mapping research at the Laboratory of Plant Breeding and the Laboratory of Nematology, Wageningen University (Roupe van der Voort et al. 1997a, b, 1998, 1999; Huang et al. 2004; van Os et al. personal communication).

Seeds of RH4X-103 progeny were sterilized in three different steps: 70% alcohol for 1 min, 1.5% sodium hypochlorite for 5 min and three times rinsing with sterilized water. Plants were generated from seeds sown *in vitro*. When the size of the *in vitro* plants was approximately 5 cm, they were multiplied for DNA isolation, resistance assay and genotype maintenance. Recombinants were maintained *in vitro* and transferred to the greenhouse for generation of leaf material for the detached leaf assays.

Accession of *S. acaule* CGN17843 and CGN20620, *S. bulbocastanum* CGN21306 and CG N17693, *S. phureja* CGN17667 and CGN18301, *S. demissum* CGN17810 were obtained from the Center of Genetic Resources in The Netherlands (CGN) and maintained *in vitro*. Potato clone Ceb44-158-4, which has *S. demissum* in its pedigree and *S. tuberosum* cultivars Titana, Alcmaria, Van Gogh, Agria, Bintje and Nicola were planted in the greenhouse, and leaves were collected for DNA isolation.

Resistance assay

The mapping population of 233 genotypes was subjected to a detached leaf assay in four replications using leaf material from the maintenance field. Fully expanded and healthy leaflets were collected and incubated in moist trays. Each leaflet was inoculated

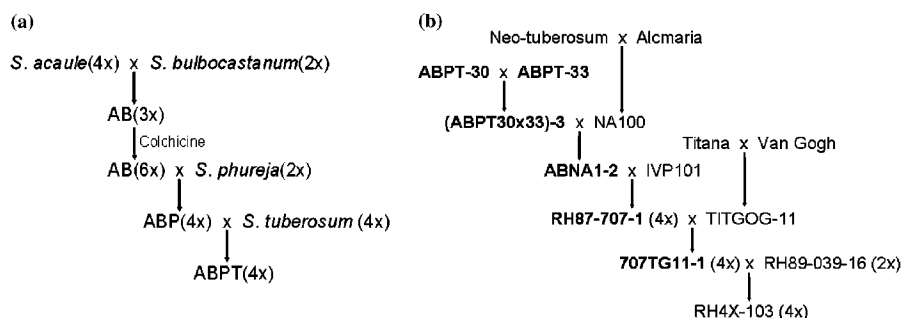


Figure 1. Plant materials used for creation of the tetraploid mapping population RH4X-103. (a) Pedigree of the tetraploid ABPT breeding clone. A, B, P and T indicate *S. acaule*, *S. bulbocastanum*, *S. phureja* and *S. tuberosum*, respectively. (b) Pedigree of RH4X-103 derived from two quadruple hybrid ABPTs. The genotypes carrying resistance against *P. infestans* are indicated in bold.

with a 10 μ l droplet of inoculum (Vleeshouwers et al. 1999). Leaves of plants were evaluated 3 and 5 days after inoculation.

P. infestans isolates 90128 (Race 1, 3, 4, 7, 8 and 11), which was kindly provided by Drs. F. Govers of Wageningen University, Wageningen, the Netherlands, USA618 (Race 1, 2, 3, 6, 7 and 11) by W. Fry of Cornell University, Ithaca, USA and IPO-82001 (Race 1, 2, 4, 5, 10 and 11) by W. Flier of Plant Research International, Wageningen, The Netherlands were used to determine the resistance. The isolates were cultured on rye sucrose agar medium (Caten and Jinks 1968) in the dark at 15 °C for 2 weeks. When mycelium was growing on the medium, it was rinsed with 5 ml ice-cold water for the release of the zoospores from sporangia. After incubating the suspension at 4 °C for 3 h, it was re-suspended to make an inoculum concentration of 5×10^5 zoospores ml^{-1} .

DNA isolation and AFLP analysis

DNA isolation was performed using a Retch protocol. Fresh leaf tissue was ground with two steel balls, in nuclear lysis buffer (0.2 M Tris-HCl, 0.05 M EDTA, 2 M NaCl, 2% CTAB, with an end pH of 7.5), DNA extraction buffer (100 mM Tris-HCl, 5 mM EDTA, 0.35 M Sorbitol, 20 mM NaBisulfite, with an end pH of 7.5) and 5% sarcosyl in 96 wells Costar plates (Corning Inc., Corning, NY, USA) using a Retsch machine (Retsch Inc., Haan, Germany). The ground leaf tissue was incubated at 65 °C for an hour followed by adding ice-cold chloroform: isoamyl alcohol (24:1). After centrifugation, the supernatant was

transferred to new tubes. Adding isopropanol and another centrifugation allowed the precipitated DNA to be extracted. After the DNA pellet was dried, the DNA was dissolved in $T_{0.1}$ E-buffer (+0.5 μ g RNase).

AFLP analysis was performed as described by Vos et al. (1995). Primary template DNA of 233 genotypes was prepared using the restriction enzymes *EcoRI*/*MseI* and *PstI*/*MseI* combinations, ligated with adaptors fitting to the *EcoRI*, *MseI* and *PstI* sites, and diluted 10 times prior to the selective pre-amplification with single nucleotide extended primers. For the selective amplification, 11 E + 3/M + 3 primer combinations were selected based on their appearance in the Ultra High Density (UHD) Map (<http://www.dpw.wau.nl/uhd/>). Extra primer combinations were selected according to the results of a bulked segregant analysis (BSA) with 204 primer combinations as described by Michelmore et al. (1991) and six primer combinations from the R2 map (Li et al. 1998).

In addition to this, AFLP marker analysis was performed to detect the wild species origin of the resistance in five different *Solanum* species as described by Li et al. (1998). The mobility of the AFLP markers, which are linked to the resistance locus, was compared.

Map construction

The AFLP markers inherited from the female parent were scored. Band intensity was discarded to use AFLP markers as dominant markers and only simplex-inherited markers [single-dose restriction fragments, (SDRFs); Wu et al. 1992]

with a simplex trait allele for the detection of linkage in the coupling phase were included in the data analysis. The linkage group containing the resistance locus was determined by JoinMap 2.0 (Stam 1993) and marker order by Record (van Os et al. 2000). The map distance was calculated based on the frequency of the recombination between AFLP markers.

AFLP marker and BAC clone analysis

Blasting of the sequences was performed on the web site <http://www.ncbi.nlm.nih.gov/BLAST/>. Genescan (<http://bioweb.pasteur.fr/seqanal/interfaces/genscan.html>; Burge and Karlin 1997) and Genemark (<http://www.ebi.ac.uk/genemark/>; Lukashin and Borodovsky 1998) were used to analyze the sequences of some BAC clones. ClustalX (Jeanmougin et al. 1998) was used to align sequences. For physical alignment of the resistance gene, the BAC library constructed from the wild species *S. bulbocastanum*, BGRC8005–8 (van der Vossen et al. 2003) was used and BAC-end sequences were performed at Greenomics (Wageningen, The Netherlands).

Marker nomenclature

AFLP markers were named with acronyms of enzyme combinations *EcoRI/MseI* and *PstI/MseI*, which were used to prepare the template DNA, followed by three selective nucleotides and the size of each marker as described in reference autodiagrams created by Keygene NV, Wageningen,

The Netherlands. Developed PCR markers, which were based on the sequence of a tomato BAC clone, were named with the original accession number followed by a defined part of the sequence, for instance right (R), left (L) and resistance gene analogue (RGA). Markers from potato BAC-end sequences were named with the plate, row and column number of the BAC library.

Results

Localization of the resistance locus

ABPT-derived clones were routinely inoculated with *P. infestans* during the breeding process and resistance was specifically noted in ABPT-30, ABPT-33, (ABPT30 × 33)-3, ABNA1-2, RH87-707-1 and 707TG11-1 (Figure 1). A tetraploid mapping population of 233 genotypes, which was generated by a cross between 707TG11-1 and RH89-039-16, was assessed for resistance to *P. infestans* isolate 90128. 707TG11-1 was assigned as resistant, RH89-039-16 as susceptible (Figure 2) and 83 and 90 offspring were unambiguously classified as resistant or susceptible, respectively. This 1:1 segregation ratio indicates a simplex inheritance of the resistance gene. The remaining 60 genotypes were tentatively classified, but excluded from the analysis for localization of the resistance locus because of less accurate observations and insufficient humidity during the incubation period.

To localize the resistance locus on the genetic linkage map of potato, 11 primer combinations

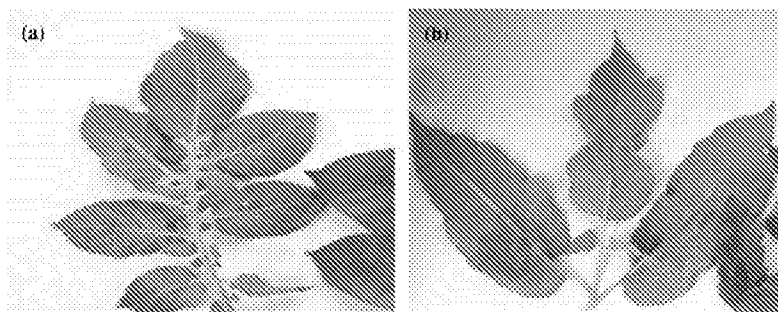


Figure 2. Detached leaf assay on resistant and susceptible parents of RH4X-103, 5 days after inoculation with *P. infestans* isolate 90128. (a) The resistant parent 707TG11-1 shows the hypersensitivity response. (b) The susceptible parent RH89-039-16 is heavily sporulating.

were selected from the UHD map. In total 1187 AFLP bands were observed in 707TG11-1 ranging between 79 and 164 per primer combination. 243 bands (20.5%) of these showed a polymorphism that was scorable with accuracy in the offspring. A subset of 191 marker loci showed a 1:1 segregation (simplex inherited markers) with the χ^2 -test ($p < 0.05$). Those markers should be considered as SDRF's. In the first round of the JoinMap analysis, two of the 191 markers, i.e. EAAC/MCAG_101 and PAC/MAAT_498 were grouped with the *Rpi-abpt* locus.

To obtain additional AFLP markers with linkage to the *Rpi-abpt* locus, BSA was applied with 128 *Eco/Mse* and 76 *Pst/Mse* primer combinations using four samples: resistant parent (Pr), susceptible parent (Ps), resistant bulk (Br) and susceptible bulk (Bs). The bulks were composed of equal amounts of pre-amplified templates of eight resistant and eight susceptible genotypes as determined by the resistance assay. AFLP markers that were present in Pr and Br and absent in Ps and Bs were selected as candidate markers with putative linkage to the *Rpi-abpt* locus. Three and five markers from *Eco/Mse* and *Pst/Mse* primer combinations, respectively, were confirmed progressively to have more definite linkage to the *Rpi-abpt* locus by analyzing the 16 genotypes of Br and Bs individuals. An example is shown in Figure 3. This analysis resulted in an efficiency of determining one marker with linkage to the resistance locus per 25 AFLP primer combinations. In the second round of JoinMap analysis, two AFLP markers from 11 primer combinations and eight AFLP markers from the BSA were grouped with the *Rpi-abpt* locus.

One of the AFLP markers, PAT/MAGA_307, was converted into a CAPS marker Th2 (Foward:

AGGATTTTCAGTATGTCTCG and Reverse: TCCATTGTTGATTGCCCT) to determine the chromosomal location of the *Rpi-abpt* locus. In the UHD mapping population, Th2 was localized on chromosome 4. *R2*, one of the *S. demissum*-derived *R* genes against *P. infestans*, was also mapped on chromosome 4 (Li et al. 1998). To confirm the chromosome number, six primer combinations selected from the *R2* map were tested on our mapping population of 233 genotypes and four *R2*-linked markers were grouped with the *Rpi-abpt* locus in the third round of JoinMap analysis. The marker order was determined by Record software (van Os et al. 2000) and genetic map distances were determined by the recombination frequencies between flanking markers. The linkage map containing 15 loci, i.e. 14 markers and the *Rpi-abpt* locus was constructed. Subsequently the map was merged with the high-resolution map in Figure 4.

Construction of a high-resolution map

From the genetic linkage map of *Rpi-abpt*, two flanking AFLP markers were selected for a large-scale recombinant analysis. The first one is EAGT/MCTT_247 mapping 3.4 cM telomeric from the *Rpi-abpt* locus and the other one is EAGA/MCAC_202 mapping 1.3 cM centromeric from the *Rpi-abpt* locus in the population with 233 genotypes. A total 1383 offspring of the population was screened with the two AFLP markers, and 98 recombinants were selected. This resulted in 7.1 cM genetic distance between the two AFLP markers. The 98 recombinants were screened with the AFLP markers located on and between EAGT/MCTT_247 and EAGA/MCAC_202 and tested for resistance to *P. infestans* isolate 90128.

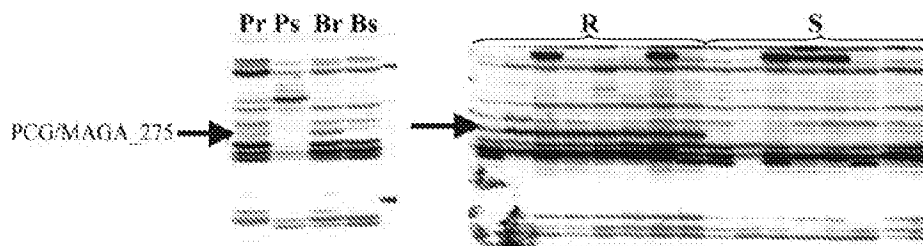


Figure 3. Part of an AFLP fingerprint showing a bulk-specific band associated with resistance using the PCG/MAGA primer combination. Pr, Ps, Br and Bs indicate the resistant parent, susceptible parent, resistant bulk and susceptible bulk, respectively. R and S represent resistant and susceptible individual genotypes of Br and Bs.

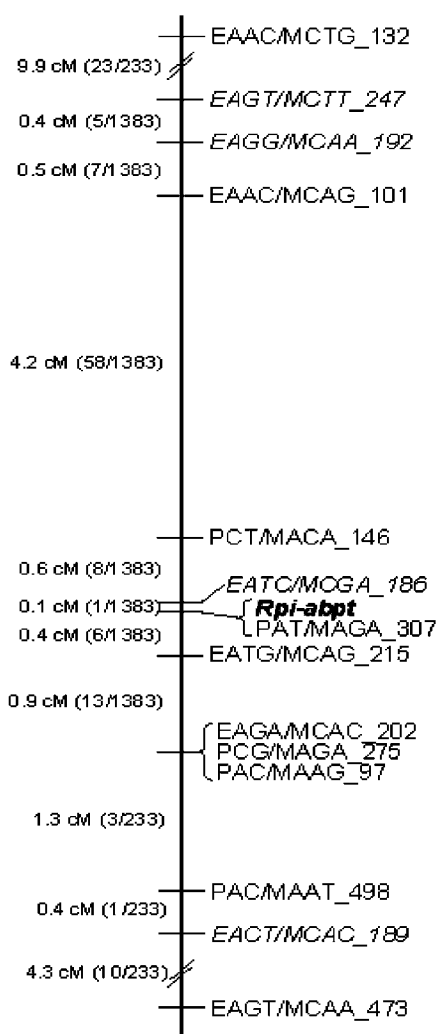


Figure 4. High-resolution map of *Rpi-abpt* on chromosome 4. The constructed map was based on 98 recombinants selected from the extended population of 1383 genotypes between the markers EAGT/MCTT_247 and EAGA/MCAC_202. The rest was based on the original map with 233 genotypes. The AFLP markers, which are also present in the map of *R2*, are indicated in italic. In the population of 86 genotypes, *R2* was co-segregating with EACT/MCAC_189 and was located 1 cM below EATC/MCGA_186 (Li et al. 1998). Genetic distance and the number of recombinants between markers are shown on the left side of the map.

The high-resolution map containing the resistance locus and six AFLP markers was constructed (Figure 4). The closest markers on both sides of the *Rpi-abpt* locus, EATC/MCGA_186 and

EATG/MCAG_215, are separated with one recombinant in 0.1 cM and six recombinants in 0.4 cM, respectively. One AFLP marker PAT/MAGA_307 was co-segregating with the *Rpi-abpt* locus.

Analysis of the *Rpi-abpt* locus

AFLP marker PAT/MAGA_307 co-segregating with the *Rpi-abpt* locus was sequenced. A blast search revealed that the protein sequence of the marker showed a high degree of similarity to several well-known plant resistance proteins that belong to the NBS-LRR class. In addition, the DNA sequence of the marker showed high similarity with sequences of *Lycopersicon esculentum* BAC clone 127E11 (Gene bank number: AF411807) derived from the tomato cultivar Heinz 1709 (van der Hoeven et al. 2002). This BAC has been localized between CT229A and TG370 at the short arm of the tomato chromosome 4 with a total length of 95,845 bp. By using Genescan and Genemark, 19 resistance gene analogues (RGA) were predicted in this clone. Three RGAs, i.e. Gene 4, 5 and 7 out of the 19 RGAs are homologous to disease resistance protein *RPP13* against *Peronospora parasitica* (Downy Mildew) in *Arabidopsis* (Bittner-Eddy et al. 1999). Marker PAT/MAGA_307 is highly similar to the 5' end of these three RGAs of the tomato BAC clone (Figure 5b). The markers AF411807L and AF411807R of the tomato BAC clone were localized at 1.2 cM and 0.8 cM distance from the *Rpi-abpt* locus, respectively. Based on the consensus sequence of the three RGAs of the tomato BAC clone, a RGA marker was designed (Forward: CCT TTGTATCATTTGCAGTT and Reverse: ACA-TCCTCCATTCTTTCTT). Analysis of the RGA marker in the mapping population revealed that the RGA marker was also co-segregating with the *Rpi-abpt* locus and PAT/MAGA_307 (Figure 5a).

For physical alignment of resistance gene homologues, we screened the BAC library from *S. bulbocastanum* (van der Vossen et al. 2003) with PAT/MAGA_307 and the RGA marker. Several BACs contained these two markers. BAC clones 139K15 and 54I8 were selected to form a small contig (Figure 5a). The BAC-end sequence marker from 139K15L is also coding for a resistance gene homologue.

Identification of the wild species origin of the resistance

The wild *Solanum* accessions CGN17843, CGN20620, CGN21306, CGN17693, CGN17667 and CGN18301 that were involved in the pedigree of RH4X-103 were inoculated with *P. infestans* isolate 90128, using an *in vitro* inoculation assay (Huang 2005). All genotypes from *S. acaule* and *S. phureja* were heavily sporulating whereas all genotypes from *S. bulbocastanum* showed a hypersensitive response. These results suggest that *S. bulbocastanum* is the most likely donor species of the *Rpi-abpt* resistance.

To identify the wild species origin of the resistance gene *Rpi-abpt* at the molecular level, the 14 AFLP markers linked to the *Rpi-abpt* locus (Figure 4) were examined in the five *Solanum* species used in this study, i.e. *S. acaule*, *S. bulbocastanum*, *S. phureja*, *S. demissum* and *S. tuberosum* (Figure 6). Four to six genotypes of each accession or species were included in this experiment. PAT/MAGA_307 generated AFLP fragments with the same mobility in *S. bulbocastanum* as in 707TG11-1, but not in the other species. The AFLP markers PCT/MACA_146, PCG/MAGA_275, EAGA/MCAC_202 and PAC/MAAG_97 were identified in the fingerprints of both *S. acaule* and *S. bulbocastanum*. The AFLP markers EAAC/MCTG_132, EAAC/MCAG_101, PAC/MAAT_498 and EAGT/MCAA_473 that were more distant from *Rpi-abpt* were only identified in *S. acaule*, *S. tuberosum* or both. Also within species, variation between genotypes was found, which can be explained by the enormous genetic variation in outbreeding *Solanum* spp. (Hawkes 1990). Marker EATG/MCAG_215 was not identified in any of the *Solanum* species we tested possibly because of the same reason. None of the AFLP markers was detected in *S. phureja*. Two of the four *R2*-linked markers from *S. demissum* were also observed in *S. bulbocastanum* and another in *S. acaule* as well. The pattern of the 14 *Rpi-abpt*-linked AFLP markers distributed over the *Solanum* species revealed that the closest markers are present in *S. bulbocastanum* that supports our previous position that the introgression fragment containing *Rpi-abpt* is likely derived from this species.

The ABPT-derived *Rpi-abpt* is located at a similar position as *R2* in *S. demissum* (Figure 4).

As described above, the *P. infestans* isolate 90128, which lacks virulence to *R2*, was unable to infect any plant carrying *Rpi-abpt*. To test whether the *Rpi-abpt* shares the same race-specificity as *R2*, the virulent *P. infestans* isolates USA618 and IPO82001 were inoculated on 55 genotypes of the segregating population RH4X-103. Both isolates showed an identical segregation for resistance as *P. infestans* isolate 90128 (data not shown). We conclude that *Rpi-abpt* functionally differs from *R2*, and thus represents a novel *R* gene to late blight.

Discussion

The mapping population RH4X-103 was derived from ABPT clones in which the wild species *S. acaule*, *S. bulbocastanum* and *S. phureja* were involved (Hermesen and Ramanna 1973). *S. bulbocastanum* is a well-known source of resistance to *Phytophthora infestans* (Umeaus and Umeaus 1994; Helgeson et al. 1998; van der Vossen et al. 2003, 2004) but unfortunately is not directly crossable with potato. In this study *S. bulbocastanum* was introgressed via ABPT bridge crosses (Hermesen and Ramanna 1973) and in the ABPT-derived mapping population RH4X-103, monogenic inheritance of *Rpi-abpt* was identified. Inoculation studies with a panel of *P. infestans* isolates revealed that the *Rpi-abpt* is a novel resistance gene with broad-spectrum resistance to all tested isolates. The definite origin of the *R* gene cannot unambiguously be determined, because the original breeding material has not been maintained during the entire process of ABPT breeding. However, several lines of research suggest that the resistance was obtained from *S. bulbocastanum*. First, the *Rpi-abpt* resistance can be traced back in the pedigree to ABPT material, and only *S. bulbocastanum* accessions were identified to contain resistance to late blight. Second, by applying a method previously described by Li et al. (1998), the mobility of AFLP markers linked to *Rpi-abpt* was examined in *S. acaule*, *S. bulbocastanum*, *S. phureja*, *S. tuberosum* and *S. demissum*. Except one marker which could not be amplified from any wild species, the six markers that were closest to *Rpi-abpt* showed an identical mobility in *S. bulbocastanum* accessions. The closest co-segregating marker was specific to *S. bulbocastanum*.

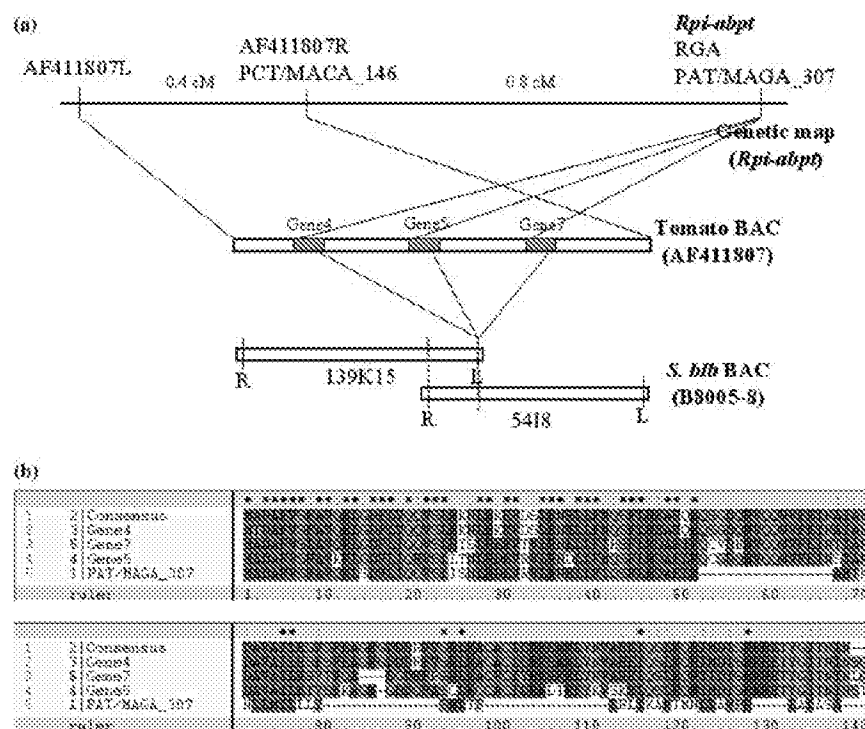


Figure 5. A physical alignment of the resistance gene *Rpi-abpt* and its homologues. (a) Graphical illustration of sequence homology among the ABPT-derived AFLP marker PAT/MAGA_307, tomato derived NBS-LRR sequences of gene4, gene5 and gene7 and *Solanum bulbocastanum* derived BAC-end sequences. GENESCAN predicted NBS-LRR genes in the tomato BAC clone with high homology to the *Rpi-abpt* candidate tagged by PAT/MAGA_307 marker. (b) Sequence alignment among the sequences of resistance gene analogues and PAT/MAGA_307 created by using CLUSTAL-X software.

only. Other markers were less specific and appeared to be conserved also on *S. acaule* or *S. demissum*. Although this indicates that this method is not providing ultimate proof about descent, the introgression fragment is most likely of *S. bulbocastanum* origin.

In the BSA analysis, eight markers linked to the *Rpi-abpt* locus in coupling phase were detected from 204 primer combinations. This observation is similar to other results in tetraploid potato. Li et al. (1998) detected 11 AFLP markers linked to *R2* from 205 primer combinations. BAS in diploid potato is more efficient than in tetraploid potato as Meksem et al. (1995) identified 29 bulk specific loci linked to *R1* from 108 primer combinations. The difference of the results between diploid and tetraploid levels can be explained by the fact that markers with linkage in repulsion cannot be used in tetraploids and that the composed bulks were

based on the genotypes with RFLP markers linked to *R1* in the diploid.

Since the molecular isolation of genes based on their map location could be applicable to any gene whose phenotypic effect can be followed in a segregating population (Ballvora et al. 2001), a reliable map position of the *Rpi-abpt* locus was successfully determined using 1383 offspring. The *Rpi-abpt* locus was localized in a 0.5 cM interval between the closest flanking AFLP markers and co-segregated with one AFLP marker. Although the relationship between genetic and physical distances is known to vary considerably in potato (Marano et al. 2002), we expect that the physical distance between AFLP markers at the region of the *Rpi-abpt* locus is relatively small because of the resistance gene homology of the co-segregating AFLP marker and the RGA marker.

Loci	Distance	a	b	p	t	d
EAAC/MCTG_132	0	—			—	
EAGT/MCTT_247	9.9		—			—
EAGG/MCAA_192	10.3					—
EAAC/MCAG_101	10.8	—			—	
PCT/MACA_146	15.0	—	—			
EATC/MCGA_186	15.6		—			—
<i>Rpi-abpt</i>	15.7					
PAT/MAGA_307	15.7		—			
EATG/MCAG_215	16.1					
EAGA/MCAC_202	17.0	—	—			
PCG/MAGA_275	17.0	—	—			
PAC/MAAG_97	17.0	—	—			
PAC/MAAT_498	18.3	—				
EACT/MCAC_189	18.7	—				—
EAGT/MCAA_473	23.0				—	

Figure 6. A representation of mobility of AFLP markers in various *Solanum* species. The symbol, '—', indicates marker presence with the same mobility on the AFLP gels in one or more genotypes within presented *Solanum* species and the dotted line indicates the expectation of the *Rpi-abpt* locus presence. Marker order and genetic distance are the same as those indicated in the high-resolution map. The AFLP markers, which are also present in the map of R2, are indicated in italic. 'a', 'b', 'p', 't' and 'd' represent *S. acaule*, *S. bulbocastanum*, *S. phureja*, *S. tuberosum* and *S. demissum*, respectively.

Comparative genomics between potato and tomato can facilitate the cloning of *R* genes (Gebhardt et al. 1991; Tanksley et al. 1992; van der Vossen et al. 2004; Huang 2005). The sequence of the co-segregating AFLP marker is highly similar with the sequence of one of the tomato BAC clones on chromosome 4 (van der Hoeven et al. 2002), which contains 19 predicted genes, some belonging to the NBS-LRR gene family. The translated protein of the sequence of the co-segregating AFLP marker is homologous to the resistance gene protein *RPP13* which belongs to the CC-NBS-LRR class in Arabidopsis (Bittner-Eddy et al. 1999) and is highly similar to RGA open reading frames of the tomato BAC clone. These results suggest that *Rpi-abpt* is a member of a resistance gene cluster. The construction of a BAC library of the resistant parent 707TG11-1 is in progress. The co-segregating and flanking AFLP markers are expected to facilitate the creation of a physical contig to span the *Rpi-abpt* locus in a few BAC walking steps. The results described in this paper predict that the novel resistance gene *Rpi-abpt* will be cloned soon.

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Two distinct potato late blight resistance genes from *Solanum berthaultii* are located on chromosome 10

Tae-Ho Park · Simon Foster · Gianinna Brigneti · Jonathan D.G. Jones

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Abstract For breeding potato varieties resistant to late blight, identification of resistance genes to *Phytophthora infestans* (*Rpi* genes) is essential. Introduction of *Rpi* genes from wild *Solanum* species into cultivated potato is likely to be a good method to achieve durable resistance to *P. infestans*. In this study, we identified two *Rpi* genes (*Rpi-ber1* and *Rpi-ber2*) derived from two different accessions of *Solanum berthaultii*. These two genes are closely linked on the long arm of chromosome 10. There are similarities between the predicted genetic locations of the previously identified *Rpi-ber* and *Rpi-ber1*, which given the common origin of these genes, may indicate that they are the same. However, the genetic positions of *Rpi-ber1* and *Rpi-ber2* are different. *Rpi-ber1* is positioned between CT214 and TG63, whereas *Rpi-ber2* is located below both of these two markers. In addition, the sequences of four linked markers to both

R genes showed different polymorphisms indicating the two *Rpi* genes could be transmitted from different haplotypes (chromosomes).

Keywords Late blight resistance · *Phytophthora infestans* · Potato · *Solanum berthaultii*

Introduction

Potato (*Solanum tuberosum* L.) is the fourth most important crop and the most important non-cereal food crop in the world. In potato cultivation, the major natural factor which limits yield is late blight caused by the oomycete pathogen *Phytophthora infestans* (Mont.) de Bary. This devastating disease can result in complete loss of crop yield (Świeżyński and Zimnoch-Guzowska 2001). Fungicide treatment is currently the most common method to control late blight. However, the high cost of fungicide application is problematic, especially in developing countries. Moreover, because fungicide application can impact on health and environmental safety, the use of chemicals is becoming restricted. In addition, the pathogen quickly evolves and some of the new variants are insensitive to commonly used fungicides (Day and Shattock 1997; Goodwin et al. 1996). Therefore, the introduction of genetic resistance into cultivated potato is a potentially valuable method to achieve durable resistance to late blight.

T.-H. Park · S. Foster · G. Brigneti · J. D. G. Jones (✉)
The Sainsbury Laboratory, John Innes Centre, Norwich
Research Park, Colney, Norwich NR4 7UH, UK
e-mail: jonathan.jones@tsl.ac.uk

Present Address:

T.-H. Park
Brassica Genomics Team, National Institute
of Agricultural Biotechnology, Suwon 441-707,
Republic of Korea
e-mail: thzoo@hanmail.net

Two main types of resistance to late blight have been described in potato (Umaerus and Umaerus 1994). First, general resistance is often based on a major quantitative trait locus (QTL) and a few minor QTLs and results in partial resistance. Second, specific resistance is based on major dominant resistance (*R*) genes. In early breeding programs during the first half of last century, 11 *R* genes (*R1*–*R11*) derived from *S. demissum* were identified. Nine *R* genes, *R3* (now separated as *R3a* and *R3b*) and *R5*–*R11* are localized on chromosome 11 (Bradshaw et al. 2006; El-Kharbotly et al. 1994, 1996; Huang et al. 2004; Huang 2005). Other *R* genes originating from *S. demissum* were mapped to different locations including *R1* on chromosome 5 (El-Kharbotly et al. 1994; Leonards-Schippers et al. 1992) and *R2* on chromosome 4 (Li et al. 1998). All *R* genes introgressed from *S. demissum* to cultivated potatoes have been overcome by the pathogen as new strains rapidly evolve that are virulent on the previously resistant hosts (Umaerus and Umaerus 1994). Consequently, partial resistance conferred by QTLs was thought to be more durable than resistance conferred by single *R* genes (Turkensteen 1993). However, partial resistance is strongly correlated with maturity type and it makes resistance breeding more difficult (Wastie 1991). Also the genetic positions of QTLs often correspond to the region of *R* gene clusters (Gebhardt and Valkonen 2001; Grube et al. 2000). Hence, recent efforts to identify late blight resistance have focused on major *R* genes conferring broad-spectrum resistance derived from diverse wild *Solanum* species. Beside *S. demissum*, other wild *Solanum* species such as *S. acaule*, *S. chacoense*, *S. berthaultii*, *S. brevidentis*, *S. bulbocastanum*, *S. microdontum*, *S. sparsipilum*, *S. spegazzinii*, *S. stoloniferum*, *S. sucrense*, *S. toralapanum*, *S. vernei*, and *S. verrucosum* have been reported as new sources for resistance to late blight (reviewed by Jansky 2000; Hawkes 1990). To date, three *R* genes, *RB/Rpi-blb1*, *Rpi-blb2*, and *Rpi-blb3* from *S. bulbocastanum* have been mapped on chromosome 8, 6, and 4, respectively (Naess et al. 2000; Park et al. 2005a; van der Vossen et al. 2003, 2005). Another *R* gene, *Rpi-abpt*, probably from *S. bulbocastanum*, has been localized on chromosome 4 (Park et al. 2005b). *Rpi1* from *S. pinnatisectum* on chromosome 7 (Kuhl et al. 2001), *Rpi-mcq1* from *S. mochiquense* (Smilde et al. 2005) and *Rpi-phul*

from *S. phureja* on chromosome 9 (Śliwka et al. 2006) have also been identified.

S. berthaultii originating from Bolivia, South America has also been identified as a source of resistance to *P. infestans* (reviewed by Jansky 2000; Hawkes 1990). Previously, *Rber* derived from *S. berthaultii* was mapped to chromosome 10 (Ewing et al. 2000). Subsequently a finer and higher-resolution genetic map of *Rpi-ber* (same as *Rber*) was constructed (Rauscher et al. 2006). In our study, we screened accessions of wild *Solanum* species *S. berthaultii* to identify new resistances to *P. infestans* and mapped two *R* genes originating from *S. berthaultii* on chromosome 10. We used several molecular markers from those references and compared genetic positions and allelic differences of these *S. berthaultii*-derived *R* genes.

Materials and methods

Plant materials

In total, 22 accessions of *S. berthaultii* were obtained from three different gene banks: two with BGRC gene bank number from Braunschweig Genetic Resources Center, Braunschweig, Germany, 17 with CGN gene bank number from Centre for Genetic Resources, Wageningen, The Netherlands and three with PI gene bank number from the US Potato Genebank (NRSP-6), Sturgeon Bay, Wisconsin, USA (Table 1). Various numbers of seeds of each accession were sown and tested for resistance to *P. infestans*. Depending on the phenotypic assignment, identified resistant plants were crossed with susceptible plants to generate mapping populations. We generated 40 different intraspecific or interspecific mapping populations.

In the present study, we selected two core populations to further characterize and identify *R* genes to *P. infestans* derived from *S. berthaultii* depending on the number of seeds produced and the results of segregation. One population is named Stenber219 and was derived from a cross between a resistant individual from accession PI473331 of *S. berthaultii* and a susceptible individual from accession CGN19035 of *S. stenotomum*. The initial mapping population for Stenber219 consisted of 44 plants. The second population is named Sphuber237 and was derived from a cross between a resistant

Table 1 Results of screening 22 accessions of *S. berthaultii* for resistance to *P. infestans*

Accessions (Phenotype ^a)	S	MS	MR	R
BGRC28006 (R) ^b	1	4	0	0
BGRC28494 (R)	3	1	0	0
CGN17716 (S)	5	3	1	0
CGN17823 (R)	0	1	1	9
CGN18042 (VR)	1	2	1	7
CGN18074 (S)	2	0	0	0
CGN18118 (VR)	7	3	1	7
CGN18189 (R)	2	2	0	1
CGN18190 (R)	3	0	1	0
CGN18194 (M)	5	0	0	0
CGN18219 (R)	2	3	0	1
CGN18228 (VR)	6	7	0	1
CGN18246 (M)	2	0	0	0
CGN18267 (VS)	2	1	1	1
CGN20635 (M)	2	0	0	0
CGN20644 (VR)	4	2	0	0
CGN20645 (VR)	1	1	0	2
CGN20650 (VR)	3	0	0	0
CGN21354 (S)	2	0	0	0
PI265857 (R)	5	9	2	11
PI265858 (R)	4	5	5	9
PI473331 (R)	3	4	0	17

^a Phenotype identified by inoculation with *P. infestans* isolates 98.170.3 and Met+. S, MS, MR, and R indicate the number of plants that were found to be susceptible, moderately susceptible, moderately resistant, and resistant, respectively

^b Phenotypes in parentheses next to each accession were as indicated in the database of the CGN gene bank. VS, S, M, R, and VR indicate very susceptible, susceptible, intermediate, resistant, and very resistant, respectively

plant from accession PI265858 of *S. berthaultii* and a susceptible line, 96H14-10 used as a *Sli* gene donor (Phumichai et al. 2006). The initial mapping population for Sphuber237 consisted of 50 plants.

Resistance assay

To identify resistance we used two different *P. infestans* isolates (98.170.3 and Met+) kindly provided by Dr. David Shaw at Bangor University, UK. Race-specificity of these two isolates was determined on the standard Black differentials (Malcolmson and Black 1966). Isolate 98.170.3 is race 1.3.4.10.11 and Met+ is race 1.3.4.7.8.10.11.

A detached leaf assay was performed using a method modified from Vleeshouwers et al. (1999). Fresh sporangia were propagated on detached leaves of a suitable sensitive plant from a maintenance plate of cleared pea broth agar (1%) supplied with 100 µg/ml carbenicillin at 18°C with 18 h light and 6 h dark condition for a week. The fresh sporangia were harvested by rinsing leaves with sterilized water and incubated at 10°C for 1–4 h to induce zoospore release. Zoospores were re-suspended to a concentration of 5×10^5 zoospore/ml. Fully expanded young leaf material was taken from greenhouse plants and placed on a 9 cm petri-dish with a piece of wet filter paper. Inoculation was performed on the abaxial leaf surface with a 10 µl droplet of the zoospore inoculum. Petri-dishes were wrapped with plastic film and incubated at 18°C with 18 h light and 6 h dark condition for 7–12 days. Experiments were repeated independently using two leaflets per plant at least three times to confirm phenotypes, classified as susceptible, moderately susceptible, moderately resistant, and resistant.

Marker development and map construction

Genomic DNA was extracted from young leaf tissue according to Bendahmane et al. (1997). To identify the genetic position of the *R* loci, both AFLP markers (Vos et al. 1995) and PCR-based markers were employed.

AFLP analysis was performed as described by Vos et al. (1995). Primary templates were prepared using a *Pst*I/*Mse*I restriction enzyme combination, followed by ligation to appropriate adaptors. Templates were diluted 10 times prior to selective pre-amplification. The pre-amplified products (secondary templates) were checked on a 1% agarose gel and diluted 10 times again. Bulk Segregant Analysis (BSA; Michelmore et al. 1991) was carried out to perform AFLP analysis on resistant and susceptible bulks comprising the secondary templates of eight resistant and eight susceptible genotypes selected based on the results of phenotypes. The bulks were screened with 24 primer combinations that decrease marker density. For the selective amplification, radioactively labeled (³³P) P+2 primers were used in combination with M+3 primers. The ³³P-labeled PCR products were loaded on the gel after 30 min of pre-run. The amplified DNA fragments were separated on a 6% polyacrylamide gel

Table 2 PCR based markers linked to *Rpi-ber1*, *Rpi-ber2* or both

Marker ^a	PCR primer (5'-3')	Type of marker ^b	
		<i>Rpi-ber1</i>	<i>Rpi-ber2</i>
CT11	F:AGATTGCTTGTGTTGGTGGTT R:CAAAAGGAATCTTGACACAG	x	a.s.
CT214	F:CGCGAAAGAGTGCTGATAG R:CCGCTGCCTATGGAGAGT	<i>Hinf</i> I	a.s.
CT238	F:TTCGATGCCAATCTCCTA R:AATTTCTCCATGTTTTTCAG	x	a.s.
TG63	F:CTGCATCAACTGGATATTCC R:GTTGAGCAGTGCAATGTAC	<i>Sau</i> 96I	<i>Sau</i> 96I[R]
U221455	F:AGGCGCTTCTTATTATCTTTCTC R:ACCACAAGCAATCATTCTACACC	<i>Alu</i> I[R]	<i>Hinf</i> I
TG206	F:AAATCGAAAAGGGGCATACC R:TTGACATCCTCCAGCAGAAAC	<i>Hha</i> I[R]	x
TG422	F:TGCATCTCTGTCCAAGCTCTATGC R:TGTGAGGCATTTTGATTTCGCAC	<i>Sau</i> 96I	<i>Sau</i> 96I[R]
T0724	F:GCTCGCAAACCACTACAAGCAG R:CTTTGAATCCTCGCCTTCTTGC	x	<i>Rsa</i> I
TG403	F:AGCTGTCAGAAAGATTGGGAG R:GCATTGTCATCAAGTGGTTC	x	<i>Dde</i> I
CT113	F:ACAACGGGCAACAGACGCAACC R:AGCTCGAGGATGGCCGCACTTT	x	a.s.[R]
BA81115t3	F:TGAAGTGAAGTATGATGTTCTTGC R:TCACAAGATTTGAAGTTGTGATGAGT	a.s.	a.s.

^a The first three markers CT11, CT214, and CT238 are from Rauscher et al. (2006)

^b The type of markers in the map of both resistance loci. x, a.s., and [R] indicate not polymorphic, allele specific, and repulsion phase marker, respectively. Restriction enzymes generate CAPS markers

in 1X TBE buffer for two and half hours. The gels were dried on Whatman papers for 2 h using a vacuum dryer and exposed to X-ray films for 4–6 days. AFLP markers were named according to the enzyme, primer combination, and the mobility of the fragment as described in reference autoradiograms by Keygene NV, Wageningen, The Netherlands. PCR-based markers were also developed to construct genetic linkage maps of the *R* genes using primers derived from published RFLP markers, tomato BAC sequences and tomato unigene sequences available in a public database (<http://www.sgn.cornell.edu/cview/index.pl>) and primers published by Rauscher et al. (2006). Genetic linkage maps were constructed according to recombination frequencies between marker loci or between marker loci and the *R* loci. The PCR-based markers used to construct the genetic linkage maps of the *R* genes are presented in Table 2.

Results

Identification of resistance

To identify resistance in wild species, we collected 22 different wild accessions of *S. berthaultii* from three different gene banks. From the seed packages of these 22 accessions, a total of 195 individual plants were tested for resistance to *P. infestans* in two replications using two different isolates, 98.170.3 and Met+. When the inoculated leaf showed sporulating lesions on the inoculated spot, it was scored as susceptible and when the leaf showed no symptoms or necrosis without sporulation, it was scored as resistant. When the two leaves did not show the same reaction or sporulation was not clear, it was classified as either moderately susceptible or moderately resistant. In the database of accessions at the CGN gene bank, the 22 accessions of

S. berthaultii have already been evaluated for resistance to late blight and most of these accessions were assigned as resistant except four accessions. However, the results of the resistance assay from our study were very different from those in the CGN database. A lot of variation was also observed between accessions and within accessions as shown in Table 1. Based on the results of screening *S. berthaultii* accessions, resistant plants were selected to generate mapping populations. These resistant plants were crossed with susceptible plants of *S. berthaultii* accessions, other susceptible wild species or cultivated diploid lines. Seeds were successfully obtained from 40 crosses. However, the number of seeds and the frequency of seed germination were variable. Eleven of these 40 populations were selected to be tested for resistance. Approximately 50 seeds from each population were sown in vitro and the in vitro plants were transplanted in the greenhouse. In the Stenber219 population derived from a cross between a resistant *S. berthaultii* (PI473331) and a susceptible *S. stenotomum* (CGN19035), 20 resistant and 24 susceptible plants were observed. In the Sphuber237 population derived from a cross between a resistant *S. berthaultii* (PI265858) and a susceptible line (96H14–10), 25 resistant and 25 susceptible plants were observed. In other mapping populations derived from different accessions, the phenotypes were not clear, segregation was skewed to resistance, or all plants were resistant (data not shown). Therefore, two mapping populations (Stenber219 and Sphuber237) were chosen to construct genetic linkage maps.

Localization of the *R* loci

The two *R* loci identified in the Stenber219 and Sphuber237 populations were named *Rpi-ber1* and *Rpi-ber2* (*R* gene to *P. infestans* derived from *S. berthaultii*), respectively. To localize these two *R* loci on the genetic linkage map of potato, we selected eight resistant and eight susceptible plants of each population to compose resistant and susceptible bulks. These bulks and both parents were tested with chromosome 10 marker TG63 as previously one *R* gene derived from *S. berthaultii* had been localized on chromosome 10 (Ewing et al. 2000; Rauscher et al. 2006). TG63 was found to be linked genetically to the resistant phenotypes in both Stenber219 and Sphuber237 populations. On subsequent analysis of individual plants, it was 10.2 and 5.0 cM distant from *Rpi-ber1* and *Rpi-ber2*,

respectively. In addition, 24 Pst+2/Mse+3 primer combinations were tested on the bulks and parents, four and five of which produced candidate AFLP markers putatively linked to the *R* loci, *Rpi-ber1*, and *Rpi-ber2*, respectively. To confirm linkage between the candidate AFLP markers and the *R* loci, the 16 individuals that comprised the two bulks were also screened for these markers. Subsequently, one and three AFLP markers were identified to be linked to *Rpi-ber1* and *Rpi-ber2*, respectively. However, all of these AFLP markers were distant from the *R* loci or linked as repulsion phase markers. PAGMAGG_324[R] was mapped 43.1 cM from *Rpi-ber1* towards the telomere. PAGMGG_227[R] and PAGMGG_228 were mapped 17 cM and PAGMGG_167[R] was mapped 11 cM from *Rpi-ber2* towards the centromere.

To obtain more markers linked to the *R* loci, we used PCR-based markers. Published RFLP markers, tomato BAC sequences, tomato unigene sequences in a public database (<http://www.sgn.cornell.edu/cview/index.pl>) and markers linked to *Rpi-ber* on chromosome 10 (Rauscher et al. 2006) were tested on both populations. In the Stenber219 population for *Rpi-ber1*, six PCR-based markers were identified to be linked to the *R* locus. CT214 mapped 16 cM towards the centromere and TG63, U221455[R], TG206[R], and TG422 mapped 10.2 cM towards telomere from the *R* locus. In the Sphuber237 population for *Rpi-ber2*, ten PCR-based markers were identified to be linked to the *R* locus. CT238, TG63[R], U221455, TG422[R], and T0724 mapped 5 cM towards centromere and TG403 mapped 12 cM towards telomere from the *R* locus. All PCR-based markers localized on both maps are described in Table 2 and the genetic linkage maps are shown in Fig. 1.

Comparative genetics

Two genetic linkage maps for *Rpi-ber1* and *Rpi-ber2* were compared to each other and to the map for *Rpi-ber* published by Rauscher et al. (2006) as shown in Fig. 1. The polymorphic pattern of PCR-based markers bridged between the *Rpi-ber1* and *Rpi-ber2* maps was also compared as shown in Table 2 and Fig. 2.

Several PCR-based markers were linked to each other on three different maps. CT214 and TG63 were present on all three maps. CT11, CT238, and TG403 were bridged between the *Rpi-ber* map and the *Rpi-ber2* map. U221455, TG422, and BA81115t3 were

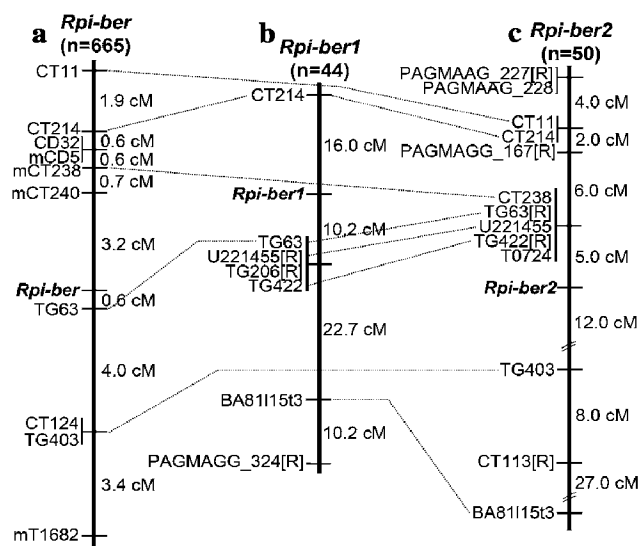


Fig. 1 Integrated genetic linkage map of the different late blight resistance genes (a) *Rpi-ber*, (b) *Rpi-ber1*, and (c) *Rpi-ber2* on chromosome 10. The map of (a) *Rpi-ber* is modified from Rauscher et al. (2006). The map distances (cM) are indicated on the right of each map

bridged between the *Rpi-ber1* map and the *Rpi-ber2* map. The order of all bridge markers was the same. However, the genetic position of the three *R* loci was not. *Rpi-ber* and *Rpi-ber1* were located on north of TG63, but genetic distances are 0.6 cM from *Rpi-ber* and 10.2 cM from *Rpi-ber1*. Although *Rpi-ber* and *Rpi-ber1* were located between CT214 and TG63, *Rpi-ber2* was located south of these two markers.

As the types and the polymorphic patterns of PCR-based markers are variable in Stenber219 and Sphuber237, we compared 11 PCR-based markers linked to *Rpi-ber1*, *Rpi-ber2*, or both (Table 2 and Fig. 2). There were more polymorphic markers in Stenber219 (10 markers) than in Sphuber237 (6 markers). One and five of these markers were allele specific in Stenber219 and Sphuber237, respectively. Five markers were bridged between genetic linkage maps of two populations as shown in Fig. 1. However, only one marker (BA81115t3) was shown to be identical in the type of marker and the polymorphic pattern. The rest of the bridge markers were all different in both populations. For instance, CT214 was a CAPS marker generated by *Hinf*I digestion in Stenber219, but was an allele specific marker in Sphuber237. TG63 and TG422 were CAPS markers generated by *Sau*96I digestion in both populations. However, these markers were linked to *Rpi-ber1* in coupling phase, whereas they were linked to *Rpi-ber2* in repulsion

phase. Also the restriction patterns of these two markers were different. Four bridge markers showing different marker types and polymorphic patterns in between two populations are shown in Fig. 2.

Discussion

In this study, we identified two *R* genes derived from the wild *Solanum* species, *S. berthaultii*. We screened 195 individual plants and 65 and 66 plants were assigned as susceptible and resistant, respectively. The rest of the plants were assigned as moderately susceptible or moderately resistant due to unclear phenotypes. Based on these results, we generated 40 mapping populations, 11 of which were screened for segregation with about 50 offspring of each population. Two populations, Stenber219 and Sphuber237, showed clear segregation. In other populations, resistance could be associated with QTLs or the resistance allele in the resistant parents could be homozygous.

The *R* genes designated as *Rpi-ber1* and *Rpi-ber2* were localized on the long arm of chromosome 10. One *R* gene (*Rber/Rpi-ber*) originating from *S. berthaultii* was previously mapped to a similar genetic region (Ewing et al. 2000; Rauscher et al. 2006). As shown in Fig. 1, all molecular markers linked to each other in these three molecular maps are colinear and there were two molecular markers, CT214 and TG63 which could bridge these three maps. The genetic positions of these two markers demonstrate that *Rpi-ber2* could be distinct from the others as *Rpi-ber* and *Rpi-ber1* are positioned between the two markers, whereas *Rpi-ber2* is located below the two markers. The accession from which *Rpi-ber2* is derived also differs from that of *Rpi-ber* and *Rpi-ber1* although all of these have been identified in the same wild *Solanum* species. However, identity of *Rpi-ber* and *Rpi-ber1* could not be excluded because these two genes originate from the same accession of *S. berthaultii*. In addition, although the genetic distances between the *R* loci and CT214 or TG63 are quite different, it is not conclusive whether *Rpi-ber* and *Rpi-ber1* are the same or not because genetic distance highly relies on the size of population. CT214 and TG63 are 8.2 and 4.8 cM away from *Rpi-ber* defined using 158 individuals in the preliminary genetic map of *Rpi-ber* (named as *Rber*) reported by Ewing et al.

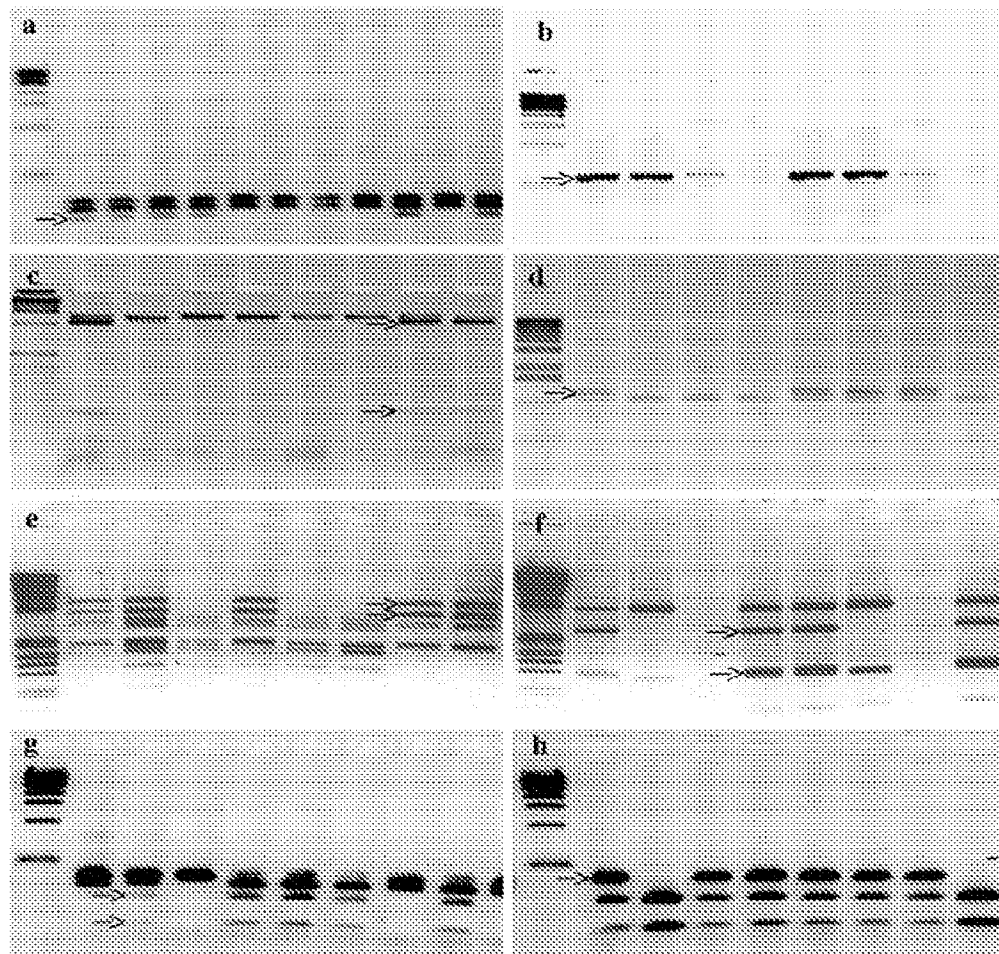


Fig. 2 Pictures of markers linked to both (a, c, e, g) *Rpi-ber1* and (b, d, f, h) *Rpi-ber2*. Four markers (a, b) CT214, (c, d) TG63, (e, f) U221455, and (g, h) TG422 show a polymorphic pattern as indicated by arrows

(2000), but 5.1 and 0.6 cM away from *Rpi-ber* defined using 665 individuals in their higher-resolution map (Rauscher et al. 2006). Similarly, two flanking markers to *Rpi-abpt* and *R2-like* were 4.7 and 3.8 cM distant in the primary maps constructed from analysing 233 and 78 individuals, but 7.1 and 6.4 cM distant in the high-resolution maps which comprised 1,383 and 1,582 individuals, respectively (Park et al. 2005b, c).

Many characterized *R* genes are clustered in the same genomic region and members of a cluster can confer resistance to not only one pathogen, but also different pathogens (reviewed by Gebhardt and Valkonen 2001; Hulbert et al. 2001). Two different *R* genes, *Gpa2* conferring resistance to potato cyst nematode and *Rx* conferring resistance to potato virus X derived from the same ancestor showed physically tight linkage (van der Rouppe Voort et al. 1999) and even one gene, *Mi-1* conferred resistance to a

nematode, potato aphid and white fly (Milligan et al. 1998; Rossi et al. 1998; Vos et al. 1998; Nombela et al. 2003). In some cases, the homology shared between *R* genes has facilitated the cloning of *R* genes such as *R3a* (Huang et al. 2005) and *Rpi-blb2* (van der Vossen et al. 2005). Clustering of *R* genes with different specificities has also been demonstrated for the *Cf-2* and *Cf-5* genes for resistance to two distinct isolates of the fungus *Cladosporium fulvum* (Dixon et al. 1996, 1998), for *R3a*, *R3b*, and *R5-R11* to *P. infestans* (Huang et al. 2004; Huang 2005) and *R2*, *R2-like*, *Rpi-abpt*, and *Rpi-blb3* to *P. infestans* (Li et al. 1998; Park et al. 2005a, b, c). This led van der Vossen et al. (2005) to conclude that intragenic recombination or unequal crossover has expanded the *Rpi-blb2* locus in *S. tuberosum* compared with that in *S. lycopersicon*, in agreement with Hulbert et al. (2001). They suggested that *R* genes showing distinct resistance specificities within the same *R* gene cluster

could be generated by intragenic or intergenic recombination and unequal crossover at *R* loci. This theory could explain that *Rpi-ber1* and *Rpi-ber2* identified in this study differ from each other although they are positioned on the genetically similar region of chromosome 10. Another evidence is that, as shown in Table 2 and Fig. 2, alleles linked to both *R* genes also showed different polymorphisms, indicating that the two *R* genes are present on distinct origin of haplotypes (chromosomes). However, no evidence has been found to indicate whether *Rpi-ber1* identified in this study is same as *Rpi-ber* or not (Ewing et al. 2000; Rauscher et al. 2006). Although the two genes originate from the same accession of *S. berthaultii* and are genetically located on the same genomic region, this does not rule out the possibility that they are distinct genes. We also detected several *R* genes showing different race-specificities in certain populations in our contemporary researches for potato late blight resistance, even though all the *R* genes belong to the same *R* gene cluster and some originate from the same accession of certain wild species (unpublished data).

As a part of our continuing efforts to identify sources of late blight resistance, two *R* genes derived from *S. berthaultii* have been identified in this study. Based on the results of the mapping positions of the two *R* genes and different polymorphic patterns of linked markers to the two *R* genes, we expect that they could be different each other. However, we cannot conclude it because they are also possibly allelic or/and same genes. Ultimately, therefore, we plan to clone these two genes. Additionally we have identified more targets to clone *R* genes derived from other wild *Solanum* species. In combination with other *Rpi* genes, such as *Rpi-mcql* from *S. mochiquense* (Smilde et al. 2005), we envisage that a polyculture approach (Dangl and Jones 2001) combined with cisgenic GM technology (Schouten et al. 2006a, b) could be applied to achieve durable resistance for potato late blight.

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Mapping and Cloning of Late Blight Resistance Genes from *Solanum venturii* Using an Interspecific Candidate Gene Approach

Mathieu A. Pel,¹ Simon J. Foster,² Tae-Ho Park,² Hendrik Rietman,¹ Gert van Arkel,¹ Jonathan D. G. Jones,² Herman J. Van Eck,¹ Evert Jacobsen,¹ Richard G. F. Visser,¹ and Edwin A. G. Van der Vossen¹

¹Wageningen UR Plant Breeding, P.O. Box 386, 6700 AJ Wageningen, The Netherlands; ²The Sainsbury Laboratory Colney Lane, Norwich, NR4 7UH, U.K.

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Late blight, caused by the oomycete *Phytophthora infestans*, is one of the most devastating diseases of potato. Resistance (*R*) genes from the wild species *Solanum demissum* have been used by breeders to generate late-blight-resistant cultivars but resistance was soon overcome by the pathogen. A more recent screening of a large number of wild species has led to the identification of novel sources of resistance, many of which are currently being characterized further. Here, we report on the cloning of dominant *Rpi* genes from *S. venturii*. *Rpi-vnt1.1* and *Rpi-vnt1.3* were mapped to chromosome 9 using nucleotide binding site (NBS) profiling. Subsequently, a *Tm-2²*-based allele mining strategy was used to clone both genes. *Rpi-vnt1.1* and *Rpi-vnt1.3* belong to the coiled-coil NBS leucine-rich repeat (LRR) class of plant *R* genes and encode predicted peptides of 891 and 905 amino acids (aa), respectively, which share 75% amino acid identity with the *Tomato mosaic virus* resistance protein *Tm-2²* from tomato. Compared with *Rpi-vnt1.1*, *Rpi-vnt1.3* harbors a 14-aa insertion in the N-terminal region of the protein and two different amino acids in the LRR domain. Despite these differences, *Rpi-vnt1.1* and *Rpi-vnt1.3* genes have the same resistance spectrum.

Late blight, one of the world's most devastating plant diseases, is caused by the oomycete *Phytophthora infestans*, causing an estimated yearly economic loss of \$3.25 billion in potato-growing areas worldwide. Despite more than 150 years of resistance breeding, disease management still relies on the enormous application of fungicides. Breeding for late blight resistance was stimulated in the mid-nineteenth century by the disastrous consequences of *Phytophthora* epidemics in the United States and Europe. Only a few of the many existing landraces survived and these formed the basis for cultivar development. However, until 1910, the reduced aggressiveness of the pathogen since 1850 probably played a more prominent role in the survival of the potato crop in Europe than the first breeding activities. The rediscovery of the Mendelian laws of

genetics subsequently directed the focus of potato resistance research on the use of wild *Solanum* spp. grown in South and Central America. Initially, special attention was given to *Solanum demissum* as the main resistance source (Black and Gallegly 1957; Malcolmson and Black 1966). To date, at least 11 specificities (*R1–R11*) have been identified in *S. demissum*, four of which (*R1*, *R2*, *R3*, and *R10*) have been introgressed by breeders up to cultivar level; however, races of the pathogen that were able to overcome these genes emerged within a few years after market introduction (Turkensteen 1993). By the end of the 1950s, most breeders switched to the use of sources of germplasm with partial or quantitative resistance, the underlying paradigm being that this type of resistance is resistant (*R*)-gene independent and assumed to be of polygenic nature and, thus, more durable (Turkensteen 1993). Extensive research in this area has led to the identification of many minor quantitative trait loci (QTL) (Bradshaw et al. 2006b; Collins et al. 1999; Costanzo et al. 2005); however, stacking of these QTL has proven to be very difficult, due to the outbreeding nature of cultivated potato. Moreover, this type of resistance is strongly correlated with late maturity under long day conditions (Howard et al. 1970). This, together with the finding that quantitative resistance is apparently also amenable to erosion (Flier et al. 2003), has stimulated breeders to reconsider the use of *R* genes, preferably through stacking of complementary genes.

A recent comprehensive survey of wild tuber-bearing *Solanum* spp. revealed that the genus *Solanum* harbors a wealth of late-blight-resistance sources that have yet to be exploited. Recent studies into the genetic basis of late-blight resistance in *S. demissum* showed that 8 of the 11 known specificities—*R3* (now known to be *R3a* and *R3b*), *R5*, *R6*, *R7*, *R8*, *R9*, *R10*, and *R11*—are located close to each other on chromosome 11 (Bradshaw et al. 2006a; El Kharbotly et al. 1994; Huang et al. 2004, 2005). Other mapped *R* genes from *S. demissum* include *R2* on chromosome 4 (Li et al. 1998) and *R1* on chromosome 5 (El Kharbotly et al. 1994; Leonards-Schippers et al. 1992). In addition to *S. demissum*, other wild *Solanum* spp. have been recognized as sources of late blight resistance. The described *R* loci include *Rpi-ber1* (initially named *R_{ber}*) from *S. berthaultii* on chromosome 10 (Ewing et al. 2000); *RB/Rpi-blb1*, *Rpi-blb2*, and *Rpi-blb3* from *S. bulbocastanum* on chromosomes 8, 6, and 4, respectively (Naess et al. 2000; Park et al. 2005; van der Vossen et al. 2003, 2005); *Rpi-pnt1* (initially named *Rpi1*) (Kuhl et al. 2001) from *S. pinnatisectum* on chromosome 7; *Rpi-mcq1* (initially named *Rpi-moc1*) (Smilde et al. 2005)

Current address for E. A. G. Van der Vossen: Keygene N.V., Wageningen, The Netherlands.

Corresponding author: H. J. van Eck; E-mail: herman.vaneck@wur.nl

Nucleotide sequence data is available in the GenBank database under accession numbers FJ423044 and FJ423046 for *Rpi-vnt1.1* and *Rpi-vnt1.3*, respectively.

from *S. mochiquense* on chromosome 9; and *Rpi-phu1* from *S. phureja* on chromosome 9 (Sliwka et al. 2006). In addition, several QTL involved in resistance to late blight have been reported, in both cultivated potato (Gebhardt and Valkonen 2001) and wild species, such as *S. microdontum* (Sandbrink et al. 2000; Tan et al. 2008), *S. paucissectum* (Villamon and Spooner 2005), and *S. phureja* on chromosome 7 and 12 (Ghislain et al. 2001).

In contrast to introgression breeding, isolation of *R* genes from *Solanum* spp. and their stable transformation into existing potato cultivars is by far the fastest means of exploiting potentially durable late-blight resistance present in the *Solanum* gene pool. Cloning of *R* genes is typically done through a positional cloning strategy. Currently, four *R* genes for late-blight resistance have been cloned and all belong to the coiled-coil nucleotide-binding site leucine-rich repeat (CC-NBS-LRR) class of plant *R* genes: *R1* (chromosome 5) and *R3a* (chromosome 11) from *S. demissum* (Ballvora et al. 2002; Huang et al. 2005) and *Rpi-blb1* and *Rpi-blb2* from *S. bulbocastanum* on chromosomes 8 and 4, respectively (Naess et al. 2000; van der Vossen et al. 2003, 2005). Once a functional gene is cloned from a specific *R* locus, one can try to clone functional alleles from the same or different species in order to determine allele frequency and allelic variation at a given locus. This was illustrated by the cloning of *Rpi-sto1* and *Rpi-ptal* from *S. stoloniferum* and *S. papita*, respectively, which were shown to be functional alleles of *Rpi-blb1* (Vleeshouwers et al. 2008). Here, we demonstrate that NBS profiling (van der Linden et al. 2004), when combined with bulked segregant analysis (BSA) (Michelmore et al. 1991), is a powerful tool to generate candidate gene markers. They can predict the position of the *R* locus under study and, upon sequence information, form a starting point for cloning of the gene through a functional allele mining strategy, avoiding the map-based cloning approach. Depending on the resolution of relevant genetic mapping studies and the size of the candidate gene family, an allele mining approach can generate many candidate genes which need to be functionally analyzed.

To date, functional analysis of candidate *R* gene homologs (RGH) typically requires stable transformation of a susceptible genotype for complementation purposes. This is a time-consuming and inefficient approach because it takes at least several months to generate transgenic plants that can functionally be analyzed. In the current study, we have exploited the finding that *Nicotiana benthamiana* is susceptible to *P. infestans* (Becktell et al. 2006) to develop a transient complementation assay for *R* genes that confer resistance against *P. infestans* (H. Rietman, I. Hein, R. G. F., Visser, E. A. G. Van der Vossen, and V. G. A. A. Vleeshouwers, *unpublished results*).

Here, we present an example of how we used the above-described approaches or techniques to clone two functionally equivalent *R* genes from *S. venturii*, a species with its origin in the Andean region of South America.

RESULTS

Genetic basis and spectrum of late blight resistance in *S. venturii*.

To determine the genetic basis of late blight resistance in *S. venturii*, 14 *S. venturii* accessions were screened in detached-leaf assays (DLA) with the *P. infestans* isolate IPO-C. Resistant genotypes selected from the *S. venturii* accessions CGN18108 and CGN18000 were used to generate the mapping populations 7698 and 7663, respectively. Following DLA with 52 F1 progeny plants of population 7698, 30 were scored as resistant and 22 as susceptible, suggesting the presence of a single dominant *R* gene (*Rpi-vnt1.1*). Of the 60 F1 progeny plants screened from population 7663, 24 were scored as resistant and 36 as susceptible, suggesting that *vnt365-1* also contained a single dominant *R* gene (*Rp-vnt1.3*). Resistance genes were named in agreement with the concurrent work of Foster and associates (2009) who identified in the *S. venturii* accessions CGN18108, CGN18279, and CGN18000 three single dominant *R* genes, *Rpi-vnt1.1*, *Rpi-vnt1.2*, and *Rpi-vnt1.3*, respectively.

The resistance spectra of *Rpi-vnt1.1* and *Rpi-vnt1.3* were analyzed by challenging them with several isolates of different complexity and aggressiveness (Table 1). Both genes were only overcome by strain EC1, suggesting that they share the same resistance spectrum.

Mapping of *Rpi-vnt1.1* and *Rpi-vnt1.3* to chromosome 9.

In an attempt to develop markers linked to *Rpi-vnt1.1* and *Rpi-vnt1.3*, we carried out a BSA in combination with NBS profiling in both mapping populations. This led to the identification of nine bulk-specific markers for *Rpi-vnt1.1* in 7698 and eight for *Rpi-vnt1.3* in 7663. On the full offspring, only two resistant bulk-specific fragments, one generated with the NBS2/*RsaI* primer-enzyme combination and the other with NBS3/*HaeIII*, cosegregated with resistance in both initial 7698 and 7663 populations of 52 and 60 F1 progeny plants, respectively. Therefore, these fragments were cloned and sequenced. When subjected to a BLAST analysis, both sequences turned out to be highly similar to the *Tm-2²* gene on chromosome 9 of tomato (Lanfermeijer et al. 2003). The cloned NBS2/*RsaI* and NBS3/*HaeIII* fragments were 350 and 115 bp in size and shared 88.3 and 80.3% DNA sequence identity, respectively, with *Tm-2²*, suggesting that *Rpi-vnt1.1* and *Rpi-vnt1.3* were *Tm-2²* related and, thus, could lie on chromosome 9. Genetic linkage of the NBS fragments to the *R* genes under study was verified by developing fragment-specific SCAR (sequence-characterized amplified region) markers in the *Rpi-vnt1.1* and *Rpi-vnt1.3* mapping populations. In this way, the NBS3-derived marker NBS3b was mapped relative to the chromosome-9-derived markers TG35, TG551, TG186, CT183, or T1421 (Fig. 1). Linkage of TG35 and TG186, and TG35 and TG551, to *Rpi-vnt1.1* and *Rpi-vnt1.3*, respectively, indicated that both genes were indeed derived from similar regions on chromosome 9 (Fig. 1). These findings were in line with the result of

Table 1. Description of *Phytophthora infestans* isolates used to determine the specificity of *Rpi-vnt1.1* and *Rpi-vnt1.3*

Isolate ID	Country of origin	Isolation year	Race	<i>Rpi-vnt1.1</i> and <i>Rpi-vnt1.3</i> phenotypes
90128	Geldrop, The Netherlands	1990	1.3.4.7.(8)	Resistant
H30P04	The Netherlands	Unknown	7	Resistant
IPO-C	Belgium	1982	1.2.3.4.6.7.10.11	Resistant
USA618	Toluca Valley, Mexico	Unknown	1.2.3.6.7.11	Resistant
VK98014	Veenkolonien, The Netherlands	1998	1.2.4.11	Resistant
IPO-428-2	The Netherlands	1992	1.3.4.7.8.10.11	Resistant
NL00228	The Netherlands	2000	1.2.4	Resistant
Katshaar	Katshaar, The Netherlands	Unknown	1.3.4.7.10.11	Resistant
F95573	Flevoland, The Netherlands	1995	1.3.4.7.10.11	Resistant
89148-09	The Netherlands	1989	0	Resistant
EC1	Ecuador	Unknown	3.4.7.11	Susceptible

concurrent work of Foster and associates (2009), who followed a map-based cloning approach to clone *Rpi-vnt1.1*, *Rpi-vnt1.2*, and *Rpi-vnt1.3* genes.

In order to develop flanking markers to screen a large offspring for recombinant events, markers linked to TG35 or TG551 were selected from Solanaceae Genomics Network (SGN) and screened in both populations. Despite low levels of polymorphism, expressed sequence tag-based markers U276927 and U270442 have been developed and mapped in populations 7698 and 7663, respectively (Table 2; Fig. 1). U276927 could be mapped 1.8 centimorgans (cM) north of *Rpi-vnt1.1* whereas

U270442 was mapped 3.5 cM south of *Rpi-vnt1.3*. Subsequently, recombinant offspring was identified using 500 individuals of population 7698 and 1,005 individuals of population 7663, using the flanking markers U276927/TG186 and NBS3B/U270442, respectively. This resulted in the mapping of *Rpi-vnt1.1* and *Rpi-vnt1.3* within a genetic intervals of 4 and 3.7 cM, respectively (Fig. 1).

Tm-2²-based allele mining.

In view of the expected high DNA sequence homology between *Rpi-vnt1.1*, *Rpi-vnt1.3*, and *Tm-2²*, we adopted a homology-based allele mining strategy to clone the former two genes. The first step was to design degenerated primers incorporating the putative start and stop codons of candidate *Tm-2²* gene homologs (*Tm2GH*). Based on an alignment of all the available potato- and tomato-derived *Tm-2²*-like sequences in public sequence databases, we designed primers ATG-Tm2F and TGA-Tm2R (Table 3). However, no amplicons of the expected size were generated when this primer set was tested on the parental genotypes of both mapping populations. Because the ATG-Tm2F primer sequence was present in the cosegregating NBS profiling-derived marker sequences, three new reverse primers (REV-A, REV-B, and REV-C) were designed 100 bp upstream of the initial TGA-Tm2R primer site, in a region that was conserved in all the aligned *Tm-2²*-like sequences. When combined with either ATG-Tm2F or NBS3BF, a single amplicon of approximately 2.5 kb was specifically amplified from the resistant parental genotypes only (i.e., vnt7014-9 and vnt365-1). These fragments were cloned into the pGEM-T Easy vector and approximately 96 individual clones from each genotype were sequenced using a primer walk strategy. All the obtained sequences shared 75 to 80% similarity to *Tm-2²*. A total of five different classes could be distinguished within the vnt7014-9-derived sequences whereas the vnt365-1-derived sequences fell into only three different classes. These different classes were subsequently named NBS3B-like or non-NBS3B-like based on the degree of homology to the NBS3B sequence.

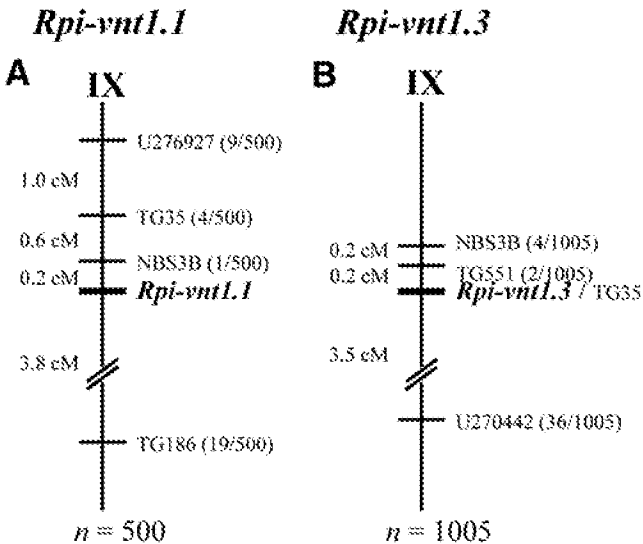


Fig. 1. Genetic linkage maps of chromosome 9 of the **a**, *Rpi-vnt1.1* and **b**, *Rpi-vnt1.3* loci mapped in the populations 7698 and 7663, respectively. Numbers on the left side indicate genetic distances (centimorgans). Relative positions of mapped loci are indicated by horizontal lines. The letter n represents the size of each population.

Table 2. Markers used to map *Rpi-vnt1.1* and *Rpi-vnt1.3*

Marker	Primer orientation	Primer sequence	Annealing temperature (°C)	Enzyme ^a
NBS3B	Forward	ccctctcctcctcacatttag	65	a.s.
	Reverse	gcattgccaactattgaaacaac	...	...
TG35	Forward	cacggagactaagattcagg	60	<i>HhaI/XapI</i> (c)
	Reverse	taaaggtgatgctgatgggg	...	...
TG551	Forward	ccagaccaccaagtgtgttc	58	<i>TaqI</i> (c)
	Reverse	aacttcagatatgctctgcag	...	...
TG186	Forward	aacggtgtacgagattttac	58	<i>HphI</i> (c)
	Reverse	acctacatagatgaacctcc	...	...
U270442	Forward	ggatattatcttgcaacatctcg	55	<i>XapI</i> (r)
	Reverse	cttctgatggtatgcatgagaac	...	...
U276927	Forward	gcattagcgcaattggaatccc	58	<i>HphI</i> (c)
	Reverse	ggagagcattagtacagcgctc	...	...

^a Abbreviations: a.s. = allele specific, c = coupling phase, and r = repulsing phase.

Table 3. Primers used for various experiments as described in this article

Marker	Primer orientation	Primer sequence	Annealing temperature (°C)	Experiments
NBS-GSP1	Forward	tccaaatattgtcgagttggg	Touch down	Genome walking
NBS-GSP2	Forward	gctttgtgcagacatgatgc	Touch down	Genome walking
REV-A	Reverse	ggttgctgaagtaacgtgcac	55	<i>Tm2</i> -based allele mining
REV-B	Reverse	tgcacggatgatgtcagatgcc	55	<i>Tm2</i> -based allele mining
REV-C	Reverse	caacttgaagtttgcatttc	55	<i>Tm2</i> -based allele mining
ATG-Tm2F	Forward	atggctgaaattctctcacagc	55	<i>Tm2</i> -based allele mining
TAA-8bisR	Reverse	ttatagtacctgtatattctcaac	55	<i>Tm2</i> -based allele mining
ATG2-Tm2F	Forward	atgaattattgtttacaagacttg	55	<i>Tm2</i> -based allele mining
TGA-Tm2R	Reverse	tgatattctcaacttgcaagc	55	<i>Tm2</i> -based allele mining
GSP1-5'race	Reverse	gaacactcaaatgatgacagacatgcc	67	5' rapid amplification of cDNA ends
GSP2-5'race	Reverse	cccaaaccggcgcatccaactattg	67	5' rapid amplification of cDNA ends

In order to retrieve the missing C-terminal part of the amplified *Tm2GH*, a 3'-genome walk was performed using primers NBS-GSP1 and NBS-GSP2 (Tables 2 and 3), which were designed approximately 100 bp upstream of the REV-A, REV-B, and REV-C primers, in order to generate an overlap of 100 bp between the cloned NBS3B-like sequences and clones generated with the genome walk. Three amplicons of approximately 200 bp were obtained from vnt7014-9 and a single clone of approximately 1 kb from vnt365-1. Following cloning, sequencing, and alignment to the cloned *Tm2GH*, all four clones seemed to fit to clone *Tm2GH*-vnt8b, because the overlapping 100 bp were an exact match. To be able to subsequently amplify full-length *Tm2GH* from the *Rpi-vnt1.1* and *Rpi-vnt1.3* loci, we designed a novel reverse primer (TAA-8bR) (Tables 2 and 3) based on the alignment of the full-length *Tm2GH*-vnt8b sequence with the *Tm-2²* sequence from tomato (Fig. 2). The original TGA stop codon was not present in the *Tm2GH*-vnt8b sequence; therefore, we included the next in-frame stop-codon (TAA) which was situated 12 bp downstream.

Full-length amplification of *Tm2GH* from vnt7014-9 and vnt365-1 was subsequently pursued with high-fidelity *Pfu* Turbo polymerase using primers ATG-Tm2F and TAA-8bR. Amplicons of approximately 2.6 kb were cloned into the pGEM-T Easy vector and sequenced. Three different types of clones were obtained from vnt7014-9, one of which harbored an open reading frame (ORF) of the expected size (*Tm2GH*-vnt1b). All the clones obtained from vnt365-1 were identical to each other and contained the expected ORF. Clone *Tm2GH*-vnt1.9 was chosen together with *Tm2GH*-vnt1b for further genetic analysis.

Before targeting *Tm2GH*-vnt1b and *Tm2GH*-vnt1.9 for complementation analysis, we needed to confirm that the selected *Tm2GH* indeed mapped to the *Rpi-vnt1.1* and *Rpi-vnt1.3* loci. When tested as SCAR markers in the initial mapping populations, both markers cosegregated with resistance. Upon amplification with ATG-Tm2F and TAA-8bisR in the set of recombinants which defined the *Rpi-vnt1.1* and *Rpi-vnt1.3* loci, amplicons of the expected size were indeed only gener-

ated from late-blight-resistant recombinants, confirming that both *Tm2GH* were indeed good candidates for *Rpi-vnt1.1* and *Rpi-vnt1.3*. However, there were resistant recombinants, two in the *Rpi-vnt1.1* mapping population and one in the *Rpi-vnt1.3* mapping population, which did not give the expected polymerase chain reaction (PCR) product, suggesting that both loci could in fact harbor a tandem of two functional *R* genes.

Transient complementation

using *Agrobacterium* transient transformation assays in *N. benthamiana*.

To investigate whether *Tm2GH*-vnt1b and *Tm2GH*-vnt1.9 were functional *R* genes, we inserted them into a Gateway binary expression vector in between the regulatory elements of the *Rpi-blb3* gene (Lokossou et al. in press). *N. benthamiana* leaves were then infiltrated with *Agrobacterium tumefaciens* cultures containing the relevant clones. Two days postinfiltration, the leaves were challenged with *P. infestans* in a DLA. Two different isolates were used, EC1 and IPO-C, which show a differential response to *Rpi-vnt1.1* and *Rpi-vnt1.3*. Both genes confer resistance to IPO-C but allow for a compatible interaction in the case of EC1. Three independent transient complementation assays were carried out in triplicate with both isolates. For each replicate, leaf numbers 4, 5, and 6 (when counting from the bottom of the plant) were agroinfiltrated and subsequently challenged with *P. infestans*. At 6 days postinoculation with IPO-C, leaves transiently expressing *Tm2GH*-vnt1b or *Tm2GH*-vnt1.9 displayed an infection efficiency between 40 and 60% (Fig. 3A and B). The resistant control plants transiently expressing the functional *Rpi-sto1* gene (Vleeshouwers et al. 2008) showed a significantly lower infection efficiency ranging between 10 and 20%, (80 to 90% of the challenged leaves showed a hypersensitive response [HR]). In contrast, leaves expressing abptGH-a, a nonfunctional paralog of *Rpi-abpt* (Lokossou et al. in press) were fully susceptible (Fig. 3A and B). In the case of EC1, all agroinfiltrated leaves were susceptible except for those infiltrated with *Rpi-sto1*, which confers resistance to EC1 (Fig. 3A and C). These

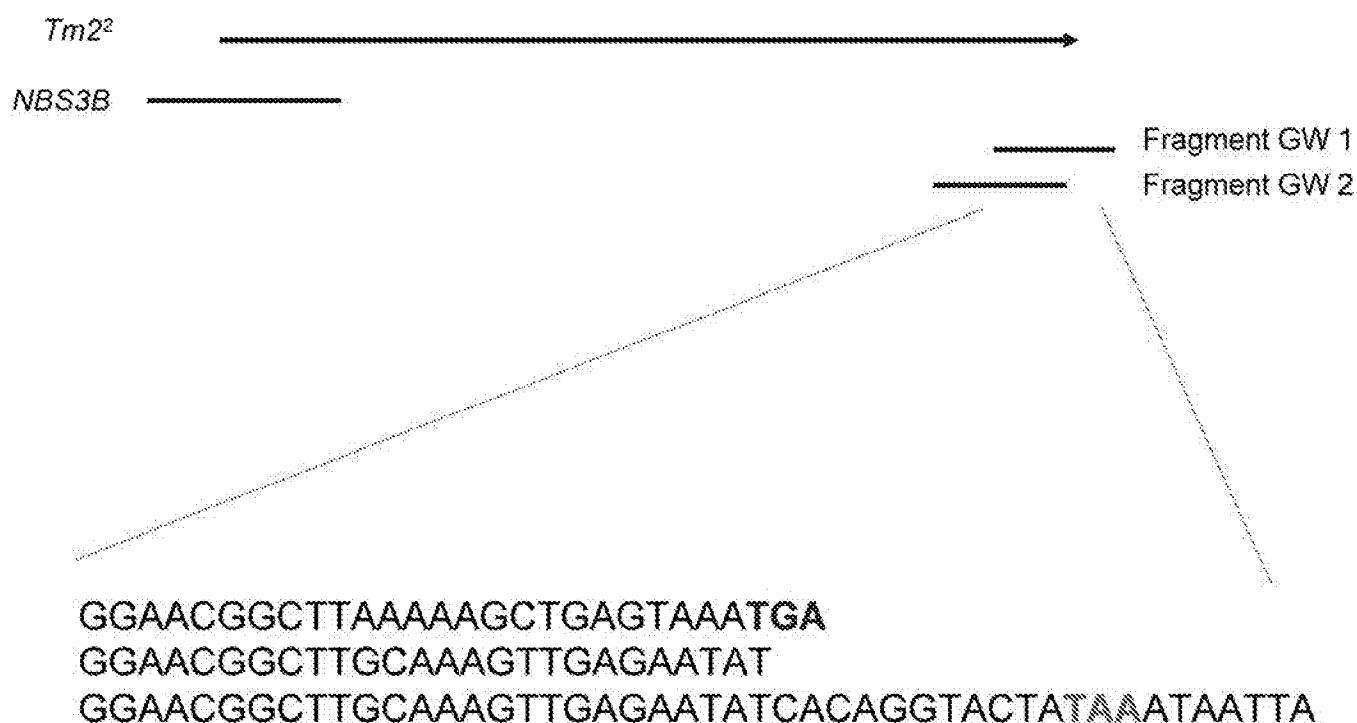


Fig. 2. Alignment of the *Tm-2²* open reading frame with two polymerase chain reaction fragments obtained with a Genome Walking kit (GW1 and GW2). The stop codons of the *Tm-2²* gene and *Rpi-vnt1.1* / *vnt1.3* are presented in blue and red, respectively.

data matched with the resistance spectrum of *Rpi-vnt1.1* and *Rpi-vnt1.3*, suggesting that *Tm2GH-vnt1b* and *Tm2GH-vnt1.9* indeed represented *Rpi-vnt1.1* and *Rpi-vnt1.3*, respectively.

Complementation analysis through stable transformation of cv. Desiree.

To confirm the results obtained with the transient complementation assays in *N. benthamiana*, the binary Gateway constructs

harboring *Tm2GH-vnt1b* and *Tm2GH-vnt1.9* were transferred to the susceptible potato cv. Desiree through *Agrobacterium*-mediated transformation. As a resistant control, we also transformed cv. Desiree with construct pSLJ21152, a binary construct harboring a 4.3-kb fragment carrying the putative *Rpi-vnt1.1* promoter, ORF, and terminator sequence (Foster et al. 2009). Primary transformants harboring the transgenes of interest were tested for resistance to *P. infestans* in DLA. Surpris-

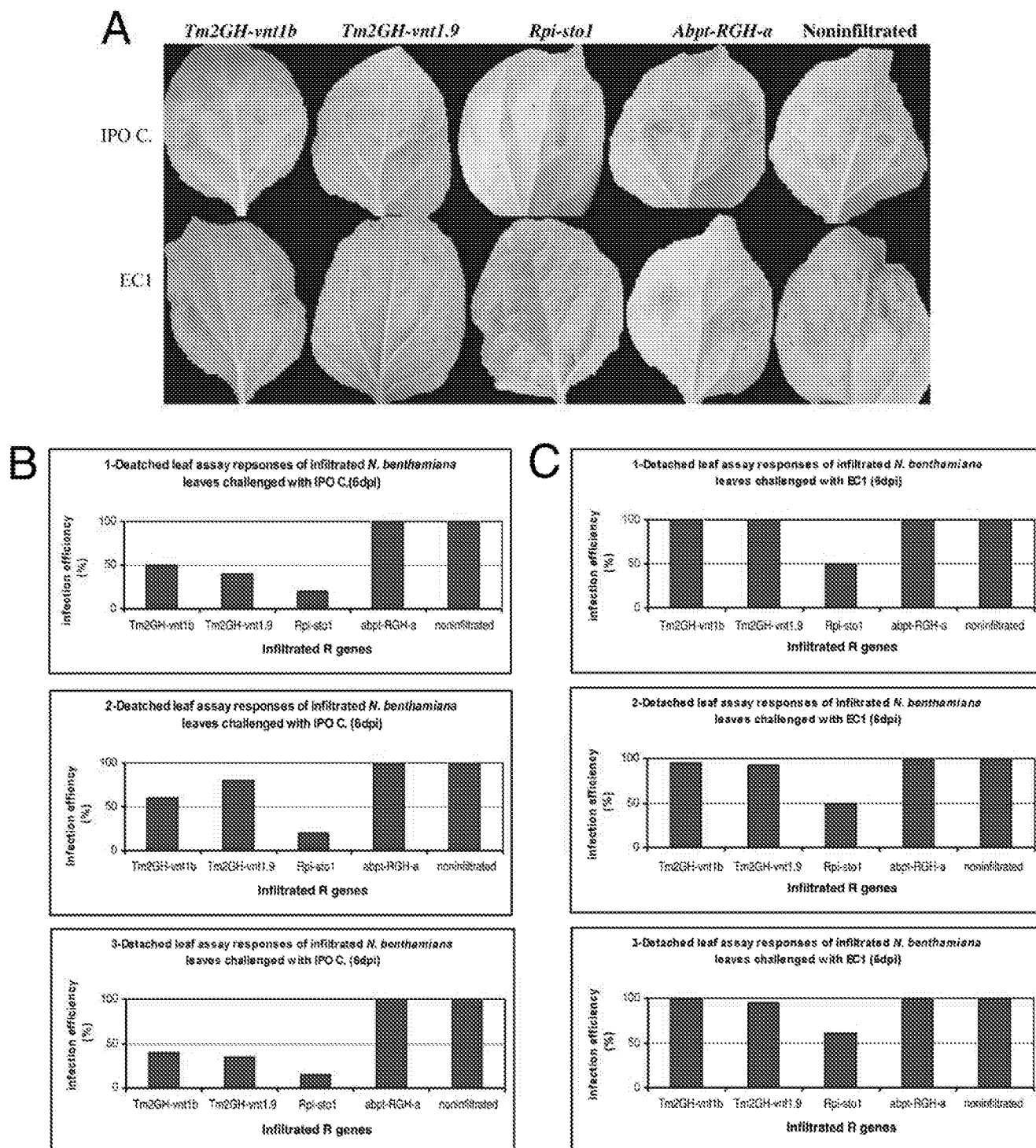


Fig. 3. Transient complementation assays in *Nicotiana benthamiana*. **A**, Typical detached leaf assay responses of *N. benthamiana* leaves infiltrated with either *Tm2GH-vnt1b*, *Tm2GH-vnt1.9*, *Rpi-sto1* (resistant control), or *abptGH-a* (susceptible control). The top row shows the response to IPO-complex (non-virulent isolate) whereas the bottom row shows the response to EC1 (virulent isolate). *Rpi-sto1* gives resistance to both isolates as expected. Pictures were taken 6 days postinoculation. **B** and **C**, Quantification of infection efficiency in the transient complementation assay as illustrated in **A**. *Tm2GH-vnt1b* and *Tm2GH-vnt1.9* showed a higher infection efficiency, ranging from 40 to 60%, than the resistant control *Rpi-sto1* construct during an incompatible interaction with IPO complex isolate.

ingly, only the genetic construct harboring the 4.3-kb *Rpi-vnt1.1* fragment was able to complement the susceptible phenotype; eight of nine primary transformants were resistant. All 22 *Tm2GH-vnt1b* and 17 *Tm2GH-vnt1.9* containing primary transformants were susceptible to *P. infestans*.

Alignment of the *Tm2GH-vnt1b* and *Tm2GH-vnt1.9* sequences to the 4.3-kb *Rpi-vnt1.1* fragment revealed the presence of an additional ATG start codon 99 nucleotides (nt) upstream from the start codon that was used as basis for the PCR-based allele-mining experiments exploiting *Tm-2²* homology. This finding, together with the negative complementation results obtained with the *Tm2GH-vnt1b* and *Tm2GH-vnt1.9* and the positive complementation result with 4.3-kb *Rpi-vnt1.1* fragment, suggest that the 5' most upstream start codon represents the actual start of the functional *Rpi-vnt1.1* and *Rpi-vnt1.3* genes.

Transient complementation assays using extended allele mining products.

In an attempt to PCR amplify the putatively full-length *Rpi-vnt1.1* and *Rpi-vnt1.3* genes from vnt7014-9 vnt365-1, respectively, genomic DNA of both genotypes was subjected to long-range PCR using the primers ATG2-Tm2F and TAA-8bR (Table 3). Amplicons of the expected size were cloned into the pGEM-T Easy vector and sequenced. Clones obtained from vnt7014-9 were all the same and identical to the corresponding sequence in pSLJ21152 (Foster et al. 2009). Clones obtained from vnt365-1 were also all identical but contained an insertion of 42 nt in the 5' extended region compared with those obtained from vnt7014-9. Both sequences were subsequently inserted into the Gateway binary expression vector in between the regulatory elements of the *Rpi-blb3* gene (Lokossou et al. in press) and targeted for transient complementation analysis in *N. benthamiana*, together with the original *Tm2GH-vnt1b* and *Tm2GH-vnt1.9* constructs and pSLJ21152. Both full-length genes showed infection efficiency comparable with the 4.3-kb genomic clone harboring the *Rpi-vnt1.1* gene (10 to 30% of the challenged leaves showed typical symptoms of late blight), whereas the shorter gene constructs again displayed significantly higher infection efficiency (65 to 75% of infected leaves), indicating that the full-length amplicons derived from vnt7014-9 and vnt365-1 represent *Rpi-vnt1.1* and *Rpi-vnt1.3*, respectively (Fig. 4A, B, and C).

Complementation analysis through stable transformation of cv. Desiree with the full-length PCR products.

To confirm the results obtained with the second transient complementation assays in *N. benthamiana*, the binary Gateway constructs harboring the full-length candidate genes *Tm2GH-vnt1.1FL* and *Tm2GH-vnt1.3FL* were transferred to the susceptible potato cv. Desiree through *Agrobacterium*-mediated transformation. Primary transformants harboring the transgenes of interest were tested for resistance to *P. infestans* in DLA. As expected, both construct were able to complement the susceptible phenotype (Fig. 5). In all, 13 of 15 and 14 of 20 primary transformants with *Tm2GH-vnt1.1FL* and *Tm2GH-vnt1.3FL*, respectively, were resistant. This result confirmed the results observed in the transient assay. Therefore, the cloned PCR products *Tm2GH-vnt1.1FL* and *Tm2GH-vnt1.3FL* were demonstrated to be *Rpi-vnt1.1* and *Rpi-vnt1.3*, respectively.

Gene structure of *Rpi-vnt1.1* and *Rpi-vnt1.3*.

The 5'-terminal structure of *Rpi-vnt1.1* and *Rpi-vnt1.3* was determined by comparing the genomic sequences with cDNA fragments generated by 5' rapid amplification of cDNA ends (RACE). For *Rpi-vnt1.1* and *Rpi-vnt1.3*, RACE identified 5' cDNA fragments comprising 5' untranslated regions of 83 and

43 nt, respectively. The ORF of *Rpi-vnt1.1* and *Rpi-vnt1.3* encode predicted peptides of 891 and 905 amino acids, respectively. In addition to the 14-amino-acid insertion in the N-terminal region of *Rpi-vnt1.3*, only two other amino acids differ between *Rpi-vnt1.1* and *Rpi-vnt1.3*. At positions 548 and 753, *Rpi-vnt1.1* harbors an asparagine and arginine residue whereas the corresponding residues in *Rpi-vnt1.3* are tyrosine and lysine, respectively (Fig. 6). However, the substituted residues have the same characteristics. Asparagine and tyrosine belong to the group of hydrophobic residues whereas arginine and lysine are positively charged residues. The protein sequences of both genes harbor several conserved motifs of the CC-NBS-LRR class of R proteins (Fig. 6). A CC domain is located in the N-terminal parts of the proteins between amino acids 1 and 183 for *Rpi-vnt1.1* and between 1 and 198 for *Rpi-vnt1.3*. In the first 183 or 198 residues, two pairs of putative heptad motifs composed of hydrophobic residues could be recognized in *Rpi-vnt1.1* and *Rpi-vnt1.3* sequences, respectively. An NBS-ARC (apoptosis, R gene products, CED-4) domain could be recognized in the amino acid stretch between residues 183 or 198 and 472 or 486, respectively (P-loop, Kinase-2, GLPL) (Van der Biezen and Jones 1998). The C terminal part of *Rpi-vnt1.1* and *Rpi-vnt1.3* comprises a series of 15 LRR motifs of irregular size that can be aligned according to the consensus sequence LxxLxxLxxLxxC/N/Sx(x)LxxLPxx (where x is any amino acid) (McHale et al. 2006). A PROSITE analysis (Hofmann et al. 1999) identified 4 N-glycosylation sites, 7 casein kinase II phosphorylation sites, 10 protein kinase C phosphorylation sites, 6 N-myristoylation sites, and 1 cAMP- and cGMP-dependent protein kinase phosphorylation site.

At the protein level, *Rpi-vnt1.1* and *Rpi-vnt1.3* share 73% amino acid sequence identity with *Tm-2²*. Interestingly, the lowest percentage of similarity was found in the LRR domain, where *Rpi-vnt1.1* and *Rpi-vnt1.3* share 62% amino acid sequence identity with *Tm-2²*. In contrast, the CC and NBS-ARC domains of *Rpi-vnt1.1* and *Rpi-vnt1.3* share 86.5% amino acid sequence identity with the corresponding regions of *Tm-2²*.

Mapping of *Tm2GH* in the ultradense genetic map of potato.

As previously described, two groups of *Tm2GH* were found based on the degree of homology to the NBS3B sequence: NBS3B-like and non-NBS3B-like. The *Rpi-vnt1.1* and *Rpi-vnt1.3* candidate genes *Tm2GH-vnt1b* and *Tm2GH-vnt1.9*, respectively, were from the NBS3B-like group and segregated with resistance. In an attempt to determine the chromosomal positions of other *Tm2GH* belonging to the non-NBS3B-like sequences group, we used the primers ATG-Tm2-F combined with REV-C to develop *Tm2GH*-specific markers in the diploid reference mapping population, a cross between the diploid potato genotypes SH83-92-488 (SH) and RH89-039-16 (RH) (SH × RH), which was previously used to generate the ultradense genetic map of potato (Isidore et al. 2003; van Os et al. 2006). Following digestion of the PCR amplicons derived from SH, RH, and five F1 progeny plants with different restriction enzymes, we identified four enzymes to generate CAPS markers that segregated from RH (*Cfr*131, *Hin*11I, *Alu*I, and *Hha*I) and one that segregated from SH (*Hpy*F10IV). Subsequent mapping of these markers in the complete SH × RH mapping population positioned them at the bottom of chromosome 9, a region of the chromosome that is similar to the late-blight R gene *Rpi-mcql* (Fig. 7A and B) (Lanfermeijer et al. 2003; Sliwka et al. 2006; Smilde et al. 2005; Trognitz and Trognitz 2005). The mapped fragments shared between 85 and 90% homology (DNA level), with *Rpi-vnt1.1*, *Rpi-vnt1.3*, and *Tm-2²* revealing the presence of a third *Tm-2²*-like cluster at

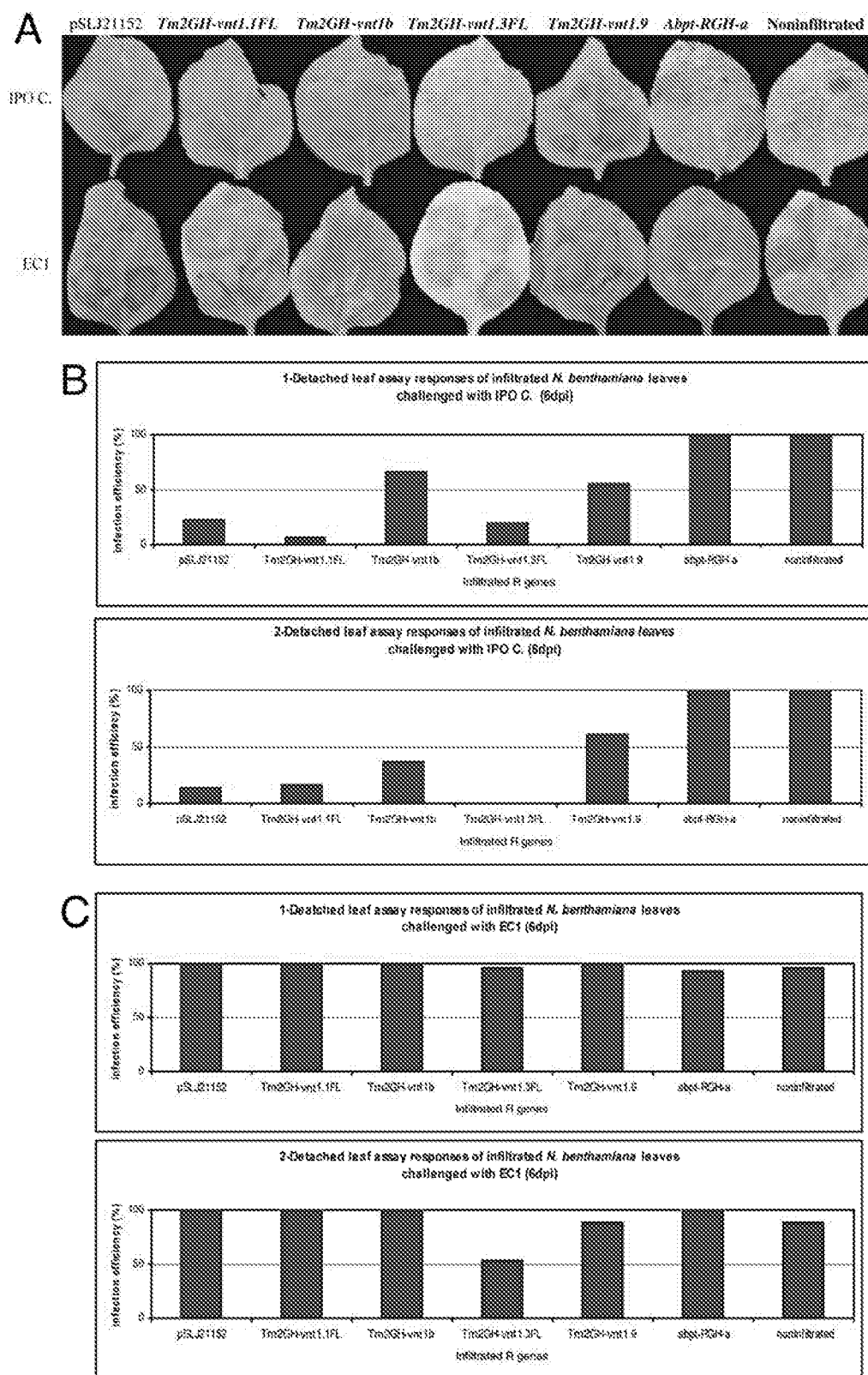


Fig. 4. Functional comparison of the N-terminal part of the truncated and full-length *Rpi-vnt1.1* and *Rpi-vnt1.3* gene constructs. **A**, Transient complementation assays in *Nicotiana benthamiana* were repeated to compare infection efficiency of the full-length candidate genes *Tm2GH-vnt1.1FL* and *Tm2GH-vnt1.3FL* with the truncated ones, *Tm2GH-vnt1b* and *Tm2GH-vnt1.9*. The construct pSLJ21152, harboring a 4.3-kb fragment carrying the putative *Rpi-vnt1.1* promoter, open reading frame (ORF), and terminator sequence was used as resistant control. The nonfunctional resistance gene *abpt-RGH-a* was chosen as susceptible control. Candidate genes *Tm2GH-vnt1.1FL* and *Tm2GH-vnt1.3FL* show an infection efficiency as low as the resistant control (construct pSLJ21152) whereas the truncated candidate genes show a higher infection efficiency. **B** and **C**, Quantification of infection efficiency in the transient complementation assay as illustrated in **A**. *Tm2GH-vnt1.1FL* (full length of *Rpi-vnt1.1* candidate gene) and *Tm2GH-vnt1.3FL* (full length of *Rpi-vnt1.3* candidate gene) showed a level of infection similar to the resistant control, pSLJ21152 (4.3-kb fragment carrying the putative *Rpi-vnt1.1* promoter, ORF, and terminator sequence).

the distal end of the long arm of the chromosome 9. Alignment of mapped fragments shows that they all cluster with *Tm2GHs* in a different group than *Rpi-vnt1.1* and *Rpi-vnt1.3*. Therefore, *Rpi-mcq1* is likely to be a *Tm2GH* as well.

DISCUSSION

Here, we report on the cloning of two putatively allelic late-blight *R* genes, *Rpi-vnt1.1* and *Rpi-vnt1.3*, from the wild potato species *S. venturii*, using a candidate gene allele-mining approach. The candidate gene family was identified through NBS profiling (van der Linden et al. 2004) in combination with a BSA approach. Sequences of NBS marker bands genetically linked to the resistance phenotypes suggested that the target genes were located in an area of the potato genome that harbored *R* gene homologs that were highly homologous to the *Tomato mosaic virus* resistance (ToMV) gene *Tm-2²* from tomato, which resides on the long arm of chromosome 9 (Ganal et al. 1989; Young et al. 1988). Putative genomic location of *Rpi-vnt1.1* and *Rpi-vnt1.3* allowed for the targeted selection of chromosome-9-specific markers (e.g., TG35 or TG551) to align both linkage groups to potato and tomato chromosome 9. Subsequently, all publicly available *Tm-2²* homologous sequences were aligned to design a set of primers with which we could PCR amplify putatively full-length gene candidates from relevant resistant genotypes. Candidate genes that were genetically closely linked to the *R* loci of interest were cloned in between the promoter and terminator sequences of the recently cloned *Rpi-blb3* gene (Lokossou et al. in press) and targeted for complementation analyses, either through *Agrobacterium* transient transformation assays (ATTA) in *N. benthamiana* or by stable *Agrobacterium*-mediated transformation of the susceptible potato cv. Desiree. Results of transient assays carried out with the initial truncated amplicons in *N. benthamiana* using the appropriate differential isolates suggested that we had indeed cloned two functional *R* genes. However, transgenic Desiree plants transformed with the same gene constructs were fully susceptible to *P. infestans*, whereas those transformed with a 4.3-kb genomic fragment, which was subcloned by Foster and co-workers from a BAC (bacterial artificial chromosome) clone that spanned the *Rpi-vnt1.1* locus (Foster et al. 2009), displayed the expected resistance spectrum. Alignment of the initial truncated amplicon sequences to those of the functional genomic fragment revealed the presence of an additional in-frame ATG start codon 99 nt upstream of the start codon that was initially used as basis for the PCR-based allele-mining experiments, suggesting that the functional *Rpi-vnt1.1* protein had an extended N-terminus of 33 amino acids compared with *Tm-2²*.

Subsequent analysis of the full-length *Rpi-vnt1.1* and *Rpi-vnt1.3* genes from the relevant parental genotypes revealed that *Rpi-vnt1.3* contained an additional 42-nt insertion in the 5' extended region compared with *Rpi-vnt1.1*. Full-length versions of both *Rpi-vnt1.1* and *Rpi-vnt1.3* were shown to confer resistance using transient and stable complementation assays. Although the 5' truncated versions of *Rpi-vnt1.1* and *Rpi-vnt1.3* were not able to confer resistance when expressed as stable transgenes, they were able to induce significant levels of resistance when expressed transiently in ATTA experiments. We speculate that absence of the N-termini of the *R* proteins may be compensated by the relatively high expression inherent to ATTA experiments. *Rpi-vnt1.1* and *Rpi-vnt1.3* belong to the CC-NBS-LRR class of plant *R* proteins. The CC domain, typically containing two or more α helices, which interact to form a super coil structure, is involved in protein-protein interactions (Liu et al. 2006). In the case of *R* proteins, it seems that the CC domain is involved in downstream signaling rather than in recognition (Van der Biezen 2002; Warren et al. 1999). Alignment of several *R* protein sequences recently led to the identification of a conserved EDVID motif among CC-NBS-LRR proteins. This motif was shown to be required for interaction between the CC domain and NBS-ARC or LRR domain (Rairdan et al. 2008). Moreover, this interaction is dependent on a wild-type P-loop motif (Moffett et al. 2002). A model proposed by Takken and co-workers (2006) suggests that the CC domain plays a role as an interactive platform for downstream signaling partners. Upon binding of ATP to the NB domain, a conformational change occurs in the CC domain, which releases the LRR signaling potential. Although the CC domain plays a role in downstream signaling, it cannot trigger the HR on its own. Another model proposed by Moffett and coworkers (2002) suggests that the CC domain may bind to an effector molecule which is released upon conformational changes within the *R* protein leading to the activation of downstream partners. The N-termini of *Rpi-vnt1.1* and *Rpi-vnt1.3* harbor four putative α helices that could interact to form two dimers. The first helix is present in the additional 99 nt upstream of the start codon that corresponds to the start codon of the *Tm-2²* gene. The truncated versions of *Rpi-vnt1.1* and *Rpi-vnt1.3* lack this first helix, thereby destabilizing a possible interaction of the CC domain with the NB or LRR domain leading to less efficient downstream signaling. Overexpression of the truncated protein may override the negative effect of destabilized intramolecular interactions and, thus, have only minor effects on the triggering of the HR upon pathogen attack. This is in line with previous findings that overexpression of functional *R* genes (e.g., *Rx*, *RPM1* and *RPS2*) using the 35S

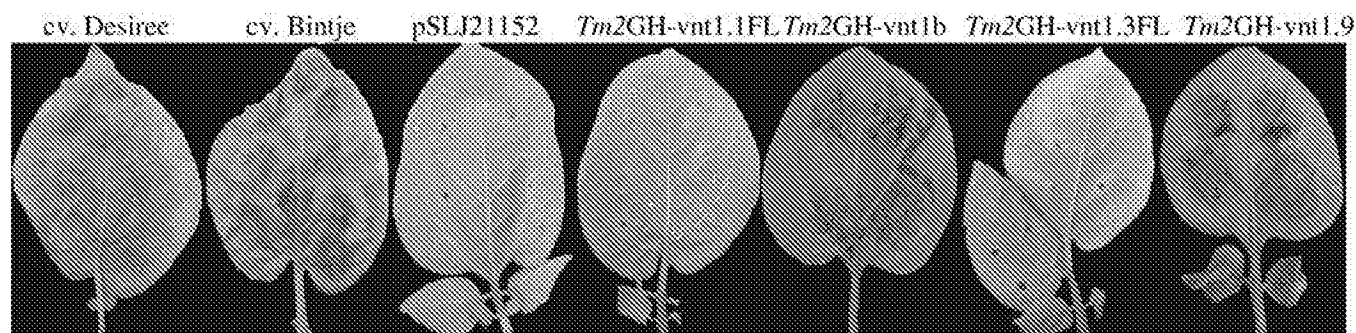


Fig. 5. Genetic complementation of cv. Desiree using full-length versions of *Rpi-vnt1.1* and *Rpi-vnt1.3* (*Tm2GH-vnt1.1FL* and *Tm2GH-vnt1.3FL*, respectively). Cv. Bintje, cv. Desiree, *Tm2GH-vnt1b*, and *Tm2GH-vnt1.9* were used as susceptible controls and pSLJ21152 (4.3-kb fragment carrying the putative *Rpi-vnt1.1* promoter, open reading frame, and terminator sequences) as resistant control. Primary transformants were challenged with IPO-complex (nonvirulent isolate) and scored 6 days postinoculation. *Tm2GH-vnt1.1FL* primary transformant -16 and *Tm2GH-vnt1.3FL* primary transformant -15 were chosen to illustrate detached leaf assay results. As expected, Bintje, Desiree, *Tm2GH-vnt1b*, and *Tm2GH-vnt1.9* showed late-blight symptoms whereas pSLJ21152 and the full-length primary transformants were fully resistant to IPO-complex.

promoter can lead to cell death responses even in the absence of the pathogen (Belkhadir et al. 2004; Tao et al. 2000).

Complementation analysis of candidate *R* genes is usually done by stable transformation of susceptible potato cultivars. This approach is time consuming, requiring several months to confirm the function of the candidate genes. In the case of an allele-mining approach, one can expect to identify many paralogous candidate resistant genes, which calls for a quick and

efficient complementation assay. Previous studies reported that resistance observed on *N. benthamiana* to *P. infestans* was mediated by the recognition of the elicitor protein INF1 (Kamoun et al. 1998). A transgenic *P. infestans* line, engineered to silence INF1 (Van West et al. 1999), was shown to be virulent on *N. benthamiana*. However, recent screens with a diverse set of *P. infestans* isolates showed that most isolates are, in fact, able to infect *N. benthamiana* (H. Rietman, I. Hein, R. G. F. Visser,

1	MNYCVYETWAVDGNKANKSTSFLLSSFGYFFLLITFRKKKFNKKLEMAHILLTAVINKS	Rpi-vnt1.3
1		Rpi-vnt1.1
1	S.....	Tm2 ²
61	IEIAGNVLFQEGTLYW LKEDIDWLQREMRH IRSYVDNAKAKEVGGDSRVKNLL KDIOQL	Rpi-vnt1.3
47		Rpi-vnt1.1
14	V....L..I...K.....A.....E.....	Tm2 ²
121	AGDV EDLLDE FLFKIQGNFFICCLFTVSFADEFAM EIEKIKRRVADID EVSTTYSITDT	Rpi-vnt1.3
107		Rpi-vnt1.1
74	D.....NY...PS.....V...I..E..N..I..	Tm2 ²
181	SNNNDDCIPLDPRPLFLNADETEVIGLEDDFNTLQAKLLDNDLPYGVVSIVGM PGLGKT	Rpi-vnt1.3
167		Rpi-vnt1.1
134	D.....VL.....I...D...M.....NQ...H.....	Tm2 ²
241	LAKMLYRHVCHQFECGGLVYVSGQQPRAGEILHDIANQVGLTEERKENLENNLSLLKIK	Rpi-vnt1.3
227		Rpi-vnt1.1
194	LIRD.....S...L.....I...QKM....D.....	Tm2 ²
301	RYVILLDGIWDVEIWDOLKLVLPEDCSKIGSRITTSRHSNVRGYIGSDFSIHVLQPLDS	Rpi-vnt1.3
287		Rpi-vnt1.1
254	...F...V.....V.....ES..L..A...E.....	Tm2 ²
361	ENSFELPTKNIENFVNON--WANASPDLVNIGRCIVER CGGIPLAIVVTAGMI PAGPTEN	Rpi-vnt1.3
347		Rpi-vnt1.1
314	DDN..S.....N..G.....E.....	Tm2 ²
420	AWNKVLESMAKIQDGGCVLALSNDLPALR PCFLY TGLYPEDHEITRAFOLTNMWIAE	Rpi-vnt1.3
406		Rpi-vnt1.1
374	G..V...A.....S.....I.....	Tm2 ²
460	KLIIVNTGNCREAESLAODVLNDLVSRNLIQVAKRTYDGRISACRINOLLNSLCVOLAKE	Rpi-vnt1.3
446		Rpi-vnt1.1
434	.F...S...R...D..E.....L.....N.....	Tm2 ²
540	SNFPKTEHYAFGDPSNVARVRRTTFYSDDNAMNEFFNLNPKPKMLRSLFCFTKDRCIFSQ	Rpi-vnt1.3
526	 N	Rpi-vnt1.1
494	A.D....G....L.....-..V.I...PS...LE...V...A..PS...N	Tm2 ²
600	MAHLNFKILQVLVVVMSQNGYQHVTFFPKNIGNMSCLRYVPLEGAIIVKLPNSIVKLECLE	Rpi-vnt1.3
586		Rpi-vnt1.1
553	..YFD...HT.....SFQAY..I..S..F...T...L...N..CG.....TR..	Tm2 ²
660	TLDIFNSSSKLP-FCVWESKILRNLCY-----TEECYCVSFASFFCRIMPPNNLQTLNW	Rpi-vnt1.3
646		Rpi-vnt1.1
613	.I...DRR.LIQ.PS.....K.....RDYQQACNS.FSI.SFY.NIYSLK.....	Tm2 ²
713	VDDKPCFEPFLHPLINLRITLCIMDVSGSTIMILSALS FV KALEVLELFFPENTSEQINL	Rpi-vnt1.3
699	 R	Rpi-vnt1.1
673	IP...F.....K..G..I..G..N..V..M..IF...L.....S..SSDP...K..	Tm2 ²
773	SSHFNIVELGLVGFSAMLLNIEAFPPNLVKLNVLGLMVDGHLAVLEKLEFLILILILNC	Rpi-vnt1.3
759		Rpi-vnt1.1
733	..Y..R..AK..H..NVNRT..A...SQS....I..T..ANFT..BYI....TF...K..KMF..	Tm2 ²
833	RHDAEKMDLSGDS---FPQLEVLVYIEDAQGLSEVTCNDMSMPKLEKFLVQGPNISPI	Rpi-vnt1.3
819		Rpi-vnt1.1
793	KYNE...A...EANGYS.....H..HSPN.....T..V.....L..T-..--FH-C	Tm2 ²
869	SLPVSEPLAKLRISQVL	Rpi-vnt1.3
875		Rpi-vnt1.1
849	GISL...K..SK	Tm2 ²

Fig. 6. Alignment of Rpi-vnt1.1, Rpi-vnt1.3, and Tm2² protein sequences. In the coiled-coil domain, four putative heptad motifs and the conserved motif EDVDID are underlined in bold or italic, respectively. Conserved motifs within the NBS-ARC (nucleotide-binding site apoptosis, *R* gene products, CED-4) domain are underlined in italic. Residues in bold represent the differences between *Rpi-vnt1.1* and *Rpi-vnt1.3* in the leucine-rich repeat domain.

E. A. G. Van der Vossen, and V. G. A. A. Vleeshouwers, unpublished results). Previous studies, focused on potato-*Phytophthora* gene-for-gene interactions, showed that co-infiltration of cognate avirulence (*Avr*) and *R* genes in *N. benthamiana* leaves leads to the triggering of an HR typical for incompatible interactions (Bos et al. 2006), indicating that all the necessary *P. infestans* resistance signaling components are present in *N. benthamiana*. We have exploited these findings to develop a transient complementation assay for late-blight *R* genes using ATTA in combination with DLA. Despite the successful use of the transient assay in the cloning of the functional *Rpi-blb1* orthologs *Rpi-sto1* and *Rpi-ptal1* (Vleeshouwers et al. 2008), the results obtained with the truncated *Rpi-vnt1.1* and *Rpi-vnt1.3* genes indicate that one needs to be careful with the interpretation of the results, and that stable transformation of a susceptible potato cultivar remains the ultimate functional assay of a gene.

Chromosomes 9 of tomato and potato seem to be hot spots for resistance because several *R* genes, conferring resistance to a broad range of pathogens, are located on this chromosome. In tomato, in addition to *Tm-2²*, four other *R* genes have been mapped on chromosome 9; *Sw5* at the distal end of the long arm, conferring resistance to *Tomato spotted wilt virus* (Spasova et al. 2001); *Ve1* and *Ve2*, conferring resistance to *Verticillium dahliae* (Simko et al. 2004); and *Frl*, conferring

resistance to *Clavibacter michiganensis* subsp. *michiganensis* (Vakalounakis et al. 1997). Interestingly, in tomato, three major QTL for resistance to *P. infestans* have been described, one of which, *Ph3*, is located at the distal end of the long arm of chromosome 9 (Chunwongse and Black 2002). In potato, *Nx-Phu*, a potato gene for hypersensitive resistance to *Potato virus X* (Tommiska et al. 1998); the late blight *R* genes *Rpi-mcq1* (Smilde et al. 2005); and *Rpi-phu1* (Sliwka et al. 2006) reside on chromosome 9. In the current study, we mapped *Tm2GHs* at the distal end of the long arm of chromosome 9, a region that coincides with the *Rpi-mcq1* locus (Fig. 6), suggesting that these two genes are likely to be *Tm2GH*. *Rpi-phu1* is also likely to be *Tm2GH* because it was mapped in the region harboring *Tm2GH* clusters. In addition to the above-described *R* loci, several QTL have also been described on chromosome 9 of potato that confer foliage resistance to *P. infestans* (*Pin9a*), resistance to the root cyst nematode *Globodera pallida* (*Gpa6*), and tuber and leaf resistance to *Erwinia carotovora* subsp. *atroseptica* (*Eca9A*) (Gebhardt and Valkonen 2001). To what extent *Tm2GHs* are also involved in quantitative resistance remains unclear.

Alignment of the *Rpi-vnt1.1* and *Rpi-vnt1.3* protein sequences to those of the currently cloned genes *R1*, *R3a*, *Rpi-blb1*, *Rpi-blb2*, and *Rpi-blb3* (Ballvora et al. 2002; Huang et al. 2005) (Lokossou et al. in press) revealed very little homol-

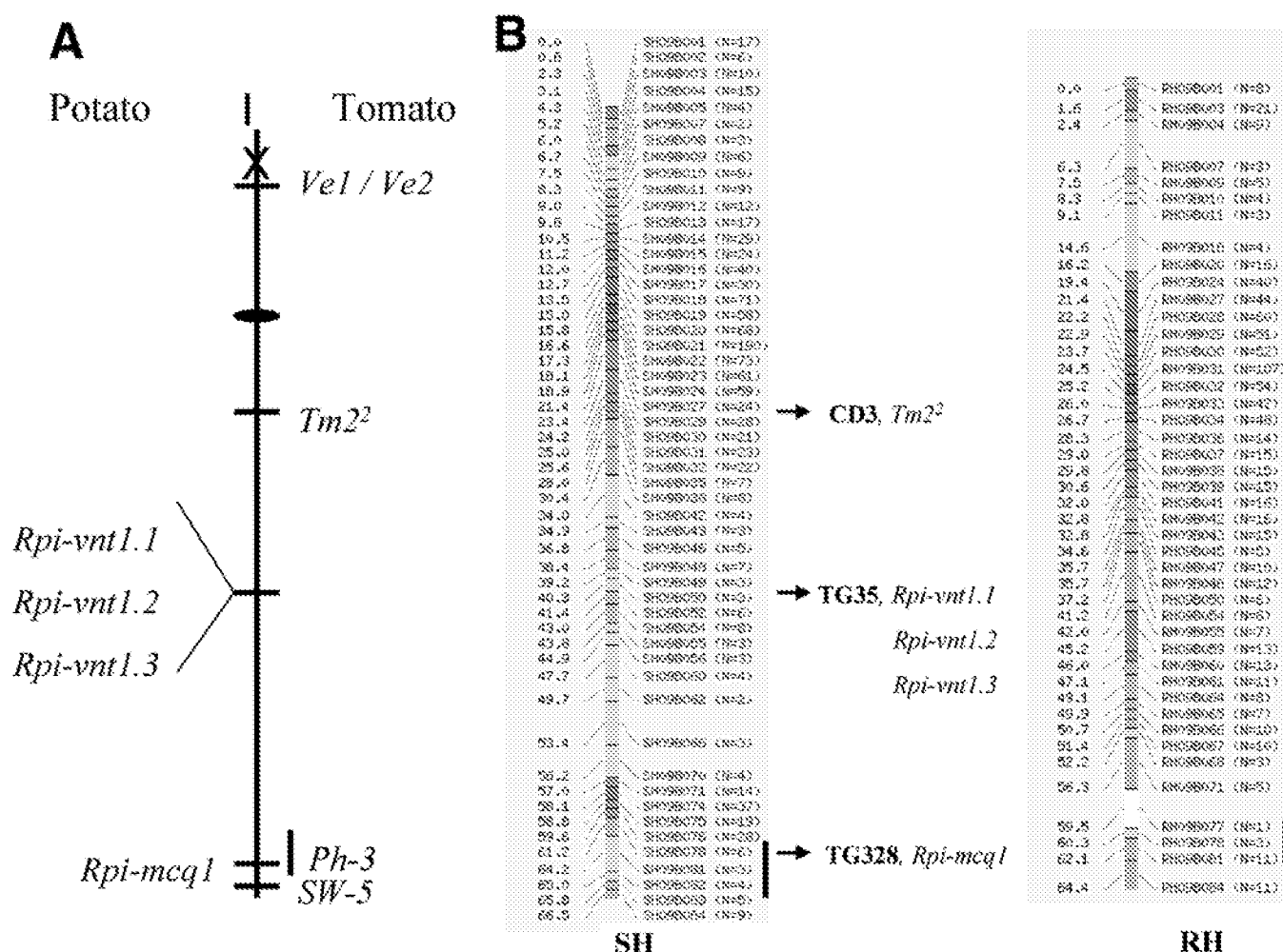


Fig. 7. Relative genetic positions of *Tm2GH* in potato and tomato. **A**, Schematic linkage maps of potato and tomato showing five resistance gene clusters. **B**, Ultra Dense potato genetic map of chromosome 9 divided in BINs from the parents SH83-92-488 (SH) and RH89-039-16 (RH). The thick vertical bars show the location of *Tm2GHs* belonging to the non-NBS3B-like group in the genome of SH and RH. The distal end of the long arm of the chromosome 9 from SH and RH harbors *Tm2GHs* in BINs 78-82 and BINs 77-81, respectively. This region coincides with the late-blight-resistant loci *Rpi-mcq1* and *Ph-3* from potato and tomato, respectively.

ogy to those genes, although all belong to the CC-NBS-LRR class of plant *R* genes. This is the first time that *Tm2GH* are shown to be involved in late-blight resistance. Alignment of the protein sequences of the chromosome-9-derived *R* genes (*Tm-2²*, *Sw5a*, *Sw5b*, *Ve1*, and *Ve2*) suggests the presence of three distinct gene families, *Tm2*-like, *Sw5*-like, and *Ve*-like. The *Ve* genes are located on the short arm of chromosome 9 whereas the *Sw5* genes are located at the distal end of the long arm (Fig. 6). *Tm-2²* and the cloned or mapped *Rpi* genes reside at three *Tm2GH* clusters that are spread across the long arm of chromosome 9 (Fig. 6), suggesting that *Tm2GH* is the predominant gene family on this chromosome arm. The overall homology between the identified *Tm2GH* ranges between 74 and 99.8% amino acid identity, suggesting that they have a common origin and that they have arisen through gene duplication events. Gene duplication and sequence exchange between *RGH* are major mechanisms that shape *R* gene diversity in plants (Kuang and Woo 2004; Meyers et al. 1999; Michelmore and Meyers 1998). Comparison of the protein sequence of *Tm-2²* with those of *Rpi-vnt1.1* and *Rpi-vnt1.3* revealed a higher homology within the CC and NB-ARC domains (86.5%) than the LRR domain (62%), which is in line with the fact that they confer resistance to completely different pathogens, ToMV and *P. infestans*, respectively, and that the LRR domain primarily determines recognition specificity and, thus, is subject to diversifying selection (Ellis et al. 2000). The functions of the CC and NBS domains, on the other hand, are likely to be more conserved, encompassing the fine tuning of intramolecular interactions (Moffett et al. 2002) and the regulation of downstream signaling (Belkadir et al. 2004; Van der Biezen 2002; Warren et al. 1999).

The rapid breakdown of the first introgressed *Rpi* genes from *S. demissum* previously stimulated breeders to reconsider their breeding goals and, subsequently, efforts toward improving late-blight resistance were focused on increasing partial resistance by using race-nonspecific sources of resistance. However, under long-day conditions, breeders using this strategy have achieved little progress, the major drawback being the correlation between foliage resistance and late foliage maturity. Nevertheless, we anticipate that breeding for late-blight resistance in potato aimed at substantially contributing to disease management requires, by one way or another, the deployment of *Rpi* genes. As more and more *Rpi* genes are identified and cloned, the chances increase that new *Rpi* genes reside at known and well-characterized loci, enabling the use of comparative genomics and, thus, the development of efficient allel mining strategies. Moreover, ongoing potato and tomato genome sequencing projects by international consortia are providing a (complete) survey of the distribution of *R* gene clusters in the family *Solanaceae*, enabling even faster cloning of *Rpi* genes. Challenges that remain are how to predict *Rpi* gene durability and how to introduce durable combinations of *Rpi* genes into existing and future cultivars in the most efficient and sustainable manner. We believe that knowledge of effector diversity may provide clues about the putative durability of *Rpi* genes (Birch et al. 2008; Vleeshouwers et al. 2008). The recent discovery of a common RXLR motif in oomycete Avr effector proteins (Birch et al. 2006; Rehmany et al. 2005) promises to accelerate the discovery and functional characterization of late-blight *Rpi* genes and of *P. infestans* Avr genes and, thus, the engineering of durable late-blight-resistant potato cultivars in the future. Efficient stacking of *Rpi* genes from one or several species is essential; however, in practice, this will enlarge the linkage drag problems considerably. Their introduction by genetic modification is a much more efficient way to improve resistance in one step and in a short period. It can even be applied to existing cultivars with a long history of safe use. Cur-

rently, *Rpi* genes of natural origin derived from sexually compatible species, so-called cisgenes (Schouten et al. 2006), can be introduced using marker-free transformation systems (Vetten et al. 2003), leading to cisgenic plants with only the gene or genes of interest and without linkage drag (Jacobsen and Schouten 2007). It implies an environmental benefit and will, therefore, hopefully meet with consumer acceptance.

MATERIALS AND METHODS

Plant material.

Accessions of *S. venturii* were provided by the Centre of Genetic Resources (CGN) in Wageningen, The Netherlands. Following screening with *P. infestans*, resistant genotypes vnt367-1 and vnt367-1 from accessions CGN18108 and CGN18000, respectively, were used to generate inter- or intra-specific mapping populations. The *Rpi-vnt1.1* mapping population 7698 was generated by crossing vnt7014-9 (resistant) (vnt367-1 [resistant] × vnt366-8 [susceptible]) × nrs735-2 (susceptible). The *Rpi-vnt1.3* mapping population 7663 was generated by crossing the resistant plant vnt365-1 (resistant) × nrs735-2 (susceptible). The genes observed in CGN18108 and CGN18000 (initially called *Rpi-okal* and *Rpi-nrs1*, respectively) (*unpublished results*) were named after the species *S. okadae* and *S. neorossi*. However, DNA fingerprinting of a wide range of wild *Solanum* spp. showed that the GenBank accessions CGN18108 and CGN18000 clustered with other accessions of *S. venturii* (Jacobs 2008; Jacobs et al. 2008). Morphological inspection of the CGN18108 and CGN18000 accessions confirmed that the labels *S. okadae* and *S. neorossi* should indeed be replaced with the label *S. venturii*. Furthermore, all available GenBank accessions of *S. okadae* and *S. neorossi* were susceptible and did not amplify a PCR product with our *Rpi-vnt1.1*- or *-1.3*-specific primers (M. A. Pel, *unpublished results*).

Disease assays and agroinfiltration.

DLA on the *Solanum* spp. were carried out as described by Vleeshouwers and associates (1999). Leaves were inoculated with 10-μl droplets of inoculum (5 × 10⁴ zoospores/ml) on the underside and incubated at 15°C for 6 days in a climate chamber with a photoperiod of 16 and 8 h, day and night, respectively. At 6 days postinoculation, leaves showing sporulation were scored as susceptible whereas leaves showing no symptoms or necrotic lesions were scored as resistant.

ATTA were performed in *N. benthamiana* followed by DLA using appropriate *P. infestans* isolates. Four-week-old plants were infiltrated with a solution of *A. tumefaciens* COR308 (Hamilton and Frary 1996) harboring putative *R* gene candidates. Agroinfiltration of the lower side of *N. benthamiana* leaves was carried according to Van der Hoorn and associates (2000) using a optical density at 600 nm of 0.1. Two days post-infiltration, a DLA was performed as mentioned above. The phenotyping was done between 4 and 7 days postinoculation.

In ATTA assays, for each construct tested, three leaves of three different *N. benthamiana* plants were infiltrated with either *Tm2GH-vnt1b* (truncated *Rpi-vnt1.1* candidate gene), *Tm2GH-vnt1.9* (truncated *Rpi-vnt1.3* candidate gene), *Rpi-sto1* (resistant control), or *abpt-RGH-a* (nonfunctional resistance gene). Assays were repeated three times. Infection efficiency was quantified by scoring 36 inoculation spots for each infiltrated constructs (three times one plant with three infiltrated leaves each harboring four inoculated spots).

Marker development.

Candidate gene markers were developed through NBS profiling as described by Van der Linden and associates (2004). Templates were generated by restriction digestion of genomic

DNA using the restriction enzymes *MseI*, *HaeIII*, *AluI*, *RsaI*, or *TaqI*. Adapters were ligated to restriction fragments. PCR fragments were generated by radioactive-labeled primers (nbs1, nbs2, nbs3, nbs5a6, or nbs9) designed on conserved domains of the NBS domain (P-loop, Kinase-2 and GLPL motifs) (Calenge et al. 2005; Syed and Srensen 2006). Additional markers from appropriate chromosomal positions were selected from the SGN database and subsequently developed into polymorphic markers in each of the relevant mapping populations.

PCR amplification of candidate *R* genes.

Long-range PCR with Taq-polymerase or *Pfu* Turbo polymerase in a 50- μ l reaction mixture was prepared containing 50 ng of genomic DNA, 1 μ l of the forward primer (10 μ M), 1 μ l of the reverse primer (10 μ M), 0.8 μ l of dNTPs (5 mM each), 5 μ l of 10 $\times$ buffer, 5 units of Taq polymerase (Perkin Elmer, Norwalk, CT, U.S.A.), or 1 μ l of *Pfu* Turbo (Invitrogen, San Diego, CA, U.S.A.). The following PCR program was used: 94°C for 3 min, 94°C for 30 s, 55°C for 30 s, 72°C for 4 min, and 72°C for 5 min during 29 cycles.

Genome walking.

Marker sequences were extended by cloning flanking DNA fragments with the Clontech Genome Walker kit (Clontech Labs, Inc., Palo Alto, CA, U.S.A.) according to the manufacturer's instructions using a blunt adapter, comprising the complementary sequences 5'GTAATACGACTCACTATAGGGCA CGCGTGGTCGACGGCCCGGGCTGGA and 5'PO4-TCCA GCCC and the adapter-specific primers AP1 (TAATACGACT CACTATAGGGC) and AP2 (5'ACTATAGGGCAGCGTG GT). A simultaneous restriction ligation was performed followed by two rounds of PCR. A 50- μ l restriction-ligation (RL) mixture was prepared containing 250 ng of genomic DNA, 5 units of blunt cutting enzyme (*Bsh1236I*, *AluI*, *DpnI*, *HaeIII*, *RsaI*, *HincII*, *DraI*, *ScaI*, *HpaI*, or *SspI*), 1 μ l of genome walker adapter (25 μ M), 10 mM ATP, 10 μ l of 5 $\times$ RL buffer, and 1 unit of T4 DNA ligase (1 U/ μ l) (Invitrogen). The digestion mix was incubated at 37°C for 3 h. Samples were diluted 50 times prior to PCR. For the first PCR round, a 20- μ l reaction mixture was prepared containing 5 μ l of diluted RL DNA, 0.6 μ l of specific forward primer 1 (10 μ M), 0.6 μ l of AP1 (10 μ M), 0.8 μ l of dNTPs (5 mM each), 2 μ l of 10 $\times$ buffer (Perkin Elmer), and 5 units of Taq polymerase (Perkin Elmer). The first PCR was performed using the following cycle program: 30 s at 94°C as the denaturation step, 30 s at 56°C as the annealing step, and 60 s at 72°C as the extension step; 35 cycles were performed. A second PCR using the same conditions as the first one was performed using specific primer 2 and AP2 and 5 μ l of 50 $\times$ diluted product from the first PCR. The second PCR product (5 μ l) was checked on gel (1% agarose) and the largest amplicons were cloned into the pGEM-T Easy Vector from Promega (Madison, WI, U.S.A.) and sequenced.

Gateway cloning of candidate *R* genes into a binary expression vector.

The Gateway cloning technique was used according to the manufacturer's instructions to efficiently clone candidate genes together with appropriate promoter and terminator sequences into the binary Gateway vector pKGW-MGW. In plasmid pKGW, the gateway cassette was exchanged against a multiple gateway cassette amplified from pDESTr4r3, resulting in pKGW-MGW. In this study, we used the promoter and terminator of *Rpi-blb3* (Lokossou et al. in press), which were cloned into the Gateway pDONR vectors pDONRP4P1R and pDONRP2RP3, respectively, generating pENTR-B1b3P and pENTR-B1b3T. PCR amplicons generated with *Pfu* Turbo polymerase were cloned into pDONR221 generating pENTR-

RGH clones and, subsequently, cloned together with the *Rpi-blb3* promoter and terminator fragments into pKGW-MGW using the multiple Gateway cloning kit (Invitrogen). The pENTR clones were made by carrying out a BP-Reaction II overnight. DH5 α competent cells (Invitrogen) were transformed by heat shock with 5 μ l of the BP Reaction II mixture. Cells were selected on Luria-Bertani (LB) medium containing of kanamycin at 50 mg/ml. Colonies were checked for the presence of the relevant inserts by colony PCR. DNA of appropriate pENTR clones was extracted from *Escherichia coli* and used to perform a multiple Gateway LR cloning reaction to generate the final binary expression clones (pVNT1 and pNRS1). DH5 α competent cells (Invitrogen) were transformed by heat shock with 5 μ l of the LR reaction mixture. Cells were selected on LB medium containing spectinomycin at 100 mg/ml. Colonies were checked by PCR for the presence of the correct inserts. Positive colonies were grown overnight in LB medium supplemented with spectinomycin at 100 mg/ml to extract the final expression vector. The final expression vector was transferred to *A. tumefaciens* COR308 through electroporation. Colonies were selected on LB medium supplemented with spectinomycin at 100 mg/ml and tetracycline at 12.5 mg/ml overnight at 30°C.

Stable transformation.

Binary vectors carrying *Tm2GH-vnt1b*, *Tm2GH-vnt1.9*, pSLJ21152, *Tm2GH-vnt1.1FL*, or *Tm2GH-vnt1.3FL* were used for *Agrobacterium*-mediated transformation in strain COR308. Internodia with a size of 2 to 5 mm were cut from explants of cv. Desiree and transferred to R3B-medium plates containing two sterile filter papers. The next day, internodia were incubated for 5 to 10 min in the bacteria solution, dried, transferred back to R3B plates, and placed at 24°C with a light period of 16 h. Two days later, internodia were transferred to selective medium containing kanamycin (100 mg/liter), zeatine (1 mg/liter), claforan (200 mg/liter), and vancomycin (200 mg/liter). Every 2 weeks, internodia were transferred to fresh selective medium. Green shoots emerging from calli were harvested and transferred to MS30 medium supplemented with kanamycin (100 mg/liter) to regenerate plantlets.

5' RACE.

The GeneRacer Kit from Invitrogen was used according to the manufacturer's instructions to determine the 5' terminal structure of *Rpi-vnt1.1* and *Rpi-vnt1.3*. Two gene-specific primers (GSP1-5race and GSP2-5race) (Table 3) were designed to perform a nested PCR using cDNA template from relevant parental genotypes and transgenic plants and High Fidelity Platinum *Taq* DNA Polymerase (HT Biotechnology Ltd., Cambridge). PCR products were cloned into the pGEM-T Easy Vector (Promega) and sequenced.

Sequencing.

Cloned fragments or PCR products generated with either Taq polymerase (Perkin Elmer) or *Pfu* Turbo polymerase (Invitrogen) were sequenced as follows: 10 μ l of sequencing reaction mixture was made using 5 μ l of PCR product or 5 ng of plasmid, 3 μ l of buffer, 1 μ l of DETT (Amersham), and 1 μ l of forward or reverse primer. The PCR program used was 25 cycles of 94°C for 20 s, 50°C for 15 s, and 60°C for 1 min. The sequences were generated on ABI 3730XL sequencers.

Mapping of *Tm2GHs* in the ultradense genetic map of potato.

The Ultra Dense genetic map of potato, which was previously made with a mapping population consisting of 130 F1 progeny derived from SH $\times$ RH (Roupe van der Voort et al.

1997), was used to map *Tm2GH*. Genomic DNA from the parents (SH and RH) were PCR amplified using the primers ATG-Tm2-F and Rev-C (Tables 2 and 3). Amplification products were digested with restriction enzymes to detect polymorphisms between the parents. A subpopulation of 50 F1 individuals was subsequently analyzed through PCR amplification and appropriate restriction digestion. PCR reaction mixtures (20 µl) contained 50 ng of genomic DNA, 1 µl of the forward primer (10 µM), 1 µl of the reverse primer (10 µM), 0.8 µl of dNTPs (5 mM each), 5 µl of 10× buffer, and 5 units of Taq polymerase (Perkin Elmer). The following PCR program was used: 94°C for 3 min, 94°C for 30 s, 55°C for 30 s, 72°C for 4 min, and 72°C for 5 min during 29 cycles. PCR product (5 µl) was digested at the required temperature for 3 h with the appropriate restriction enzymes. Segregating polymorphisms were mapped in the ultradense potato map relative to 10,000 amplified fragment length polymorphism markers.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- Andreas Untergassen Lab's BP-Reaction protocol webpage:
www.untergassen.com/lab/protocols/bp_gateway_reaction_ii_v1_0.htm
- European Union project FAIR5-PL97-3565 ultra-dense genetic map:
www.plantbreeding.wur.nl/potatomap

Identification of RFLP markers linked to the *H1* gene conferring resistance to the potato cyst nematode *Globodera rostochiensis*

OMAIRA PINEDA, MERIDETH W. BONIERBALE, AND ROBERT L. PLAISTED

Department of Plant Breeding and Biometry, Cornell University, Ithaca, NY 14853, U.S.A.

BILL B. BRODIE

United State Department of Agriculture - Agricultural Research Service, Department of Plant Pathology, Cornell University, Ithaca, NY 14853, U.S.A.

AND

STEVEN D. TANKSLEY

Department of Plant Breeding and Biometry, Cornell University, Ithaca, NY 14853, U.S.A.

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The *H1* gene from *Solanum tuberosum* ssp. *andigena* confers high levels of resistance to the potato cyst nematode *Globodera rostochiensis* and is used extensively in potato breeding. Using a dihaploid segregating population, a search was conducted for linkage between this gene and markers on the potato/tomato RFLP map. A total of 60 RFLP markers covering the entire genome were screened on bulk resistant and susceptible segregants. Linkage was indicated for eight markers on chromosome 5. Individual plant analysis placed the closest marker, *CD78*, at a maximum map distance of 2.7 cM from *H1*. A molecular marker for the *H1* should be useful both as a correlative screening tool for incorporation of resistance into new cultivars and as starting point for map-based cloning of this important gene.

Key words: *Solanum tuberosum*, potato, resistance genes, *Globodera rostochiensis*, *H1*, RFLP, cyst nematodes.

PINEDA, O., BONIERBALE, M. W., PLAISTED, R. L., BRODIE, B. B., et TANKSLEY, S. D. 1993. Identification of RFLP markers linked to the *H1* gene conferring resistance to the potato cyst nematode *Globodera rostochiensis*. *Genome*, **36** : 152-156.

Le gène *H1* de *Solanum tuberosum* ssp. *andigena* confère des niveaux élevés de résistance aux kystes causés par un nématode, le *Globodera rostochiensis*, et il est grandement utilisé dans les programmes d'amélioration de la pomme de terre. Utilisant une population dihaploïde en ségrégation, une recherche a été entreprise pour détecter des linkages entre ce gène et des marqueurs sur la carte des PLFR de pomme de terre/tomate. Un total de 60 marqueurs, couvrant le génome entier, a été détecté chez les ségrégeants regroupés en sensibles et résistants. Le linkage sur le chromosome 5. a été révélé par huit marqueurs. L'analyse des plantes individuelles a permis de situer le marqueur *CD78*, le plus rapproché du gène *H1*, à une distance cartographique maximale de 2,7 cM. Un marqueur moléculaire pour le chromosome *H1* devrait s'avérer utile à la fois comme outil corrélatif de sélection pour l'incorporation de résistance dans de nouveaux cultivars et comme point de départ de clonage, d'après la cartographie de ce gène important.

Mots clés : *Solanum tuberosum*, pomme de terre, gènes de résistance, *Globodera rostochiensis*, gène *H1*, PLFR, kystes de nématode.

[Traduit par la rédaction]

Introduction

The potato cyst nematodes *Globodera rostochiensis* and *G. pallida* cause serious problems in potato growing areas worldwide (Ross 1986). Yield losses in countries with general infestation average approximately 9%, but left uncontrolled, they are capable of causing 100% crop loss (Brodie 1984; Brodie and Mai 1989). Potato cyst nematodes originated in the Andes of South America and were introduced to Europe in the 1850s with potato collections used in breeding for late blight resistance. Since then this pathogen has spread to several major potato growing areas around the world (Evans and Brodie 1980).

Five pathotypes, *Ro1* to *Ro5*, have been distinguished within *G. rostochiensis*, which differ in their ability to multiply on certain tuberbearing *Solanum* clones and hybrids (Kort *et al.* 1977; Canto-Saenz and Scurrah 1977). Resistance to pathotypes *Ro1*, *Ro2*, and *Ro4* has been shown to be mediated by single dominant genes, including *H1* (Huijsman 1955), *H2* (Dunnet 1963), *A* and *B* (Huijsman

1960), and *Fa* and *Fb* (Ross 1986). The gene designated *H1*, detected from the accession CPC1673 of *Solanum tuberosum* ssp. *andigena*, confers high levels of resistance to pathotype *Ro1* (often referred to as the golden nematode). Later it was demonstrated that this gene also confers resistance to *Ro4* (Ross 1986).

H1 is active against the feeding sites of developing females of *G. rostochiensis* (Piegat and Wilski 1965; Hoopes *et al.* 1978). The female larvae are most affected by insufficient feeding and remain small, contain fewer eggs, and often die (Ross 1986). Mullin and Brodie (1988a, 1988b) also reported reduction in the infective potential of second stage juveniles (J2s) emerging from a resistant host, relative to those emerging from a susceptible host. In addition, the number of males and females that reach maturity is reduced in the resistant host.

Resistant cultivars are the most efficient and effective means of crop protection from the golden nematode. The level of crop protection provided by *H1* is equivalent to that

obtained by soil fumigation. As a result, several countries in Europe and in North America have developed one or more high yielding commercial cultivars containing the *H1* gene. In the United States *H1* is the only source of resistance to *G. rostochiensis* in commercial production and more than 20 cultivars released in the last 20 years carry this gene. Resistance conferred by *H1* appears to be stable (Brodie 1984), since potato cultivars that carry this gene have been grown for many years in infested research plots and farmers fields, and new races of *G. rostochiensis* have not been detected (Mullin and Brodie 1988b).

Over the past few years linked molecular markers have been identified for genes controlling resistance to several plant diseases (Young *et al.* 1988; Martin *et al.* 1991). These markers provide a powerful tool for indirect selection in breeding programs and open the possibility of cloning resistance genes via chromosome walking. In potato, RFLP markers linked to another gene controlling resistance to *Globodera rostochiensis* (Barone *et al.* 1990), as well as genes for resistance to PVX (Ritter *et al.* 1991) and *Phytophthora infestans* (Leonards-Schippers *et al.* 1992), have been identified using diploid segregating populations. In this paper we present the use of bulked DNA analysis described by Micheltore *et al.* (1991) in a dihaploid population derived from a resistant tetraploid cultivar for the detection of RFLP markers linked to the *H1* gene.

Materials and methods

Plant material

A dihaploid population ($2n = 2x = 24$) was used for mapping *H1*. This population was previously derived via parthenogenesis from 'Atlantic', which is resistant to *G. rostochiensis* and known to be simplex for the *H1* gene. Tuber progeny of this population were kindly provided by Dr. S. Peloquin, University of Wisconsin. For confirmatory results a second population ($2n = 4x = 48$) from the Cornell University breeding program was used. This was a cross between 'Steuben' $\times$ 'Monona'. The female parent, 'Steuben', is resistant to *G. rostochiensis* and the pollen parent is susceptible. The resistant parent was a selection from a simplex by nulliplex cross and is simplex for *H1*.

Screening for resistance

One hundred and one clones from the dihaploid population and 50 clones from the tetraploid population were screened for resistance to the golden nematode by staff of the Federal Nematology Laboratory at Cornell University. The procedure used is described in Brodie *et al.* (1991). Individual tubers were planted in 5-cm clay pots containing a sterile 1:1, soil - sand, mixture inoculated with enough *G. rostochiensis* (*Ro1*) cysts to provide 5000 viable eggs per pot. For initial evaluations two pots of each clone were planted and for confirmatory evaluation two more pots were planted in a separate screening trial. The pots were maintained at 21–24°C, and 8 weeks after emergence the plants were removed from the pots and their roots examined for number of cysts. In this test a clone was considered resistant if it allowed the development of five or fewer cysts. Clones bearing more than five cyst were considered susceptible (Plaisted *et al.* 1984). Subsequent to the resistance screening, 37 clones from the dihaploid population and 30 from the tetraploid were selected for mapping *H1*.

DNA isolation, restriction digests, electrophoresis, and blotting

DNA was extracted from each of the potato clones as described by Bernatzky and Tanksley (1986), except that sodium bisulfate was used instead of mercaptoethanol. Two pools of DNA were made to represent resistant and susceptible genotypes by combining DNA from 20 resistant (pool R) and 17 susceptible (pool S)

dihaploid clones. DNA from the pools, and also from each individual clone, was digested with five different restriction enzymes: *EcoRI*, *EcoRV*, *HindIII*, *DraI*, and *XbaI*. Eight micrograms of digested DNA were loaded and separated on 0.9% agarose gels. Electrophoresis and Southern blotting were performed as described by Bernatzky and Tanksley (1986) with two modifications of the above protocols: the substitution of Hybond N+ filters for Zetabind and the use of capillary blots instead of dry blotting.

Two different sets of replicate filters were made for the dihaploid progeny. The first set consisted of bulked DNA from the resistant and susceptible genotypes digested with the five different restriction enzymes. The second set consisted of individual DNA from each of the 37 selected clones digested with the same restriction enzymes. For the tetraploid progeny the filters consisted of the parental DNA of the cross as well as DNA from pools of 15 susceptible and 15 resistant genotypes, digested with the same restriction enzymes.

Probes

The filters were probed with cDNA and genomic clones from the tomato/potato RFLP maps (Bonierbale *et al.* 1988; Tanksley *et al.* 1992). Markers from 20-cM intervals throughout the potato genome were applied in succession to the filters containing pools of DNA from the resistant and susceptible dihaploid plants. Putatively positive markers identified by this bulked segregant analysis were hybridized against filters containing bulked DNA from the tetraploid population as well as the individual (dihaploid) DNA filters.

Results and discussion

Segregation for resistance

Of the 101 dihaploid progeny, 51 were found to be resistant to *Globodera rostochiensis* pathotype *Ro1*, 38 clones were susceptible, and 13 plants were not tested because of poor growth. This ratio fits the expectation for a 1:1 segregation of resistance from the simplex maternal parent ($\chi^2 = 1.899$; $p < 0.25$). Haploid families obtained by parthenogenesis (Hougas *et al.* 1964; Taylor 1978) are expected to be a random sample of the female parental gametes. 'Atlantic' is understood to be simplex (*Hhhh*) for the dominant *H* allele conferring resistance and to produce gametes *Hh* and *hh* in a 1:1 ratio. This expectation was fit in our observation of a 1:1 segregation for resistant-susceptible dihaploid progeny.

RFLP analysis

Sixty probes from throughout the genome were hybridized with the bulked DNA filters from the dihaploid population. Polymorphism between the resistant and the susceptible pools (digested with *HindIII* and *XbaI*) was initially detected with *TG69* mapping to chromosome 5. Flanking markers close to *TG69* were then used to hybridize against the same filters to confirm the linkage. Polymorphism between the two pools was also detected with the following markers from chromosome 5: *CT172*, *TG351*, *TG60*, *CD78*, *TG413*, *CT80A*, and *CD74A* (Fig. 1A). Markers from the remaining 11 chromosomes failed to detect polymorphism between the resistant and susceptible pools (Fig. 1B).

The putatively positive markers identified by this bulked segregant analysis were then verified on filters containing digested DNA of 37 individual resistant and susceptible plants from the dihaploid population (Table 1). The *CD78* marker was found to be tightly linked with *H1*. Cosegregation between the nematode resistant phenotype and this marker is shown in Fig. 2 and Table 2. One individual (plant 64) was classified as resistant in the nematode test but

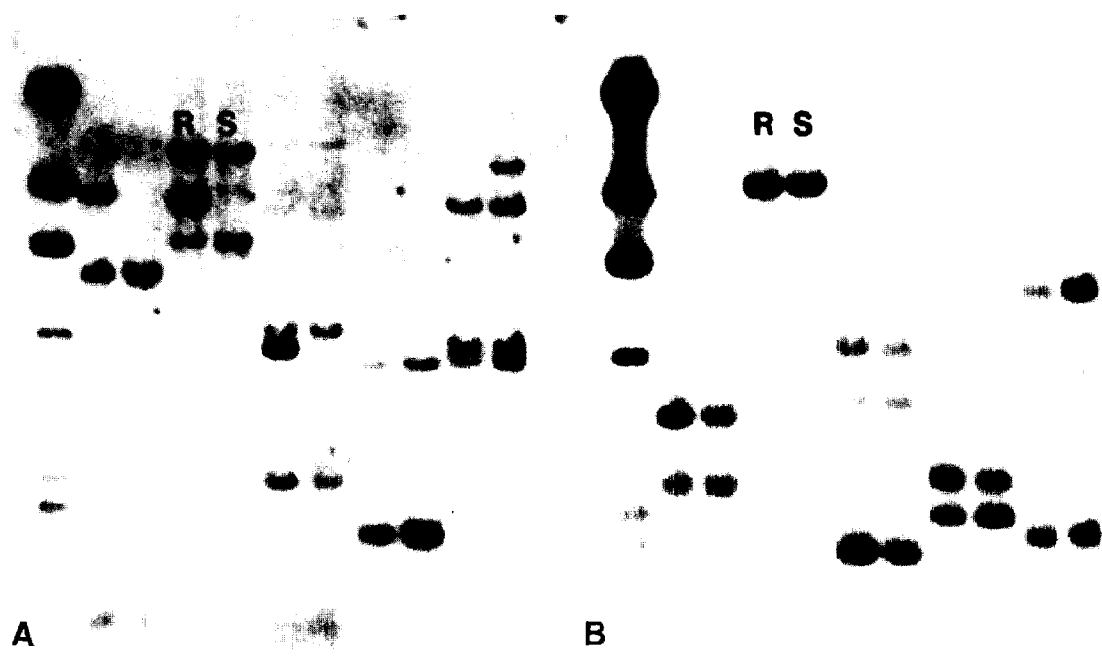


FIG. 1. Autoradiograms of bulked segregant analysis of cv. Atlantic haploid (2X) progeny. (A) Polymorphic probe *CD78*, chromosome 5. (B) Monomorphic probe *TG61* chromosome 7. Paired lanes are digested with different restriction enzymes in the order *EcoRI*, *EcoRV*, *HindIII*, *DraI*, and *XbaI*. Each pair of lanes consists of a resistant (left) and a susceptible (right) bulked DNA sample.

TABLE 1. Recombination frequencies observed in the dihaploid population between chromosome 5 markers and *H1*

Marker	Recombination/total	Map distance
CD78	1/37	2.7
TG60	2/37	5.4
TG413	2/37	5.4
CT80A	2/36	5.5
TG69	3/37	8.1
CD74A	3/37	8.1
TG351	3/34	8.8
CT172	6/36	16.6

TABLE 2. Contingency table showing the distribution of the genotype at *CD78* and the resistance reaction against Ro1 in the dihaploid population

	Ro1 reaction		
	R	S	Total
0	1	17	18
1	19	0	19
Total	20	17	37
χ^2 (for independent assortment) = 33.20			$p < 0.005$

NOTE: R, resistant; S, susceptible; 0, absence of fragment; 1, presence of fragment.

showed the RFLP genotype of susceptible individuals. One recombinant among 37 individuals would indicate a map distance of 2.7 cM. When the same 37 plants were hybridized against the other seven polymorphic markers from chromosome 5, this putative recombinant individual always showed the susceptible RFLP genotype. Since this plant was not in the second screening, we hold the possibility that it was a susceptible plant that escaped inoculation. In the case of a single misclassification, *H1* cosegregates perfectly with *CD78*. Alternately, the map distance is 2.7 cM in the diploid population.

To confirm these results in the tetraploid population, the bulk DNA filters from 'Steuben' $\times$ 'Monona' were hybridized against the polymorphic markers implicated in the dihaploid analysis. Polymorphisms between the resistant parent ('Steuben') and the susceptible ('Monona'), as well as between the resistant and the susceptible pools, were detected with *CD78* (Fig. 4), *TG60*, *TG69*, and *TG413*, confirming the linkage of the *H1* gene to the molecular markers in both diploid and tetraploid populations.

Relationship of H1 to other genes conferring resistance to the golden cyst nematode

In addition to the *H1* gene from *S. tuberosum* ssp. *andigena*, three other genes (*Fa*, *Fb*, and *Fc*) have been described that also confer necrotic or hypersensitive resistance to different pathotypes of *G. rostochiensis*. These genes from *S. spegazzinii* have been shown to be simply inherited (Momeni *et al.* 1969) but often these major genes are strongly influenced by a number of minor genes as has been shown for *Fb* (Ross 1986). In such cases, it is often noticed that after several generations of backcrossing, the complex of minor genes is lost and the level of resistance diminishes (Ross 1986). This is not the case with the *H1* gene, which has the highest level of expressivity and least epistatic effects of all the major genes conferring resistance to *G. rostochiensis*.

Barone *et al.* (1990), working with a diploid cross involving *S. tuberosum* and *S. spegazzinii*, reported linkage between markers from potato chromosome 7 and a resistance gene designated *Gro1*, which may correspond to the previously described *Fb* gene. We demonstrate in this report that *Gro1* and *H1* are independent and map to different chromosomes.



FIG. 2. Autoradiogram of *Eco*RI digest of 37 individuals from the cv. Atlantic dihaploid progeny, probed with *CD78*. R, resistant; S, susceptible individual. Arrow indicates putative recombinant.

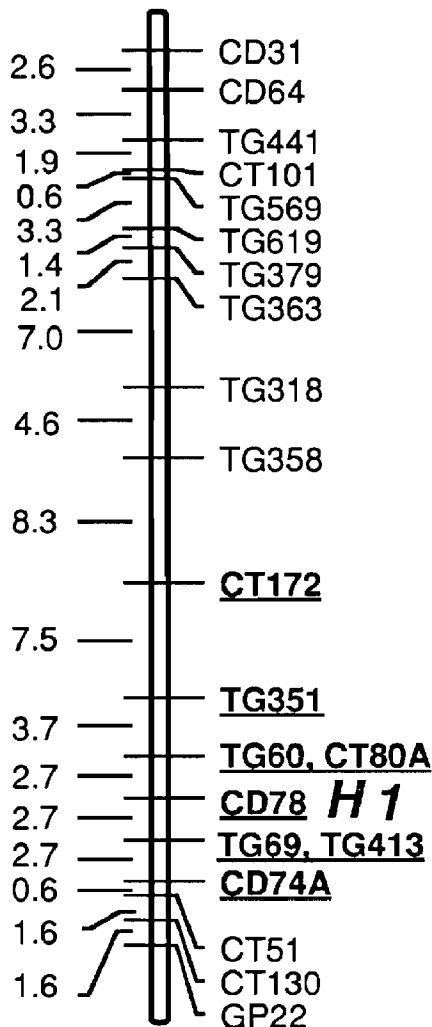


FIG. 3. Composite genetic map of potato chromosome 5 showing position of the *H1* gene. Map distances are based on segregation in an interspecific diploid cross ((*S. tbr*-*S. ber.*)/*S. ber.*), except the markers underlined which correspond to linkage analysis in the cv. Atlantic dihaploid family. LOD > 2.5.

Applications

The localization of *H1* to an interval of potato chromosome 5 is the first step in developing a molecular probe for this important gene. Susceptibility to *G. rostochiensis* is one

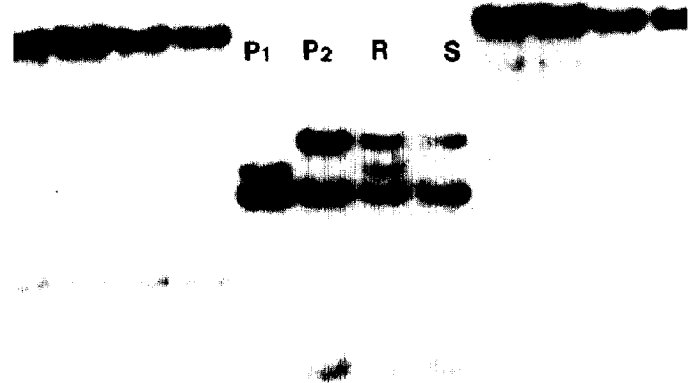


FIG. 4. Autoradiogram of bulked segregant analysis of 'Steuben' × 'Monona' tetraploid progeny digested with three restriction enzymes and probed with *CD78*. P1, 'Steuben'; P2, 'Monona'; R, pool of 15 resistant individuals; S, pool of 15 susceptible individuals.

of the few simply inherited characteristics upon which strict negative selection is exercised in potato improvement programs. Standard methods of screening for *H1* involve the use of live nematode and require several months for results. A rapid and inexpensive early generation screening tool would therefore be very attractive. Moreover, a marker linked to *H1* will provide a means of testing for resistance without risking the introduction of the nematode into new areas. Another application will be the provision of an efficient means for determining the allelic "plex" level of resistant clones. Parents that are triplex (*HHHh*) or quadruplex (*HHHH*) will provide only resistant progeny eliminating the need for screening. Information about the plex level of resistant clones may also be useful in basic research into the mechanisms and stability of resistance. Currently the molecular and biochemical basis of the resistance conferred by *H1* is not understood. The identification of linked RFLP markers may ultimately lead to the isolation of this gene via map-based gene cloning. Finally, testing for nematode

resistance (*G. rostochiensis* and *G. pallida*) often involves both qualitative and quantitative types of resistance from different sources. A marker that could distinguish one type of resistance from others in populations with complicated pedigrees could be useful in the breeding process.

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	Filing Date		2019-08-14
	First Named Inventor	Willem Hendrik Lindhout	
	Art Unit	1663	
	Examiner Name	Russell T. Boggs	
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1	Uitdewilligen et al., PLoS ONE 8(5), e62355 (2013)	☐
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A Next-Generation Sequencing Method for Genotyping-by-Sequencing of Highly Heterozygous Autotetraploid Potato

Jan G. A. M. L. Uitdewilligen^{1,2}, Anne-Marie A. Wolters^{1,2}, Bjorn B. D'hoop¹, Theo J. A. Borm^{1,2}, Richard G. F. Visser^{1,2,3}, Herman J. van Eck^{1,2,3*}

¹ Laboratory of Plant Breeding, Wageningen University, Wageningen, The Netherlands, ² The Graduate School for Experimental Plant Sciences, Wageningen, The Netherlands, ³ Centre for BioSystems Genomics, Wageningen, The Netherlands

Abstract

Assessment of genomic DNA sequence variation and genotype calling in autotetraploids implies the ability to distinguish among five possible alternative allele copy number states. This study demonstrates the accuracy of genotyping-by-sequencing (GBS) of a large collection of autotetraploid potato cultivars using next-generation sequencing. It is still costly to reach sufficient read depths on a genome wide scale, across the cultivated gene pool. Therefore, we enriched cultivar-specific DNA sequencing libraries using an in-solution hybridisation method (SureSelect). This complexity reduction allowed to confine our study to 807 target genes distributed across the genomes of 83 tetraploid cultivars and one reference (DM 1–3 511). Indexed sequencing libraries were paired-end sequenced in 7 pools of 12 samples using Illumina HiSeq2000. After filtering and processing the raw sequence data, 12.4 Gigabases of high-quality sequence data was obtained, which mapped to 2.1 Mb of the potato reference genome, with a median average read depth of 63× per cultivar. We detected 129,156 sequence variants and genotyped the allele copy number of each variant for every cultivar. In this cultivar panel a variant density of 1 SNP/24 bp in exons and 1 SNP/15 bp in introns was obtained. The average minor allele frequency (MAF) of a variant was 0.14. Potato germplasm displayed a large number of relatively rare variants and/or haplotypes, with 61% of the variants having a MAF below 0.05. A very high average nucleotide diversity ($\pi = 0.0107$) was observed. Nucleotide diversity varied among potato chromosomes. Several genes under selection were identified. Genotyping-by-sequencing results, with allele copy number estimates, were validated with a KASP genotyping assay. This validation showed that read depths of ~60–80× can be used as a lower boundary for reliable assessment of allele copy number of sequence variants in autotetraploids. Genotypic data were associated with traits, and alleles strongly influencing maturity and flesh colour were identified.

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* E-mail: Herman.vanEck@wur.nl

Introduction

DNA variants such as single nucleotide polymorphisms (SNPs), multinucleotide polymorphisms (MNP), and insertions and deletions (indels) are differences at the nucleotide sequence level among individuals or alleles and represent the basic units of genetic diversity. They can be assayed and exploited as high-throughput molecular markers and are widely used for marker assisted and genomic selection, association analysis and mapping of quantitative trait loci (QTL), and haplotype and pedigree analysis in several crops and model plants [1–7]. Genotyping of DNA sequence variants in highly heterozygous polyploid species, such as potato (*Solanum tuberosum*), is more challenging than in diploid species, because a given gene may be represented by up to four different alleles per locus per genotype. Therefore, genetic analysis in tetraploid potato requires a genotyping system that can distinguish among alleles and quantify the allele copy number. In tetraploid species, the possible allele copy number (zygosity)

categories include nulliplex (0), simplex (1), duplex (2), triplex (3), and quadruplex (4).

Several studies have shown that direct resequencing of amplicons by Sanger sequencing is sufficiently quantitative to allow the simultaneous discovery and genotyping of sequence variants in polyploids [8,9]. Amplicon sequencing is a reasonable method to analyse a single or small number of target gene(s), but beyond that scale, more effort is required to design unique primers and optimize PCR parameters to ensure equal amplification of all alleles. Furthermore, in species like potato that exhibit high nucleotide diversity, the many indels usually create uninterpretable sequence reads that further reduces the throughput of Sanger-based amplicon resequencing. With the introduction of the next-generation massively-parallel sequencing (MPS) technologies, several novel approaches have been developed to discover, sequence and genotype not tens, but hundreds to thousands of genes simultaneously [2,10–14]. Similar to Sanger sequencing, MPS can be used directly to genotype sequence variants. Using the

former technology, genotype data are retrieved from chromatogram peak intensities at variant positions. For MPS, accumulation of sequence reads provides allele copy number estimates for each variant. Accuracy of this allele copy number estimate is dependent on sequencing depth. Genotyping-by-sequencing (GBS) of tetraploids will require higher read depth compared to diploids. In humans, for example, a sequence read depth of 30–35× is considered appropriate for accurate genotyping [15]. In order to identify each allele in tetraploids a simulation model suggested a read depth of at least 15× [13]. However, accurate genotyping ($p=0.95$) of an autotetraploid organism implies also to discriminate digenic-simplex (*aaab*) as opposed to digenic-duplex (*aabb*) zygosity. Based on a binomial distribution this will require a sequence depth of at least 48×.

To achieve an increase in read depth, the portion of the genome that is sequenced can be reduced by applying, for example, RNAseq [2,16], restriction enzyme based complexity reduction [17,18], or sequence capture methods such as SureSelect, Nimblegen, and Raindance [19,20,21]. RNAseq is less suitable for GBS, since alleles may vary in transcription level across genes, tissues and stages, and thus may generate inaccurate genotyping data. Methods based on restriction enzyme treatments, on the other hand, are more likely to target non-coding parts of the genome, producing less useful data for functional gene analysis. Furthermore, restriction-based methods cannot target specific regions of interest, and nucleotide variants in the restriction site may interfere with digestion and cause null alleles. Sequence capture methods like SureSelect use oligonucleotide baits designed to bind to regions of interest, which can be specifically selected and enriched before sequencing [22]. For example, whole exomes [23] or regions associated with particular traits [19,24] can be targeted. Sequence capture approaches require *a priori* availability of sequence data to design DNA capture probes. The recently sequenced genome of *Solanum tuberosum* group Phureja is appropriate for this purpose in potato [25].

Here we describe DNA resequencing results obtained from dozens of autotetraploid potato cultivars and one monoplod accession after genome complexity reduction using hybridisation-based in-solution enrichment. We subsequently used this data to identify sequence variants within and across cultivars and to call the genotypes of resequenced individuals. The accuracy of genotyping-by-sequencing was validated using a SNP genotyping assay. The resulting marker dataset is useful for describing allele frequencies, nucleotide diversity, and population structure in potato, and for validating QTLs via association analysis. Our approach is an efficient means of producing data for the design of both high and low-density SNP genotyping assays applicable to a wide range of potato cultivars, and the resulting tools can be used to address questions in population genetics and marker-trait association research.

Materials and Methods

Design of the Capture Library

A custom-designed SureSelect capture library containing 57,054 RNA oligonucleotide baits of 120-bp length each was developed. Genes targeted for enrichment were selected from the PotCyc metabolic pathway database [26], the Potato Maps and More database (PoMaMo) [27], an in-house QualitySNP marker database [28,29], and a subset of single-copy genes homologous to the Conserved Ortholog Set II (COSII) [30]. Functional genes widely used as genetic markers for carbohydrate metabolism [31] and secondary metabolism [32–34], and a number of additional putative candidate genes for potato quality traits, were also

included as targets. In addition to these functional genes, a number of intergenic sequences corresponding to AFLP markers, and a number of chloroplast and mitochondrial genes were included (Table 1). To avoid intron spanning baits initial cDNA targets were aligned to the *S. tuberosum* Group Phureja DM whole genome assembly v.1 and baits were designed based on the genomic reference sequence with highest homology. Genome annotation for the DM sequence was not yet available at the time the baits were designed, so genomic coding regions and intron/exon boundaries were estimated using GeneSeqer [35]. Other reference sequences used for bait design included the chloroplast genome of *Solanum tuberosum* cv. Desiree (NC008096), *Solanum tuberosum* mitochondrial sequences (S66866, X74826, X80386, X83206, X93576) and sequenced BAC clones of genotype RH89-039-16 [25].

During bait design, we aimed to optimize the in-solution hybridisation enrichment by avoiding targets exhibiting repeat elements and paralogous sequences in the reference genome, which can affect target-bait hybridization during enrichment and add difficulty to read mapping after resequencing. Stretches of repetitive sequences within the target regions were excluded from bait design using RepeatMasker (<http://www.repeatmasker.org>). BLAST homology search against the *S. tuberosum* Group Phureja DM whole genome assembly v.1 [25] was conducted to avoid the use of targets with paralogous and/or duplicated sequences. Except for a small number of target gene families of specific interest (e.g. polyphenol oxidases), target sequences having a secondary hit with E-value $<10^{-10}$ were excluded from bait design. Regions consisting mainly of introns (>200 –1000 bp) were avoided as targets. For each gene target sequence, we used an average of 3–4 continuous regions (contigs) for bait design, each region having an average length of 475 bp and, where applicable, including both exons and introns. OligoTiler [36] was used to tile the reference strand of each target region, with baits (of 120 bp in length) starting approximately every 20 bp. This produced a 6× bait tiling coverage and resulted in 57,054 unique baits for the SureSelect capture library (ELID 0274451). In total, the library targets 2,945 contigs (1.44 Mb, GC-content 39%; Table 1). Complete lists of sequencing targets and oligonucleotide bait sequences are available in XLS-file S1 and FASTA-files S1&S2.

Plant Collection

A subset of 83 tetraploid potato cultivars (Table A in Supporting Information S1) was selected from a larger collection [37] using marker-based genetic distance estimates. The panel represents the global gene pool of commercial potato, both heirloom and contemporary, with emphasis on cultivars with high value in breeding and/or use. We also included a monoplod potato clone DM 1–3 511, derived by anther culture of the heterozygous diploid *S. tuberosum* group Phureja, clone BARD 1–3 of accession PI225669 [38]. The DM 1–3 511 clone is highly related to the recently sequenced clone DM 1–3 516R44 (CIP801092) [25], a doubled monoplod derived from the same BARD 1–3 clone [38].

Extraction and Fragmentation of DNA

DNA was extracted from leaves ground in liquid nitrogen using KingFisher Genomic DNA Purification Kit (Thermo Scientific) and the KingFisher MI magnetic nucleic acid extraction system (Thermo Scientific) according to the manufacturer's procedures. DNA concentrations were quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and diluted to 35 ng/μl. Of each DNA sample, 3.5 μg was fragmented by Adaptive Focused Acoustics on a Covaris S2 instrument (Covaris, Inc.), using a 10% duty cycle at intensity 4 for 120 seconds with 200

Table 1. Targets for SureSelect bait library development.

	Source	Genes	Target Contigs	Target Sequences	# of Baits (1 bait/20 bp)	Proportion of baits in library
Nuclear genome	COSI database	248	853	439.6 kb	17,618	30.9%
	PotCyc database	149	467	142.5 kb	4,940	8.7%
	PoMaMo & Candidate genes	116	523	283.1 kb	11,481	20.1%
	In-house database	249	789	424.7 kb	17,174	30.1%
	AFLP sequences	45	202	87.8 kb	3,400	6.0%
Sub-total		807	2,834	1377.7 kb	54,613	95.8%
Organelle genome	Chloroplast	64	99	55.9 kb	2,272	4.0%
	Mitochondrial	4	12	4.7 kb	169	0.3%
Total		875	2,945	1,438.3 kb	57,054	100%

For most targets, (cDNA) sequences were aligned to the potato reference genome, and the genomic sequence of each locus was used to design the baits.
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cycles per burst. Fragmentation of DNA to an average size of about 300 bp was verified using Bioanalyzer High Sensitivity DNA chips (Agilent).

Indexed Sequence Library Preparation

Custom-ordered, HPLC-purified, indexed adapters consisted of complementary Illumina adapters PE1 and PE2 [39], with PE1 extended by a 4-bp index sequence and an extra terminal T to facilitate sticky-end ligation. The reverse (PE2) adapter was extended by the reverse complement of the PE1 index (Table B in Supporting Information S1). The twelve indices had a balanced base composition and a minimal edit distance (i.e. the number of mutations required to change one index to another) of 2 bp to detect sequencing errors in the index region. To pair the adapter strands, mixtures of the forward and reverse strands (each 50 μ M in TE) were incubated at 95°C for 2.5 minutes, followed by a cool down (−1°C/30 sec.) to 25°C and subsequently diluted to a working concentration of 12.5 μ M. DNA sequencing libraries were prepared using the NEBNext DNA Sample Master Mix Set 1 (New England Biolabs). Purifications were carried out between end-repair, dA-tailing, and adapter ligation steps using AMPure XP beads (Agencourt Bioscience). To index the 84 potato DNA extracts, each of the twelve unique adapters was ligated to seven different DNA samples by mixing adapter and DNA in a molar ratio of approximately 20:1. After the initial ligation of 15 minutes at 20°C, samples were held at 4°C overnight and purified using AMPure XP beads. Adapter ligation was verified by fragment size analysis using Bioanalyzer High Sensitivity DNA chips.

In-solution Hybridization and Target Enrichment

The 84 indexed paired-end sequencing libraries were hybridized to the SureSelect capture library according to the manufacturer's instructions (Agilent SureSelect Target Enrichment System for Illumina Paired-End Sequencing Library Protocol, Version 1.0 May 2010), with minor modifications. Size selection on gel was omitted, allowing us to skip the standard pre-amplification step prior to in-solution enrichment and reduce the number of clonal reads in the later generated sequences. The use of index-specific blocking oligos to reduce non-specific pull-down due to adapter-adaptor hybridisation can also be avoided when pre-amplification is excluded, as non-amplified Y-adapters do not concatenate. We therefore excluded from the hybridization mix Block #1 (Human C0t-1 fraction) [40], which is irrelevant for plants, and excluded

Block #3 (PE adapter block) [40]. We used half of the specified hybridization volumes. Of each indexed DNA sample, 50–400 ng was mixed with 1.25 μ g salmon sperm DNA (Block #2), denatured, and hybridized to 100 ng SureSelect biotinylated RNA baits developed from the capture library. The hybridization mix was held at 65°C for 24 h for hybridization, then added to 250 ng (25 μ l) T1 streptavidin Dynabeads (Invitrogen), and pulled down. Bait-selected DNA was purified using AMPure beads, and amplified for 14 cycles (T_m 60°C) using 1 U Herculase II Fusion proofreading DNA polymerase (Agilent) and 25 μ M each of custom primers PE1.0 and PE2.0 (Table C in Supporting Information S1). The alternative DNA polymerase was chosen after the polymerase included in the NEBNext DNA Sample Master Mix Set 1 was seen to cause a negative shift in average fragment size.

Amplified, enriched DNA libraries were purified using AMPure XP beads and evaluated by agarose gel electrophoresis, NanoDrop, and Bioanalyzer High Sensitivity DNA chips. Library subsamples were also quantified by qPCR using a two-step amplification protocol [95°C activation for 2 min, 40 $\times$ (95°C 10 sec., 60°C 30 sec.)] and primers qPCR_1.1 and qPCR_2.1 (Table D in Supporting Information S1).

Hi-Seq2000 Sequencing and Data Preprocessing

Seven distinct resequencing pools were made by combining equimolar amounts of twelve unique, differently-indexed, enriched DNA library samples (Table A in Supporting Information S1). Pooled libraries were sequenced on a Hi-Seq2000 (Illumina) lane using 100-base paired-end sequencing at the Genome Analysis Facility of the University Medical Center Groningen (UMCG). Four sequenced pools that generated low cluster numbers and/or partly failed reverse read sequences were repeated. Initial quality checks (average read quality per cycle, average read quality, base call % per position) were performed using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Sequences in each pool were categorized to their biological sources according to the 4-bp index using NovoBarCode (Novocraft) with the average edit distance set to 3. The first 5 bp of each de-multiplexed sequence read (i.e., the 4-bp index plus an extra T used for ligation) were removed.

Sequence read-pairs containing ≥ 63 nucleotide sequences with 100% match to chloroplast genome sequences of *Solanum tuberosum* cv. Desiree (NC008096) were extracted from the de-multiplexed

sequences and kept for separate analysis. Remaining sequence reads were aligned using the Burrows-Wheeler Alignment tool (BWA) [41] with default alignment parameters, except for the maximum edit distance, which was relaxed to seven due to the expected high sequence divergence between potato alleles. Sequences were aligned to the annotated superscaffolds (DMGv3.4, comprising 705.5 Mb of sequence in 1,419 superscaffolds and 43 Mb of non-ACGT bases) of the *S. tuberosum* Group Phureja DM whole genome assembly v.3 reference genome [25]. The 120-bp SureSelect bait sequences of genomic origin were correspondingly mapped to the annotated superscaffolds, and baits with a mapping quality (MQ) ≥ 37 were used to define the genomic target regions.

Alignment data was processed with SAMtools and Picard [42] to mark duplicate reads and estimate the average insert size of the paired-end reads. The Genome Analysis Toolkit (GATK) [43] was used for indel realignment, base-score recalibration, and extraction of read depth information. Read depth and coverage data were processed with custom Perl scripts and BEDTools [44]. The raw sequencing reads and mapping assembly are available on our server (<http://datarelease.plantbreeding.nl/>).

Sequence Variant Detection and Genotype Calling

For covered regions (see Results for definition), sequence variants were identified simultaneously among the aligned reads from all 83 tetraploids and the single monoploid using the FreeBayes polymorphism discovery algorithm [45]. Sequence variants included binary SNPs, MNPs, and small indels, as well as allelic series of tri-SNPs and tetra-SNPs, multi-allelic MNPs, and indels with a variable number of (repetitive) nucleotides. Reads marked as duplicate, with more than seven base mismatches, more than three separate gaps, or with MQ < 30 were excluded for variant calling. The expected mutation rate or pairwise nucleotide diversity was set to 0.01. In order to include an alternate allele as a variant, supporting bases required a minimum base quality (BQ) of at least 13, and at least one supporting alignment was required to have BQ ≥ 20 . Furthermore, the alternate allele had to be observed in at least 5 reads and represent at least 12.5% of the observations of reads at that locus within a single potato sample. Mapping quality of alleles was included when calculating posterior probabilities, and variants were only called for sites that had a probability of polymorphism greater than 0.95. Sequence variants adjacent to indels, which were likely due to local misalignment, were filtered using GATK [43].

For genotype calling, zygosity of all sequence variants were resolved by allele-specific read depths for all non-duplicated reads with MQ ≥ 13 using FreeBayes. This resulted in nulliplex (aaaa), simplex (aaab), duplex (aabb), triplex (abbb) and quadruplex (bbbb) genotype calls relative to the reference sequence for the tetraploid potato samples, while variants in the monoploid sample were genotyped as either absent or present. Hence a variant is called not only if a genotype differs from the reference, but also if a genotype differs from any other genotype. Only variants previously identified by variant calling were used for genotyping, and samples required a minimum read depth of 15 $\times$ at the variant position to yield a genotype call. In addition to this 15 $\times$ coverage threshold we applied a threshold GQ ≥ 26 . Genotyping Quality (GQ) was calculated by a likelihood estimate to determine the probability that a genotype call was different from the true genotype using FreeBayes. It was encoded as a phred quality score ($-10 \times \log_{10}(p)$) and included read depth at a variant position as a parameter.

Diversity Analysis and Prediction of Functional Consequences of Allelic Variants

To calculate the nucleotide diversity, we first calculated the gene diversity (heterozygosity) of each binary variant; $GD = 1 - \sum P_i^2$, where P_i is the frequency of the i th allele and GD is summed across n alleles. Nucleotide diversity (π) was calculated by averaging GD over all nucleotide sites – or all coding and/or non-coding nucleotides sites – on a contig or gene.

The functional effect (codon mutations, splice site mutations, frame shift mutations) of sequence variants was predicted using snpEff [46] and gene annotation DMGv3.4 of the DM genome.

In order to identify population structure, principal components analysis (PCA) was performed on genotype scores using the FactoMineR library of R [47]. Only genotype scores of variants called in all 84 cultivars were included. Since all genotype scores were measured in units of allele copy number, the data were not scaled. K-mean clustering of the first three principal components was used to identify genotype clusters.

Association Analysis

Adjusted phenotype means for plant maturity and tuber flesh colour in each of the 83 tetraploid cultivars, measured previously over a period of five years [37,48], were used as trait values for conducting association analysis. Additive and dominant genotype models were each tested both with and without correction for population structure. The genotype clusters identified by PCA analysis were used as the adjustment factors for population structure. For dominant association models, linear regression models were used as implemented in PLINK [49], with tetraploid data recoded into diploid homozygous/heterozygous scores, using binary allelic variants only. Adjustment of p-values to correct for multiple testing effects was carried out using step-up FDR control as implemented in PLINK. For the additive tetraploid genotype models, we applied linear regression models implemented in Genstat. For each trait and each marker the model fitted was: response = allele copy number (+ structure)+error.

Validation of Genotype Calling

The accuracy of GBS genotype calls was validated using the Kbioscience Allele-specific Polymorphism Assay (KASP) SNP genotyping platform. Binary SNPs identified in the sequence data that exhibited a minor allele frequency between 0.15 and 0.35 were selected as candidate for assay design. To assure independence among all SNPs in the KASP assay (i.e., use of a unique SNP from each haplotype block), SNP data from GBS were clustered using hierarchical cluster-analysis, and a single SNP from each cluster having a correlation coefficient of $r^2 \geq 0.16$ was used for the KASP assay, yielding 768 SNPs in the final design. We KASP-assayed DNA from 65 potato cultivars included in GBS, with two of these cultivars measured in duplicate to assess KASP genotyping consistency. A number of additional diploid potato clones were assayed (~96) and used to examine the signal ratio of the nulliplex, duplex and quadruplex genotype signals. The software package fitTETRA [50] was used for full tetraploid zygosity genotype calling. In total, 270 of the 768 KASP assayed SNPs were selected for validation of the GBS calls. This selection was based on (1) sharp clustering of the signal ratios of discrete genotype classes, (2) clustering of signal ratios of heterozygous diploids with duplex tetraploids, and (3) clustering of signal ratios of homozygous diploids with nulliplex or quadruplex tetraploids.

Results

Sequencing and Mapping of Enriched Libraries of 84 Potato Genomes

We designed an in-solution hybridisation capture library targeting primarily introns and exons of nuclear coding genes, but also including intergenic, chloroplast, and mitochondrial sequences. Baits targeting nuclear genome sequences in the enrichment library covered approximately 1.3 Mb of the potato DM reference genome sequence, scattered across all 12 chromosomes (Figure 1 & BED-file S1).

Genomic DNA sequencing libraries from 83 diverse tetraploid potato cultivars and progenitor lines, and a single monoploid potato clone, were indexed by cultivar with 12 distinct 4-bp index sequences and individually enriched using the capture library. Indexed and enriched samples were multiplexed in pools of 12 unique indices and paired-end sequenced. In total, 592,100,112 read-pairs were obtained, representing approximately 100 Gigabases of sequencing data. The cultivar-specific sequence index could be identified in 96% of the read-pairs (Figure A in Supporting Information S1). For 19% of the read-pairs, cultivar identification was based on the index from only one read of the pair. In most cases, this resulted from technical failure of the reverse-read sequencing.

Within each multiplexed pool, the cultivar-specific read counts rarely reached a twofold difference between cultivars, indicating approximately equivalent amounts of DNA were added to a pool from each cultivar present. Initial inspection of the cultivar-tagged sequences showed that chloroplast-derived sequences were highly abundant (60% of the read-pairs). These sequences were filtered using broad homology criteria and saved for a later analysis of its own. The remaining 227,263,706 read-pairs were aligned to the DM reference genome, with 80% mapping. This resulted in 23.9 Gigabases of high quality ($MQ \geq 13$) genome-aligned sequence data. Among mapped reads, 8% were marked as duplicate reads aligning with identical start and end positions on the reference genome.

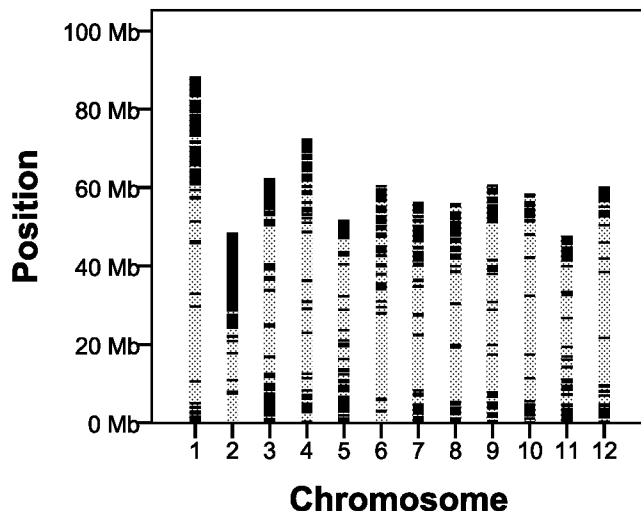


Figure 1. Mapping of resequencing targets onto the potato genome. Physical position of the approximately 800 target genes scattered across the 12 potato chromosomes. Physical positions are ordered according to the latest pseudomolecule order (v.3 beta). The distribution of the ~800 genes reflect the physical proportions of gene rich chromosome arms and gene poor heterochromatic regions. doi:10.1371/journal.pone.0062355.g001

Genome and Target Coverage

As a consequence of the enrichment method sequences aligned not only to target regions but also to flanking and off-target regions. To define the “accessible” part of the genome, i.e., the part with adequate coverage to allow identification of sequence variants, we used the following criteria: (1) at least five cultivars had to be sequenced at the base position, with a threshold criterion of at least 20 component reads with $MQ > 30$ per cultivar; (2) in view of the fragment sizes of mapped reads (261 ± 94 bp, mean $\pm$ SD), regions defined as covered were at least 261 bp long; (3) regions were discarded when more than 5% of reads within the region aligned to multiple locations with equal probability ($MQ = 0$); and (4) finally, regions with $> 80\%$ homology to chloroplast and mitochondrial sequences were removed. This resulted in 2,445 sequenced contigs with a total length of 2.1 Mb (XLS-file S2 and BED-file S2). A total of 12.5 Gigabases of high quality sequence data aligned to these contigs, providing an average read depth of $5,871 \times$ when all cultivars were considered together. Median depth per cultivar within this accessible genome was $63 \times$, ranging from $15 \times$ to $177 \times$ (Table 2 and Table A in Supporting Information S1).

Almost all genomic regions and genes targeted by the enrichment library fell within the accessible regions, with 97% of target sequences and 793 of 807 nuclear target genes covered. In total, 10.7 Gigabases of high quality sequence data aligned to target regions with a median depth per cultivar of $88 \times$, ranging from $20 \times$ to $240 \times$. Accessible flanking regions had an average length of 150 bp and comprised 554 kb of additional sequence. Regions flanking target sequence but interrupted by poor coverage for a small number of nucleotides, and more remote off-target regions, accounted for 288 kb (13.5%) of the accessible genome.

DNA Sequence Variants

A total of 129,156 putative sequence variants (SNPs, MNPs and indels) were identified in the accessible genome (Table 3, CSV-file S1, and VCF-file S1). The density of substitution variants (SNPs and MNPs) was 1.6 times higher in non-coding regions than in coding regions, and the indel density was 12 times higher in non-coding regions. The transition/transversion ratio (T_s/T_v), calcu-

Table 2. Summary of target enrichment sequence coverage for 84 potato cultivars.

“Accessible” genome parameter	Value
Number of covered regions (contigs)	2,445
Sequence length	2,136,143 bp
Coding sequence (DMGv.3.4)	655,930 bp
Sequencing depth per cultivar at contigs ^{b,c}	$70 \pm 36 \times$ (Median $63 \times$)
Sequencing depth per cultivar at target sequence ^{b,c}	$95 \pm 48 \times$ (Median $88 \times$)
Genes covered	977
Target genes covered	793 out of 807
Target sequence covered	1,294,097 bp (97%) ^a
Target+directly flanking sequences	1,848,192 bp
Off-target sequence	287,951 bp (13%)

^aPercentage of target bait sequence mapped to the DM genome.

^bOnly sequenced nucleotides that aligned with high quality ($MQ \geq 13$).

^cMean and standard deviation.

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Table 3. Overview of DNA variants observed across 84 cultivars in the accessible potato genome.

Variant type	Number of sequence variants called		
	Accessible genome (2136 kb)	Non-coding (1480 kb)	Coding (656 kb)
Di-nucleotide SNPs	105,812	84,454	25,358
Tri-SNPs	5,304	4,097	1,207
Tetra-SNPs	96	66	30
Indels	13,094	12,641	453
MNPs	4,850	4,084	766
Total	129,156	101,342	27,814
Average variant density	1/16.5 bp	1/14.6 bp	1/23.6 bp

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lated only for biallelic SNPs, was 1.55, and the ratio of non-synonymous to synonymous SNPs (pN/pS) was 0.64.

Across all cultivars, an average variant density of 1/24 bp in coding regions and 1/15 bp in non-coding regions was observed. Within a single tetraploid cultivar, on average 52,233 sequence variants (1/42.5 bp) were observed. On average each cultivar had 116 cultivar-unique variants, ranging from 0 to 2,688. Cultivars like Vitelotte Noir, the only cultivar with purple flesh colour in our samples, and those with wild species introgression segments contained a relatively high number of cultivar-unique variants (e.g., up to 2.0% of all variants within cv. Vitelotte Noir). Cultivars without unique variants either had ancestors that were widely used in breeding of novel potato cultivars or had themselves been used for this purpose (e.g., cv. Agria and cv. Katahdin respectively).

To evaluate the increase in variant density per additional sequenced cultivar, we permuted the order of the cultivars a thousand times and calculated the variant frequency at each incremental step (Figure 2). More than half of all variants were detected by selecting three random cultivars. When 16 random cultivars were selected, 84% of all variants were detected, and the number of novel variants that could be identified by sequencing an additional cultivar dropped below 1% of the variants already discovered. To detect 95% of all the identified variants, 46 random cultivars were required.

Genotype Calling and Allele Frequencies

To be assigned a valid genotype call for a specific variant position, a cultivar required a minimal read depth of 15× at that position. Using this criterion, 86.6% of all possible genotype calls were valid (i.e., of the matrix of 84 cultivars by 129,156 sequence variants, 13.4% had insufficient read depth to make a call), equivalent to a per-locus average of 73 out of 84 cultivars receiving a genotype call. For 42,625 sequence variants (33% of all identified sequence variants), all 84 cultivars were genotyped, and more than 90% of all sequence variants were genotyped in at least half of the cultivars. Population-level allele frequencies were calculated using all valid ($\geq 15\times$) calls. The distribution of minor allele frequencies (MAF) is shown in Figure 3. The average MAF was 0.14; 17.4% of all sequence variants had $MAF < 0.01$, and 60.9% had $MAF > 0.05$. For 13,458 sequence variants, 10.4% of all identified, the allele in the DM reference genome differed from the major allele in the population.

Nucleotide Diversity

We next calculated the nucleotide diversity (π) for each of the 2,445 sequenced contigs, for each gene, and for each chromosome. Mean π of the covered genome was 1.07×10^{-4} (Table 4). As

expected due to functional constraints on evolutionarily tolerable mutations, π was lower for coding regions than for non-coding regions (Table 4 and Figure 4). The physical position of contigs on the potato pseudomolecules was used to plot π over the twelve potato chromosomes (Figure B in Supporting Information S1). Mean nucleotide diversity for chromosomes 5 and 11 was significantly higher relative to other chromosomes (Figure 5). In contrast, the mean π for chromosome 10 was significantly lower than for other chromosomes. Individual genes with low nucleotide diversity were observed on all chromosomes (XLS-file S3).

Population Structure

Population structure was analysed using the sequence variants that were genotyped in all 84 cultivars. The first three components of a principal component analysis described respectively 6.4%, 4.5% and 3.8% of the variance. In the centre of the PCA plot, a cluster of cultivars of diverse origin was located (Figure 6). Three groups diverging from this set of cultivars were noted, which consist of (a) heirloom British cultivars, (b) a number of typical frying cultivars from continental Europe, and (c) progenitors of potato cyst nematode (PCN) resistance and cultivars bred for the

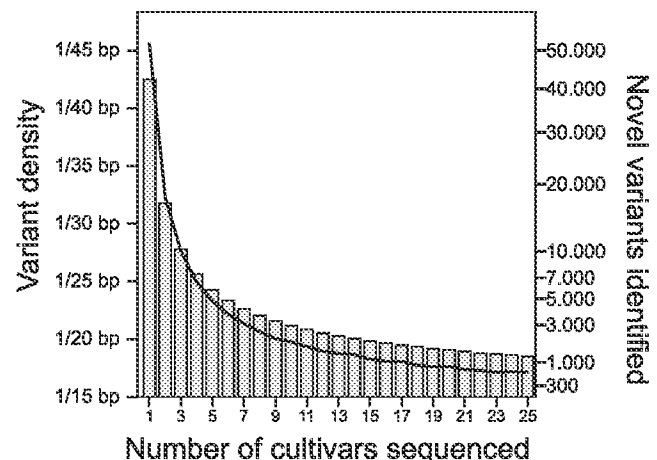


Figure 2. Sequence variant density as the number of randomly-added cultivars increases. The bars show variant density (primary Y-axis), and the black line shows the number of newly-identified variants (secondary Y-axis) as a function of the number of sequenced cultivars. Data is not shown after the 25th cultivar, but continues to drop to a variant density of 1/16.4 bp and an average of 116 novel variants at the 84th cultivar.

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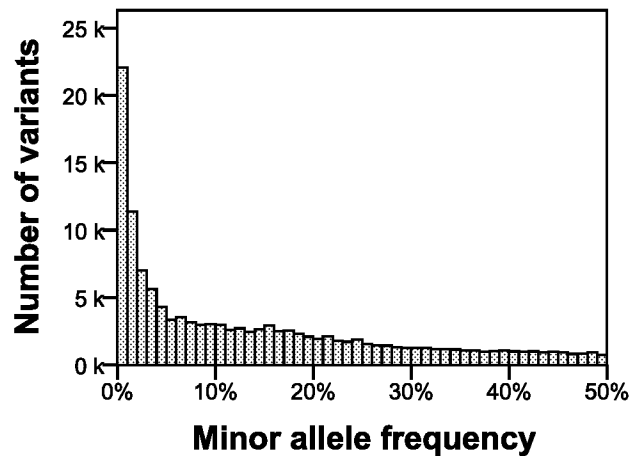


Figure 3. Distribution of minor allele frequencies (MAF) of all 129,156 genotyped sequence variants.
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starch industry with resistance to PCN. The heirloom cultivar group is closest to the more distant monoplod *S. tuberosum* Group Phureja clone, which fell into a cluster of its own.

Association Analysis

The genotype dataset was used for a genome wide association study (GWAS) to validate the sufficiency of this data for identifying known QTLs for plant maturity and tuber flesh colour. Association analysis was performed using both additive and dominant genotype models with separate tests with and without correction for population structure. To a large extent, the results of the dominant and additive models overlapped. The results of the dominant allele models are shown in Figure 7. Even with the small population size of 83 phenotyped cultivars, the well-known QTL for early plant maturity on potato chromosome 5 was clearly detected ($-\log_{10}(p)=6.0$) and explained 44% of the observed phenotypic variance. Using the current data, this QTL mapped to a region of approximately 371 kb within superscaffold PGSC0003DMB000000192 containing 28 strongly associated variants ($-\log_{10}(p)\geq 5$). For tuber flesh colour, a major QTL was observed on chromosome 3, mapping to a region of approximately 683 kb containing 27 strongly associated variants ($-\log_{10}(p)\geq 7$). These sequence variants were located within and near *CHY2* (β -carotene hydroxylase), a well-known gene influencing flesh colour via carotenoid synthesis, and explained 61% of the phenotypic variance. Two additional minor-effect QTLs for flesh colour were found on chromosomes 4 and 12. When corrected for population structure, the QTL on chromosome 12 was not significant. The flesh colour QTL on chromosome 4 ($-\log_{10}(p)=3.7$) explained 9% of additional phenotypic variance beyond that explained by the major QTL.

Validation of Genotyping-by-sequencing Data

A subset of 270 binary SNPs was selected to validate genotype calls made by GBS using KASP genotyping assays. For one of the two cultivars duplicated in the KASP assay, all genotype calls were identical between replicates, and for the other cultivar, only two genotype calls varied between replicates. The expected KASP genotyping error rate of this subset of SNPs is thus very low (0.4%). For concordance analysis, expected genotype calls were obtained from these 270 KASP assay SNPs and observed genotype calls were obtained by the GBS results. Scored as either

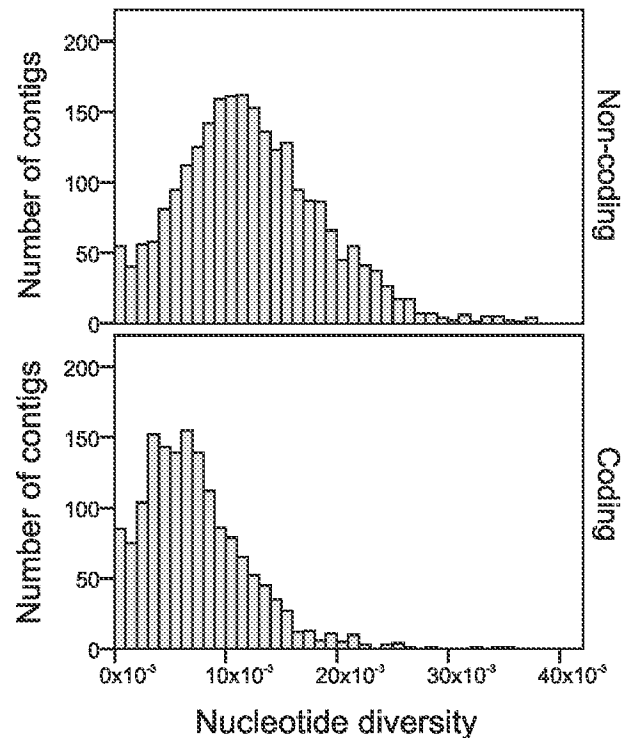


Figure 4. Distribution of nucleotide diversity across sequenced contigs for coding and non-coding regions.
doi:10.1371/journal.pone.0062355.g004

homozygous (i.e., nulliplex or quadruplex) or heterozygous (i.e., simplex, duplex, or triplex), 97.9% of the homozygous and 99.9% of the heterozygous GBS calls were concordant with the results of KASP genotyping. When heterozygous GBS calls were split into simplex, duplex and triplex categories, overall concordance with KASP genotyping dropped to 94.4%. For duplex calls, which were most difficult to classify, only 90.3% of the calls were concordant. We therefore applied a genotype quality (GQ) filter to validate GBS/KASP concordance in the five discrete zygosity classes. A

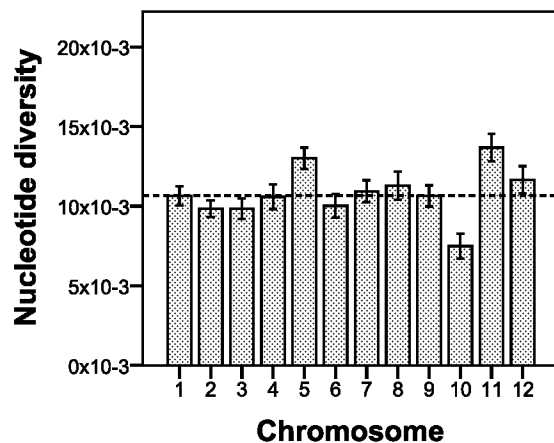


Figure 5. Mean nucleotide diversity across sequenced contigs per chromosome. Dashed line represents genome wide nucleotide diversity (both coding and noncoding sequences), and error bars represent 95% confidence intervals.
doi:10.1371/journal.pone.0062355.g005

Table 4. Nucleotide diversity in potato (mean value and standard deviation across contigs).

Nucleotide diversity ($\times 10^{-3}$)	10.7 $\pm$ 5.4
Nucleotide diversity ($\times 10^{-3}$), coding sequence	7.3 $\pm$ 4.8
Nucleotide diversity ($\times 10^{-3}$), non-coding sequence	12.4 $\pm$ 6.7

doi:10.1371/journal.pone.0062355.t004

threshold of $GQ \geq 26$ was required to reduce the number of discordant duplex calls below 5%. Applying the GQ26 filter to the complete set of 129,156 sequence variants yielded 74.8% of variants with an assigned genotype call, with an average of 63 out of 84 cultivars genotyped per variant position. This is equivalent to 25.2% of unassigned genotype calls, yielding approximately two-fold more unassigned genotype calls compared to the set subject only to the $15\times$ read-depth threshold. Median read depth of all GBS genotype calls meeting the GQ26 threshold was $61\times$, and for duplex calls this was $81\times$. Overall concordance between the GQ26 filtered set and KASP genotyping was 98.4%, with 96.2% concordant duplex calls (Table 5).

Analysis of Chloroplast Reads

As an initial analysis of chloroplast reads, 100,000 paired-end reads per cultivar, with index sequences on both sides, were mapped to the chloroplast reference genome. A total of 241 sequence variants, covering the chloroplast genome, were identified (VCF-file S2). Since chloroplast sequences are monomorphic, the chloroplast haplotypes could be directly inferred. Four main chloroplast types, with a number of sub-types, were found using a phylogenetic approach (Figure 8 and Table A in Supporting Information S1). A number of cultivars contained distinct chloroplast genomes resembling those of *S. demissum*, *S. vernei* and *S. tuberosum* Group Phureja, but most resembled those commonly found in *S. tuberosum* Group Tuberosum cultivars.

Discussion

Target-enriched Genome Sequencing

This paper presents evidence that GBS in complex polyploid genomes can be performed accurately, especially with regard to the specific requirement of accurate allele dosage detection. Below, we will argue that our method of target enrichment was of great importance for genotyping accuracy. Target enrichment allowed the achievement of sufficient read depth. Furthermore, bait-hybridisation enrichment allowed exclusion of complex multi-copy or repetitive regions of the genome, and avoidance of potential difficulties in distinguishing allelic variants from paralogous sequences [51]. We focussed on a single copy genes, e.g. from the set of conserved orthologous sequence genes (COSII) [30] and used uniquely mapped genes to define the genomic target regions. It is not excluded that individual potato genotypes may be polymorphic for specific gene duplications (Copy Number Variants). Indeed, for some genes read depths were observed twice or more than average in specific cultivars, suggesting gene duplications in these cultivars (data not shown). In these rare cases the duplication maps to the same location on the reference genome and could affect genotype calling. However, we feel confident that this is a relatively rare phenomenon, because the validation of genotype calling with KASP assays showed high concordance.

The SureSelect enrichment method yielded sequences of which 45% aligned to the target sequences. Other in-solution DNA

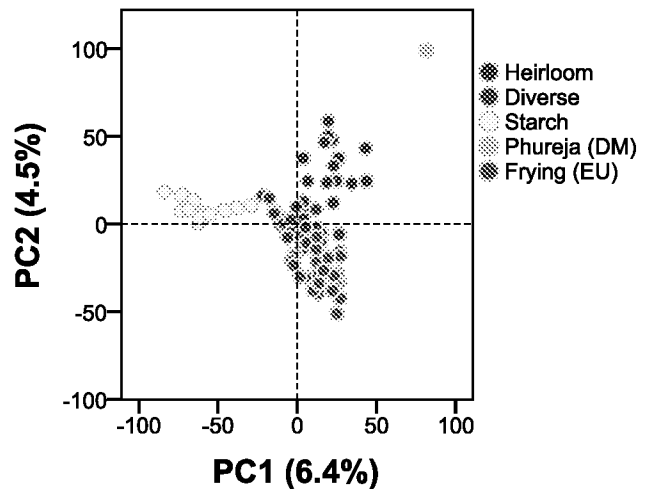


Figure 6. First and second components from principal component analysis of potato sequence variant genotypes. Population structure was analysed using ~43 K sequence variants genotyped in all 84 cultivars. The first three components describe 14.7% of the variance. Based on these three components, the cultivars were clustered into five groups. The most distant cultivar is the monoplod *S. tuberosum* Group Phureja clone. In the centre of the PCA plot cultivars of diverse, worldwide origins are observed. Three additional divergent groups can be observed, consisting of heirloom cultivars, frying cultivars from continental Europe and cultivars and germplasm used in starch industry.

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enrichment studies in plant species have not yet been published, but this on target percentage is consistent with the 40–50% reached in human and animal studies that have used the SureSelect system for target enrichment [19,40].

To reduce sequencing costs we multiplexed twelve cultivars in a sequencing pool. The custom index adapters have proven valuable as multiplex adapters; no index-specific bias in read counts was observed and over 96% of the generated reads were assigned to cultivars. The inclusion of these indices permits tracking of the alleles' cultivar source so that zygosity can be determined in individual genotypes, allowing GBS of many individuals. Larger numbers of indices can be easily created [17] and allow multiplexing of tens to hundreds of individuals. We found consistent enrichment across all indexed samples, and virtually all target sequences were covered covered at a sufficient depth, with a median average read depth of $88\times$ per cultivar for nuclear target sequences ($63\times$ for the extended "accessible" genome). The read depth of nuclear sequences was reduced by the large number of cpDNA reads (60% of total reads) generated in this study. This large share of cpDNA reads was unforeseen in view of the 4% share of cpDNA baits used for target enrichment. In retrospect, this large proportion is almost certainly due to the copy number variation between nuclear DNA and cpDNA. The latter is present in about 100 copies per plastid and about 100 plastids per leaf cell [52]. Hence, bait library design should avoid sequences from, or homologous to extra-nuclear DNA.

In the estimate of allele copy number we assume no ascertainment bias; i.e., the relative number of allele-specific sequencing reads is proportional to the allele copy number. However, in-solution hybridisation based target enrichment may bias the pool of captured DNA targets that are sequenced towards those variants which preferentially hybridize and have higher sequence similarity to the reference bait sequence [19]. Anticipating this bias, we used a high probe tiling redundancy ($6\times$, one

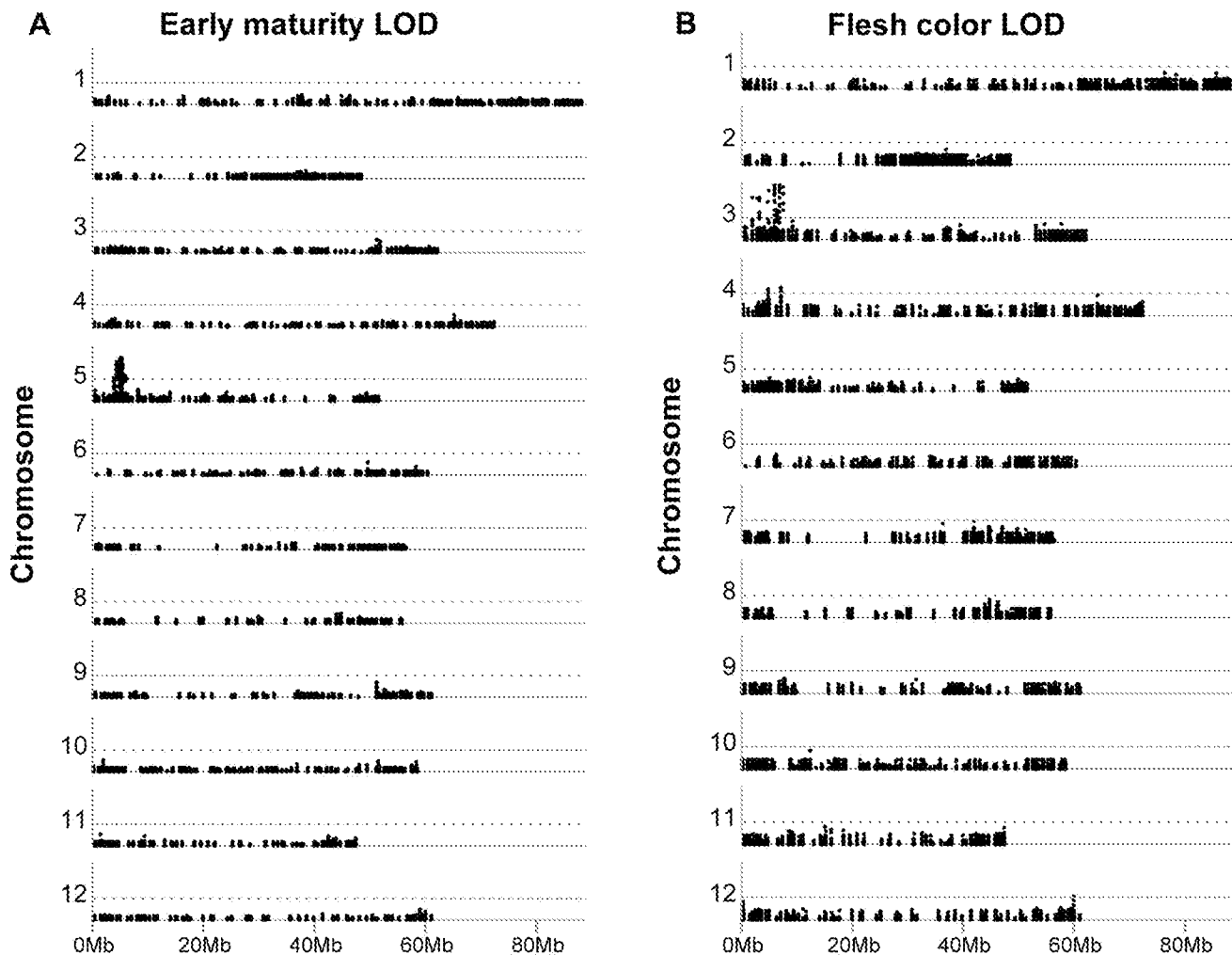


Figure 7. Manhattan plots of p values for associations between DNA sequence variants and two phenotypic traits in potato: (A) plant maturity and (B) tuber flesh colour. The FDR corrected $-\log_{10}(p)$ values from GWAS analysis are plotted relative to the physical position on each of 12 potato chromosomes. The horizontal dashed line is plotted at the FDR-corrected significance threshold of $\alpha=0.001$ ($-\log_{10}(p)=3$). doi:10.1371/journal.pone.0062355.g007

RNA bait every ~20 bp) to reduce allele-specific bias during hybridization. Furthermore, we relaxed mapping quality settings for counting the reads mapped to the reference genome. In a

preliminary genotype analysis performed after sequence variant calling, only reads with a very high mapping quality ($\geq MQ30$) were used to determine the relative number of allele-specific

Table 5. Concordance between genotyping-by-sequencing and KASP genotyping calls.

Genotyping-by-sequencing	Expected genotype call (KASP)					Total
	Nulliplex	Simplex	Duplex	Triplex	Quadruplex	
Concordant calls	4,975	4,861	2,204	1,373	1,272	14,685
Discordant calls	67	25	86	34	25	237
Percentage of concordant calls	98.7%	99.5%	96.2%	97.6%	98.1%	98.4%
Median read-depth of concordant calls	85×	91×	101×	92×	75×	89×
Median read-depth of discordant calls	52×	56×	73×	83×	47×	61×
Percentage of alternative reads for concordant calls	0%	23%	49%	76%	100%	—
Percentage of alternative reads for discordant calls	4%	36%	34%	56%	97%	—

The genotype calls derived from each method for 270 binary SNPs were compared. Sequencing calls were filtered by a minimum read depth of 15× and a minimum genotype quality score of GQ26.

doi:10.1371/journal.pone.0062355.t005

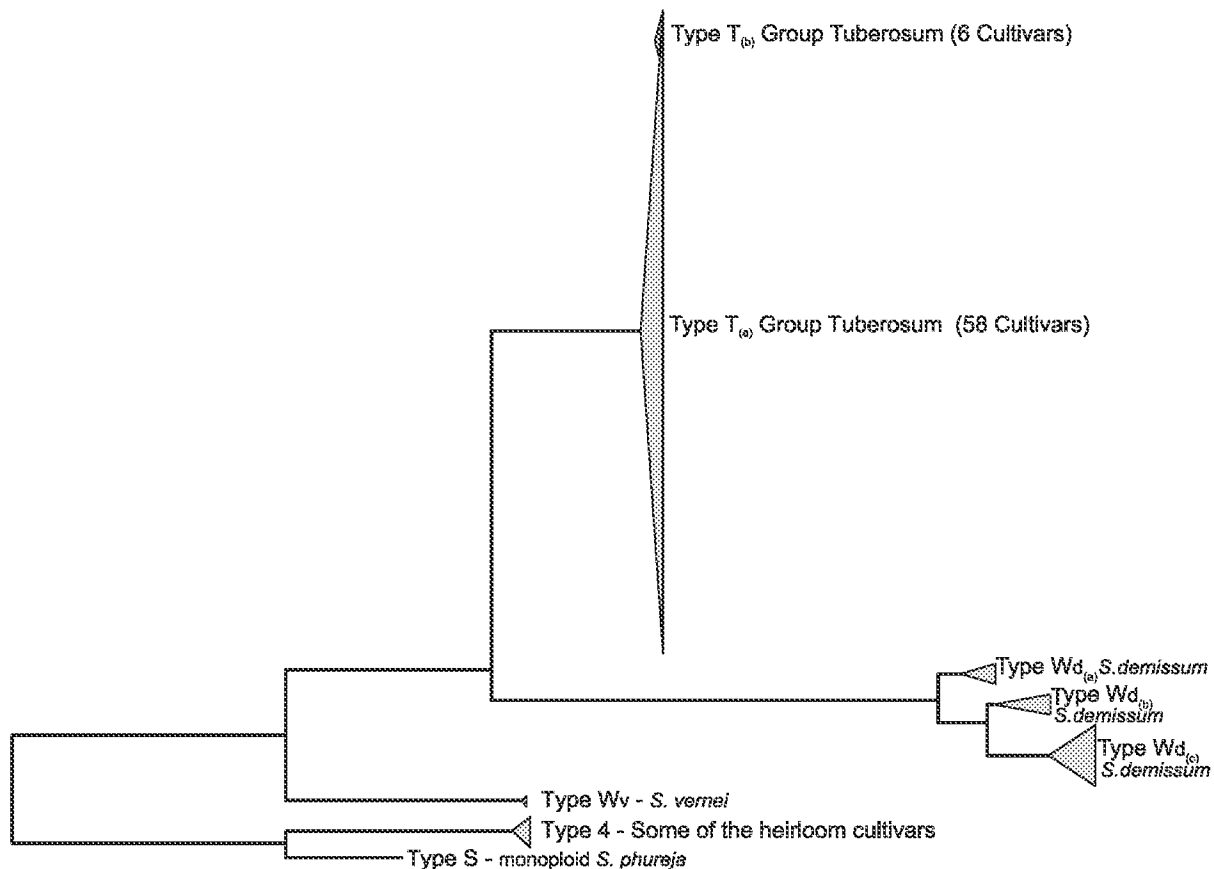


Figure 8. Neighbour-Joining tree of chloroplast haplotypes. The distances of 241 sequence variants for the 84 cultivars were computed using the Jukes-Cantor method and the tree inferred using the Neighbor-joining method.
doi:10.1371/journal.pone.0062355.g008

sequencing reads. This caused a large ascertainment bias due to severe underrepresentation of non-reference alleles. Inclusion of reads with a lower mapping quality ($MQ \geq 13$) strongly reduced this ascertainment bias. When the GQ-filter was applied, 95% of the sequence variants were concordant, as shown by the KASP validation (Table 5). The average read ratio for these calls shows no bias. We conclude from this study that hybridisation based capture bias hardly affected genotype calling, even in potato germplasm where reference and non-reference reads often have only about 95% sequence homology. We have not examined the genotyping accuracy of MNPs or indels, but larger indels are likely to reduce capture and mapping efficiency.

We used a mapping approach to align the sequencing data to the potato genome sequence [25]. An advantage of alignment to an annotated reference genome is that it allows prediction of whether a sequence variant falls within or near a gene of interest, and whether it is expected to cause a functional change in the protein product (e.g. synonymous vs. non-synonymous) that might alter the enzyme activity of the protein. This can be very useful in determining whether a particular sequence variant is likely to be responsible for a phenotype of interest. We observed approximately twenty-five thousand non-synonymous variants, with 50 variants that cause premature stop codons in 43 out of 807 genes. Hence, natural diversity of potato offers a substantial resource for the identification of knockouts for gene-function studies.

A disadvantage of mapping sequence reads toward a reference sequence is that structural variation like chromosome rearrangements, inversions and large (transposon) insertions are likely to be

missed. Structural variation between reads and reference genome complicates the mapping of reads. This disadvantage can be partly avoided by *de novo* assembly, but computational difficulties associated with the assembly of highly diverse polyploid species like potato makes mapping sequence reads to a reference sequence a more straightforward approach. In this study we did not systematically look for structural variants, nor did we consider gene copy number variants (CNVs). Methods for detecting both structural variants and CNVs using a mapping approach have however been developed [53,54] and could be applied. Fortunately, structural variants can frequently be detected using sequence variants in linkage disequilibrium (LD). For example, orange potato flesh colour due to zeaxanthin accumulation, caused by a transposon insertion in the zeaxanthin epoxidase gene was initially detected using SNPs [34].

Sequence Diversity Analysis

This study confirms the high sequence diversity of potato, albeit using a much higher number of potato cultivars and on a more genome-wide scale than previous studies [55,56]. We found an overall frequency of one variant every 17 bp in the cultivar population, 1/15 bp in non-coding and 1/24 bp in coding regions. This is somewhat higher than the one variant per 21–23 bp reported in previous studies on potato [55,56], reflecting the differences in the number of potato cultivars used. Compared to other crops where both coding and noncoding regions were analyzed for nucleotide diversity, total nucleotide diversity in potato cultivars ($\pi = 10.7 \times 10^{-3}$) is larger than that in sugar beet

(7.6×10^{-3}) [57], maize elite lines (6.3×10^{-3}) [58], and soybean (1.25×10^{-3}) [59]. Simko et al. (2006) presented a somewhat higher nucleotide diversity for potato of $\pi = 14.6 \times 10^{-3}$, but their sample included a number of distantly related wild potato species [56].

For discovery of common sequencing variants (i.e. variants with a high MAF), only a limited number of potato cultivars have to be sequenced, as more than half of all variants were already detected by sequencing three random cultivars. In a previous study in potato, SNPs have been identified in cDNA of a set of three to six – mainly North-American – potato cultivars, using Sanger EST-sequences available in GeneBank from three potato cultivars, and using high-throughput transcriptome sequencing on three additional cultivars [2]. The SNPs identified in this relatively small sample, are expected to mainly represent the common SNPs from all coding sequences, whereas this study included introns and sampled rare alleles. Based on exons sequenced in both studies an overlap of only 2,572 variants was expected between our 129,156 variants and the 69,586 mapped SNPs identified by Hamilton et al. (2011). We detected 2,362 (92%) of these SNPs. As only one cultivar was included in both studies (cv. Bintje), the 8% of variants undetected in our study might represent rare variants more specific to the North American cultivars predominantly sampled by Hamilton et al. (2011), or may be false positive/negatives in either study. As a result of the at least 14-fold larger sample size and larger geographic diversity targeted by our study, we find approximately 15,000 extra variants in the exon sequences covered by both studies. These include multi-allelic sequence variants at 6% of the base positions where SNPs were called by Hamilton et al. (2011). When these SNPs are assumed bi-allelic, they may complicate genotype calling in a SNP genotyping array.

An important feature affecting the application of sequence variants as molecular markers is their MAF, which influences the type of information provided by the marker in different populations. Moderate-frequency alleles are valuable for mapping studies, where it is desirable to maximize the number of polymorphic markers between two parental lines. Rare variants can, if not assayed, cause ascertainment bias in phylogenetic reconstructions, overestimations of mean diversity [60], and spurious correlations in association mapping [61]. This paper presents an L-shaped distribution of the MAF (Figure 5) which points to an abundance of rare alleles in the cultivated gene pool. In comparison with another vegetatively propagated outbreeder, *Vitis vinifera*, the average MAF of potato (0.14) is lower. *Vitis vinifera* has an average MAF of 0.24 [62]. In a population of 80 grapevine cultivars, over 80% of SNP variants had a MAF above 0.10, while in potato we found only 48% of the variants had a MAF above 0.10. We expect that wild species introgressions with low MAF contribute significantly to the high variant density in potato.

To investigate whether we could identify potential signatures of selection in the potato genome, we examined the nucleotide diversity of loci along the physical position of each chromosome. Nucleotide diversity values were very variable both between and within chromosomes. Chromosomes 5 and 11 exhibited highest nucleotide diversity. Introgression of resistance genes from wild species is a likely explanation, as chromosome 5 and 11 contain the largest clusters of resistance genes, conferring resistance to a wide variety of pathogens [63,64]. In contrast, overall nucleotide diversity was reduced on chromosome 10. Some of the most conserved genes in the dataset are located near the position of the skin colour, tuber shape and eye depth QTL on chromosome 10 [65–67]. An eye-catching difference between commercial western germplasm and Latin American land races are the highly variable tuber shapes and anthocyanin pigmentation patterns in tuber skin

and flesh. Hence this reduced nucleotide diversity could well reflect a selective sweep.

Within all chromosomes, individual genes were found with reduced nucleotide diversity as well. It is not possible at this stage to say whether these genes themselves, rather than closely linked loci are under selection, but some of these genes are good candidate genes for phenotypic traits under strong selection like day-length dependent tuberisation and some resistance traits. An example of a good direct candidate gene is the *CONSTANS* gene that has been shown to affect the day-length regulation of tuber induction in potato [68] and is the second most conserved gene we sampled on chromosome 2.

Chloroplast Types and Population Structure

The presence of an extreme cytoplasmic bottleneck in cultivated potato has been described before [69]. Five main cpDNA types (A, S, C, W and T) and a number of sub-types have been described [70]. The A haplotype is most frequent in Group Andigena and the T haplotype in Chilean *S. tuberosum* and modern cultivars. Diploid *S. tuberosum* Group Phureja is assigned to the S-type and wild material like *S. vernei* and *S. demissum* to the W-type. Our phylogeny of chloroplast haplotype data supports previous work suggesting that most modern cultivars have chloroplasts resembling those of the *S. tuberosum* Group Tuberosum genepool (T-Type) [71,72]. Two cultivars have a W-type haplotype originating from *S. vernei* (VE71–105 and VTN62–33–3) and thirteen cultivars have a W-type haplotype originating from *S. demissum* [73]. These chlorotypes have been introduced during introgression of resistance traits. More remarkably, four heirloom cultivars in our sample (cv. Belle De Fontenay, cv. Keppelstone Kidney, cv. Home Guard, and cv. Shamrock) have chloroplast haplotypes phylogenetically close to that from the *S. tuberosum* Group Phureja (S-Type). This chlorotype might represent the *S. tuberosum* Group Andigena type (A-Type) that was more common in cultivars from before the 1840s late blight (*Phytophthora infestans*) epidemic [74].

The genome-wide population structure of this study largely corresponds with the structure observed using AFLP markers [48]. While the AFLP study distinguished five divergent cultivar groups, we identified four, with one outlier forming its own group. Both analyses support genetic similarity within heirloom cultivars and within starch cultivars, but the high-throughput approach used here did not support separate groups of fresh consumption, processing (crisps or fries), and additional miscellaneous cultivars. Instead, PCA analysis suggested the existence of a cluster of cultivars originating from continental Europe, consisting mainly of processing cultivars, along with a cluster containing cultivars of mixed, world-wide origin. Given that the first three components of the principal component analysis account for less than 15% of the total variation, it seems that there is little population structure within the cultivated potato gene pool, as was also observed by D'hoop et al. (2010).

Concordance between GBS and KASP Genotyping in an Autotetraploid

In diploids it is known that the accuracy of variant detection and genotyping depends on sequence depth and the mode of sexual reproduction (homozygous selfers vs. heterozygous outbreeders) [75,76]. Genotyping variants in polyploid species, such as potato, is more challenging than in diploids, because a given gene may be represented not only by a number of different alleles, but also by different zygosity levels. We therefore tested the accuracy of genotype calls made by GBS by testing a small subset of binary SNPs using an independent KASP genotyping platform. The genotype calls of GBS were found to be over 99% consistent with

KASP when scored as homozygous or heterozygous. We applied a genotype quality (GQ) parameter to account for differences in read depth requirement across all zygosity classes (nulliplex, simplex, duplex, triplex, and quadruplex). Over 95% of duplex calls (the zygosity class with lowest consistency between GBS and KASP) were consistent between the two genotyping methods at a threshold of GQ26. Overall concordance of the five zygosity classes was 98.4% at this threshold. In conclusion, for genotyping autotetraploids a GQ26 is recommended to achieve reliable genotype calls using our pipeline. In a more general sense, our results indicate that read depths of 60–80x are sufficient for reliable, dosage diagnostic genotype calls in GBS in autotetraploid potato.

Association Analysis using GBS

The GBS dataset was tested for its performance in GWAS. Even though the data do not represent a complete genome, but a focussed survey at 807 loci randomly distributed across the genome, and although the number of 83 potato cultivars severely limits statistical power, several interesting traits could be mapped. It appeared that the 129,156 sequence variants provide a robust dataset to detect alleles influencing monogenic traits such as plant maturity and tuber flesh colour (this paper) and various other monogenic traits such as tuber shape and pathogen resistance (unpublished data). The sequence variants associated with tuber flesh colour and plant maturity are indeed located in and near the respective candidate genes *CHY2* [34] and *StCDF1*. The latter was recently shown to be the causal gene involved in potato day-length signalling [77]. We observed that a simplistic dominant model (0/1 data reflecting absence/presence of an allele) gave similar GWAS results as the more detailed additive model (0/1/2/3/4 copies of the allele). This makes sense, since 61% of the sequence variants have a MAF below 0.05. If such variants follow Hardy-Weinberg expectations, in practice the minor allele will be absent in ~81% of the cultivars and simplex in ~17% of the cultivars, while a duplex cultivar is hardly expected (1.4%). This notion, that many sequence variants have a low allele frequency, points to the advantage that the input GBS data does not require the more stringent genotype quality filtering necessary for accurate estimation of allele copy number.

GWAS requires marker densities surpassing the decay in LD. In this study only a fraction (2.1 Mb, 0.25%) of the 840 Mb potato genome was re-sequenced, but in most cases the genetic distance between our 807 candidate genes will be less than the estimated LD decay of 4–10 cM [48,56]. For the data generated here, both short- and long-range LD still needs to be analysed. Although short-range LD could have been analysed, long-range LD requires the robust ordering of superscaffolds of the DM reference genome into physical chromosomes, preferentially in combination with an aligned high-resolution genetic linkage map. These maps and pseudo-molecules are currently being developed to estimate LD decay on both genetic (cM) and physical (bp) scale in the near future. We expect that the identified sequence variants and regions covered in this study are in LD with most functional alleles. We also expect that identification of haplotypes and phasing of the sequence variants can be achieved by using a ‘read-backed phasing’ approach (unpublished data) for use in a multi-allelic GWAS analysis. Since a large number of genes in our study were either known candidate genes, or primary- and secondary metabolism genes, the annotated biochemical pathway may

suggest a biological link between the gene underlying the sequence variants, and the QTL. Alternatively, the potato genome browser (<http://solanaceae.plantbiology.msu.edu>) will support the identification of candidate genes in proximity to QTL.

Supporting Information

Supporting Information S1 Supporting Tables and Figures.

(DOC)

BED-file S1 SureSelect baits mapped (MQ≥37) to the superscaffolds of the DM reference genome.

(BED)

BED-file S2 Accessible genome regions of the DM reference genome.

(BED)

CSV-file S1 Annotations for sequence variants identified in the accessible genome, including allele copy numbers of each of the 84 samples.

(GZ)

FASTA-file S1 SureSelect RNA bait sequences.

(FASTA)

FASTA-file S2 SureSelect target sequences.

(FASTA)

VCF-file S1 Sequence variants and genotypes identified in the accessible potato DM genome of 84 samples.

(GZ)

VCF-file S2 Sequence variants identified in the chloroplast genome.

(VCF)

XLS-file S1 Annotations for genomic SureSelect targets, including observed coverage in resequencing data.

(XLS)

XLS-file S2 Annotations for accessible genome regions, based on sequence data collected in this study.

(XLS)

XLS-file S3 Annotations of nucleotide diversity for accessible genes.

(XLS)

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Author Contributions

Conceived and designed the experiments: JU AW BD HvE. Performed the experiments: JU. Analyzed the data: JU TB. Contributed reagents/materials/analysis tools: TB. Wrote the paper: JU RV HvE.

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Generation of self-compatible diploid potato by knockout of *S-RNase*

Mingwang Ye^{1,2,5}, Zhen Peng^{1,2,5}, Die Tang², Zhongmin Yang³, Dawei Li³, Yunmei Xu¹, Chunzhi Zhang^{2,4*} and Sanwen Huang^{2,4*}

Re-domestication of potato into an inbred line-based diploid crop propagated by seed represents a promising alternative to traditional clonal propagation of tetraploid potato, but self-incompatibility has hindered the development of inbred lines. To address this problem, we created self-compatible diploid potatoes by knocking out the self-incompatibility gene *S-RNase* using the CRISPR–Cas9 system. This strategy opens new avenues for diploid potato breeding and will also be useful for studying other self-incompatible crops.

Potato (*Solanum tuberosum*) is the most important tuber crop worldwide. Unlike other major crops, potato is an autotetraploid plant that is propagated from tubers. Potato researchers and breeders have long been challenged by the tetrasomic inheritance of potato and the need for vegetative propagation. The genetic gain from population improvement in major crops such as maize is approximately 1% per year¹. By contrast, breeding has done little to increase yields in potato. Some century-old potato varieties, such as Russet Burbank (released in 1902)² and Bintje (bred in 1904)³, are still widely cultivated. Thus, an increasing number of studies has focused on reinventing potato as an inbred line-based diploid crop that is propagated by seed^{4–6}. Indeed, diploids were very prevalent among wild species and landraces, accounting for 70% of the potato germplasm⁷. Re-domestication of these diploid potatoes using modern agricultural technologies would represent a breakthrough in potato breeding. However, most tuber-bearing diploid potato species are self-incompatible, which has hampered the development of inbred lines.

Self-incompatibility in potato is controlled by the highly polymorphic *S*-locus⁸. The identification of a dominant *S*-locus inhibitor (*Sli*), originating from the wild potato species *Solanum chacoense*, has paved the way for exploring diploid breeding in potato^{9,10}. This gene has been widely used in genetic research and breeding^{9,11–13}. However, wild *S. chacoense* accessions produce long stolons, approximately 1 m in length¹⁴, which will lead to excessive loss of energy in stolon growth and difficulty in harvesting. Moreover, the tubers of *S. chacoense* contain high levels of toxic steroidal glycoalkaloids^{15,16}. Owing to these undesirable traits, a viable alternative to the deployment of the *Sli* gene is required. Thus, in the current study, we used clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein 9 (Cas9) to create a self-compatible diploid potato line without introducing any wild DNA fragments.

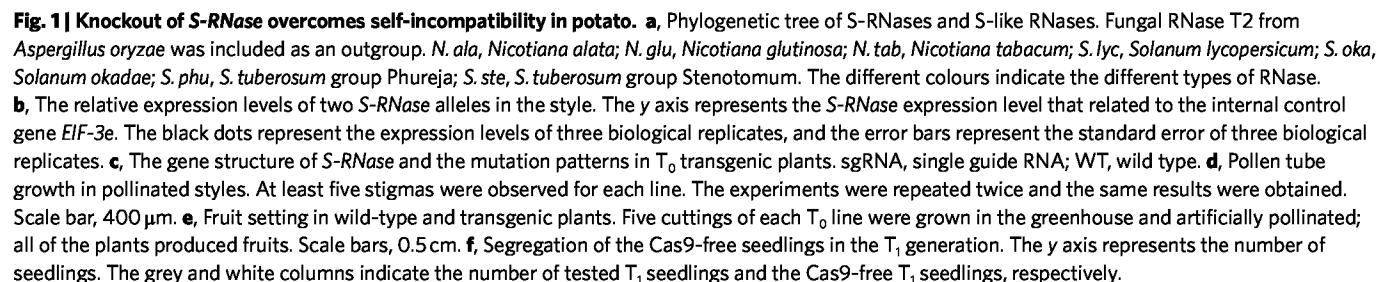
The *S-RNase* gene, which controls self-incompatibility in potato, has high allelic diversity, with amino acid similarity varying from

32.9% to 94.5%¹⁶, and is highly expressed in styles¹⁷. The traditional method used to obtain the full-length sequences of genes such as *S-RNase* is rapid amplification of cDNA ends. However, high rates of polymorphism make it difficult to design the appropriate primers for successful amplification by rapid amplification of cDNA ends. The presence of relatively conserved domains in *S-RNase* and its tissue-specific expression pattern motivated us to isolate the full-length *S-RNase* sequence by RNA sequencing (RNA-seq).

To verify the utility of this method, we generated RNA-seq data using RNA from the styles of the diploid potato clone *S. tuberosum* group Phureja S15-65. We carried out de novo assembly of the RNA-seq data using Trinity (v2.2.0) and calculated the expression levels of all transcripts using RSEM (v1.2.9). In total, 83,545 transcripts were obtained, which greatly exceeded the number of genes (39,000) in the potato reference genome, as some genes had different isoforms or their complete transcripts were divided into different fragments. Using the sequence of the *S-RNase* protein from the potato reference genome DM as a query, five target sequences were found among the assembled transcripts, all of which were highly expressed in the style (Supplementary Table 1). The target sequences contained two 5' untranslated regions and two 3' untranslated regions; thus, we designed PCR primers to amplify the full-length *S-RNase* genes (Supplementary Table 2). We ultimately obtained two complete *S-RNase* alleles (*S_{p3}* and *S_{p4}*, named in accordance with the naming conventions proposed by Dzidzienyo et al.¹⁸) (Supplementary Fig. 1). The amino acid sequences of *S_{p3}* and *S_{p4}* share a similarity of 59%, indicating that they are different *S-RNase* alleles. Phylogenetic analysis showed that they are located in the same clade as known functional *S-RNase* genes (Fig. 1a). We also validated their expression by quantitative PCR (Fig. 1b). Our results demonstrate that de novo assembly of RNA-seq data is an efficient, low-cost way to clone genes, especially for genes with high diversity in a population.

The self-incompatibility mechanism is conserved in Solanaceae^{9,18}. Although most wild tomatoes are self-incompatible, cultivated tomato is self-compatible due to the loss of function of *S-RNase* and other self-incompatibility-related genes¹⁹. Thus, we reasoned that we could re-invent self-incompatible diploid potato by knocking out the *S-RNase* alleles using the CRISPR–Cas9 system. To induce mutations in *S-RNase*, we designed a CRISPR–Cas9 construct targeting the first exon of *S-RNase*. The single guide RNA was designed to target the relatively conserved domain of the *S-RNase* protein and could target *S_{p3}* and *S_{p4}* simultaneously (Supplementary Fig. 1). We generated 96 plantlets that grew on medium containing

¹The CAAS–YNNU Joint Academy of Potato Sciences, Yunnan Normal University, Kunming, China. ²Genome Analysis Laboratory of the Ministry of Agriculture, Agricultural Genomics Institute at Shenzhen, Chinese Academy of Agricultural Sciences, Shenzhen, China. ³College of Horticulture, Northwest Agriculture and Forest University, Yangling, China. ⁴Key Laboratory of Biology and Genetic Improvement of Horticultural Crops of the Ministry of Agriculture, Sino-Dutch Joint Laboratory of Horticultural Genomics, Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing, China. ⁵These authors contributed equally to this work: Mingwang Ye, Zhen Peng. *e-mail: zhangchunzhi01@caas.cn; huangsanwen@caas.cn



Moreover, the corresponding in vitro-grown plants were much stronger than the wild-type clones. Thus, the ploidy of these plants might have doubled during tissue culture, which is a common phenomenon in potato³⁸. Flow cytometry analysis revealed that some plants were tetraploid (Supplementary Fig. 2); these clones

were excluded from further analysis. We ultimately obtained five mutant diploid clones, each containing mutations in at least one *S-RNase* allele (Fig. 1c and Supplementary Table 3). As *S-RNase* is a co-dominant gene, the mutation of either allele would enable pollen containing the same *S*-haplotype to extend into the style, thereby resulting in self-compatibility.

To investigate the self-compatibility of the transgenic plants, we transferred all mutant clones to the greenhouse under a 16-h light/8-h dark cycle. We performed artificial pollinations at the open flower stage, with at least 50 flowers self-pollinated per line. We observed the growth of pollen tubes in styles by aniline blue fluorochrome staining. In wild-type styles, pollen tube growth was suppressed and was restricted to the upper part of the style, whereas in mutant styles, many pollen tubes extended to the ovary (Fig. 1d). After self-pollination, all mutant lines produced fruits (Fig. 1e). Although the rate of fruit setting varied among lines, the number of seeds per fruit was sufficient for further propagation, ranging from 67 to 288 seeds per fruit. These results demonstrate that inducing loss-of-function mutations in *S-RNase* genes resulted in self-compatibility in potato. The growth vigour and plant morphology of the mutant lines did not differ from those of the wild type, indicating that they can directly be used for breeding.

To determine whether the mutations could be transmitted to the T_1 generation, we sowed the T_1 seeds on the MS medium. The germination rate was >95% for all five families. The ratio of T_1 plants without the Cas9 cassette varied from 3.6% to 24.5% (Fig. 1f and Supplementary Table 3). The Chi-square test showed that lines 42 and 44 contained a single copy of the Cas9 cassette and lines 32 and 57 contained two copies. However, as the ratio of the Cas9-free T_1 plants of line 66 is lower than 1/4 but higher than 1/16, we infer that the number of T_1 plants used for the test might not be enough or the genomic region harbouring the Cas9 cassette might exhibit segregation distortion in the selfed progeny. We detected mutations in the *S-RNase* genes in all Cas9-free plants. The chimeric mutations in lines 32 and 44 did not inherit into the T_1 generation and only a single mutation of *S_{pd}* in line 57 was transmitted to the T_1 generation (Supplementary Table 3), suggesting that the mutations in the leaves and germ cells were different. All T_1 plants harboured mutations in at least one *S-RNase* allele (Supplementary Table 3), indicating that the pollen containing the wild-type *S-RNase* was rejected during selfing.

In summary, we designed an efficient approach for overcoming self-incompatibility in potato without introducing any exogenous DNA. Using the same method, we also obtained *S-RNase* mutants using two *S. tuberosum* group Phureja clones (S15-47 and S15-76) and two *S. tuberosum* group Stenotomum clones (S15-48 and S15-107) (Supplementary Table 2). More self-compatible clones are needed to increase the genetic diversity of the *S*-locus to avoid a potential genetic bottleneck in future diploid breeding efforts. These self-compatible diploid potatoes can be used to produce inbred lines, which can be further used to generate mutant libraries, recombinant inbred lines, near isogenic lines and introgression lines. These populations will be a powerful tool to accelerate basic research and genetic improvement in potato. Furthermore, the strategy presented should be beneficial for researchers and breeders of other self-incompatible crops, such as plants in the Cruciferae and Rosaceae families, allowing them to make more rapid progress in genetic improvement.

Methods

De novo assembly of RNA-seq data. Total RNA was isolated from potato styles using an RNAPrep Pure Plant Kit (polysaccharides and polyphenolics rich) (Tiangen). The RNA was subjected to whole-genome resequencing using the Illumina HiSeq X Ten platform (reads length = 150 bp) (Novogene). The insert size of the library was 300 bp and 13 million reads were generated. The RNA-seq data were de novo assembled using Trinity (v2.2.0) without a reference guide with default parameters. Transcript qualification and analysis of the differential

expression of genes and isoforms in the samples were performed using RSEM (v1.2.9). The assembled transcripts were built as the local database and known *S-RNase* protein was aligned to the database using the tblastn program of BLAST (v2.5.0) with default parameters on the Linux system; those with a low E-value ($<1 \times 10^{-5}$) and a high FPKM (fragments per kilobase of transcript per million mapped reads) value (>200) were identified as candidate *S-RNase* sequences.

Quantitative PCR. The RNA was reverse transcribed using a FastQuant RT Kit (with gDNase) (Tiangen). Gene-specific oligonucleotides were designed using the Primer Premier 5 software (Applied Biosystems). *EIF-3e* was used as the internal control gene. PCRs were performed on a BIO-RAD CFX96 using SYBR Premix (Roche) according to the manufacturer's instructions. The relative gene expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method³³.

Plasmid construction and transformation. The 19-nt single guide RNA sequence for *S-RNase* in the diploid clone S15-65 was selected manually from the conserved domain and used for BLAST analysis against the potato reference genome version 4.03 (ref. ³³); no other potential matches in the genome were identified. The target sequence was incorporated into the CRISPR-Cas9 vector pKSE401 (ref. ³³). The diploid self-incompatible *S. tuberosum* group Phureja S15-65 clone was used in this study. Three-week-old plantlets were used for transformation. *Agrobacterium*-mediated transformation of potato internodes was conducted as previously described with some modifications³³; after 2 days of pre-culture, the explants were co-cultured with *Agrobacterium* harbouring pKSE401 with the target sequence for 2 days in the presence of 2 mg l^{-1} α -naphthaleneacetic acid and 1 mg l^{-1} zeatin, followed by callus induction and regeneration mediated by 0.01 mg l^{-1} α -naphthaleneacetic acid and 2 mg l^{-1} zeatin until shoot proliferation. Positive transformants were screened based on growth on the medium containing 50 mg l^{-1} kanamycin.

Detection of mutations in the transgenic lines. To detect the mutations in *S-RNase*, the full-length *S-RNase* sequences were amplified from all regenerated plantlets and sequenced. As chromosome doubling occurred at high frequency during potato callus regeneration³³, the ploidy of the mutated plants was determined by flow cytometry (BD FACSDiva v7.0 Software).

Aniline blue fluorochrome staining of pollen tubes. Pollinations were performed using mature self-pollen. Carpels were collected 48 h post-pollination and immediately placed in 3:1 95% ethanol:glacial acetic acid. After fixation, the carpels were submerged in 5 M NaOH softening solution for 24 h. After washing with double-distilled water, the carpels were incubated in 0.005 mg ml^{-1} aniline blue fluorochrome in $0.1 \text{ M K}_2\text{HPO}_4$ (pH 10) buffer for 24 h and viewed under a fluorescence microscope.

Analysis of selfed progeny. The T_1 seeds were sown in modified MS medium (2.2 g l^{-1} MS powder, 10 g l^{-1} sucrose and 6 g l^{-1} agar (pH 5.8)). Four weeks after sowing, DNA was extracted from fresh leaves. The detection of the Cas9 cassette was performed for all T_1 plants. The two *S-RNase* alleles in all clones lacking the Cas9 cassette were amplified and sequenced.

Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The RNA-seq data have been deposited in the NCBI with the accession number SRX3978417.

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Author contributions

S.H. and C.Z. conceived and designed the experiments. M.Y. and Z.Y. performed the potato transformation. Z.P. and Y.X. conducted the genotyping and phenotyping of T₀ and T₁ plants. D.T. performed the bioinformatics analyses. D.L. made the CRISPR–Cas9 construct. C.Z., Z.P., M.Y. and S.H. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Data collection

No software was used.

Data analysis

Trinity (v2.2.0) was used for de novo assembly of RNA-seq data. RSEM (v1.2.9) was used to calculate the expression of genes and isoforms. The program tblastn of BLAST (v2.5.0) was used to search for the S-RNase homologs. BD FACSDiva (v7.0) was used to analyze the results generated by flow cytometry.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research [guidelines for submitting code & software](#) for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The RNA-seq data has been deposited in NCBI with accession number SRX3979417.

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/authors/policies/ReportingSummaryFinal.pdf](https://www.nature.com/authors/policies/ReportingSummaryFinal.pdf).

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Based on our previous studies, there are usually one or two copies of Cas9-cassette transformed into the T0 regenerated plants in potato. According to the Mendelian genetic law, the segregation of Cas9-containing and Cas9-free plants in T1 generation should be 3:1 or 15:1. To screening the Cas9-free T1 plants, we screened 135 or 192 T1 seedlings for each T0 family, and obtained the Cas9-free plants for all families.
Data exclusions	No data was excluded for analysis.
Replication	In this study, we developed a method to generate self-compatible diploid potato. We tested this method in five other diploid potato clones, and it worked well for all of them.
Randomization	This is not relevant to our study. No allocation was involved in this study.
Blinding	Blinding was not relevant to this study, because all data are objective.

Reporting for specific materials, systems and methods

Materials & experimental systems	Methods
n/a	n/a
☒ Involved in the study	☒ Involved in the study
☒ Unique biological materials	☒ ChIP-seq
☒ Antibodies	☐ Flow cytometry
☒ Eukaryotic cell lines	☒ MRI-based neuroimaging
☒ Palaeontology	
☒ Animals and other organisms	
☒ Human research participants	

Flow Cytometry

Plots

Confirm that:

- ☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- ☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- ☒ All plots are contour plots with outliers or pseudocolor plots.
- ☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	The fresh leaves from in vitro plants were chopped and incubated in lysate. Then the cell nucleus were collected by centrifugation.
Instrument	BD LSRII Fortessa™ X-20
Software	BD FACSDiva v7.0 Software
Cell population abundance	The proportion of target cell was higher than 70%.

Gating strategy

We used known diploid and tetraploid potato clones as control to deduce the ploidy of transgenic plants.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Willem Hendrik Lindhout *et al.*

Serial No.: 16/541,071

Filed: August 14, 2019

For: Hybrid Seed Potato Breeding

Group Art Unit: 1663

Examiner: Russell T. Boggs

Atty. Dkt. No.: VONL:004USC1

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 Forms 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).

No fees are believed to be due in connection with the filing of this Supplemental Information Disclosure Statement. However, should any fees under 37 C.F.R. §§ 1.16 to 1.21 be deemed necessary for any reason relating to these materials, the Commissioner is authorized to deduct the appropriate fees from Dentons US LLP's Deposit Account No.: 19-3140/VONL:004USC1.

Respectfully submitted,

/Amy Dunstan/

Amy Dunstan
Reg. No. 61,069
Agent for Applicants

Dentons US LLP
2000 McKinney Avenue, Suite 1900
Dallas, Texas 75201
(214) 259-0965

Date: March 29, 2021

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 et al.	
	Art Unit	1663	
	Examiner Name	Russell T. Boggs	
	Attorney Docket Number	VONL:004USC1	

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

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

U.S. PATENT APPLICATION PUBLICATIONS						
Examiner Initial*	Cite No	Publication Number	Kind Code ¹	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear
	1	20210029956		2021-02-04	Lindhout et al.	
	2	20210137041		2021-05-13	de Vries et al.	

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

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

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NON-PATENT LITERATURE DOCUMENTS								
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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 et al.
Art Unit	1663
Examiner Name	Russell T. Boggs
Attorney Docket Number	VONL:004USC1

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
	1		☐

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

EXAMINER SIGNATURE

Examiner Signature		Date Considered	
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*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.

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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 et al.
Art Unit	1663
Examiner Name	Russell T. Boggs
Attorney Docket Number	VONL:004USC1

CERTIFICATION STATEMENT

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

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

OR

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

☐ See attached certification statement.

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

☒ A certification statement is not submitted herewith.

SIGNATURE

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

Signature	/Kristi D. Wilson/	Date (YYYY-MM-DD)	2021-06-28
Name/Print	Kristi D. Wilson	Registration Number	77,672

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.**

Electronic Acknowledgement Receipt

EFS ID:	43105770
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:	Kristi D. Wilson
Filer Authorized By:	
Attorney Docket Number:	VONL:004USC1
Receipt Date:	28-JUN-2021
Filing Date:	14-AUG-2019
Time Stamp:	14:12:23
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	VONL004USC1SIDSTRANS0628 2021.pdf	20719	no	2
			2c8b629f555c0523982df64b1a08e12e416 8b87a		

Warnings:

Aardevo Exhibit 1010

Information:					
2	Information Disclosure Statement (IDS) Form (SB08)	VONL004USC1SIDS06282021. pdf	60135	no	3
			8edb2de3f2137bb0b28e181d3b091cd0bf d86b11		
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
Total Files Size (in bytes):			80854		
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. <u>New Applications Under 35 U.S.C. 111</u> 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. <u>National Stage of an International Application under 35 U.S.C. 371</u> 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. <u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.					

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Willem Hendrik Lindhout, *et al.*

Serial No.: 16/541,071

Filed: August 14, 2019

For: HYBRID SEED POTATO BREEDING

Group Art Unit: 1663

Examiner: Russell T. Boggs

Atty. Dkt. No.: VONL:004USC1

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).

This Supplemental Information Disclosure Statement, in accordance with 37 C.F.R. § 1.97(b)(4) is being filed prior to the mailing of a first Office Action on the merits after filing a Request for Continued Examination. No fees are believed due. However, if any fee is due, the Commissioner is authorized to deduct the appropriate fee from Dentons US LLP's Deposit Account 19-3140/VONL:004USC1.

Respectfully submitted,

/Kristi D. Wilson/

Kristi D. Wilson
Reg. No. 77,672
Agent for Applicants

Dentons US LLP
2000 McKinney Avenue, Suite 1900
Dallas, Texas 75201
(214) 259-1872

Date: June 28, 2021



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/541,071	08/14/2019	Willem Hendrik Lindhout	VONL:004USC1	4348
125771	7590	07/23/2021	EXAMINER	
DENTONS US LLP			BOGGS, RUSSELL T	
P.O. Box 061080			ART UNIT	PAPER NUMBER
Chicago, IL 60606			1663	
			NOTIFICATION DATE	DELIVERY MODE
			07/23/2021	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

<i>Examiner-Initiated Interview Summary</i>	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 of Record	
ROB HANSON	Attorney	

Date of Interview: 13 July 2021

Issues Discussed:

35 U.S.C. 112

The parties discussed the meaning of "essentially heterozygous" in claim 45. Agreement was reached to delete limitation via an examiner's amendment. (Pending the application owner's approval.)

35 U.S.C. 102

The composition claims, 40, 45 and 48, under a product-by-process analysis, can be rejected under the prior art of record. The parties agreed to amend the claims as per attached draft. (Pending the application owner's approval.)

Non-statutory Double Patenting

A terminal disclaimer needs to be filed as per the Office action on page 39 et seq.

☒ Attachment

/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: It is not necessary for applicant to provide a separate record of the substance of 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.

Doc code: IDS

Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)

Approved for use through 11/30/2020. OMB 0651-0031

U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

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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					

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

U.S.PATENT APPLICATION PUBLICATIONS						
Examiner Initial*	Cite No	Publication Number	Kind Code ¹	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1					

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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)

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

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

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

EXAMINER SIGNATURE

Examiner Signature	/RUSSELL T BOGGS/	Date Considered	06/26/2021
--------------------	-------------------	-----------------	------------

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

CERTIFICATION STATEMENT

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

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

OR

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

☐ See attached certification statement.

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

☒ A certification statement is not submitted herewith.

SIGNATURE

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

Signature	/Sheila Walker/	Date (YYYY-MM-DD)	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.**

Doc code: IDS

Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)

Approved for use through 11/30/2020. OMB 0651-0031

U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

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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 et al.		
	Art Unit	1663		
	Examiner Name	Russell T. Boggs		
	Attorney Docket Number	VONL:004USC1		

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

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

U.S. PATENT APPLICATION PUBLICATIONS						
Examiner Initial*	Cite No	Publication Number	Kind Code ¹	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear
/R. T. B./	1	20210029956		2021-02-04	Lindhout et al.	
/R. T. B./	2	20210137041		2021-05-13	de Vries et al.	

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

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

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

NON-PATENT LITERATURE DOCUMENTS								
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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 et al.
Art Unit	1663
Examiner Name	Russell T. Boggs
Attorney Docket Number	VONL:004USC1

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
	1		☐

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

EXAMINER SIGNATURE

Examiner Signature	/RUSSELL T BOGGS/	Date Considered	07/17/2021
--------------------	-------------------	-----------------	------------

*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.

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

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OR

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☐ See attached certification statement.

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Signature	/Kristi D. Wilson/	Date (YYYY-MM-DD)	2021-06-28
Name/Print	Kristi D. Wilson	Registration Number	77,672

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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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First Named Inventor	Willem Hendrik Lindhout
Art Unit	1663
Examiner Name	Russell T. Boggs
Attorney Docket Number	VONL:004USC1

/R.T.B/	1	Uitdewilligen et al., PLoS ONE 8(5), e62355 (2013)	☐
/R.T.B/	2	Ye et al., Nature Plants 4(9): 651-654 (2018)	☐

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Examiner Signature	/RUSSELL T BOGGS/	Date Considered	06/26/2021
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CERTIFICATION STATEMENT

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

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SIGNATURE

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Signature	/Amy Dunstan/	Date (YYYY-MM-DD)	2021-03-29
Name/Print	Amy Dunstan	Registration Number	61,069

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/R.T.B/	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.	☐
/R.T.B/	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.	☐
/R.T.B/	3	Ritte et al. (2002) "The starch-related R1 protein is an α -glucan, water dikinase", Proc. Nat. Acad. Sci. USA. 99:7166-7171.	☐
/R.T.B/	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.	☐
/R.T.B/	5	Rommens et al. (2008) "Engineered native pathways for high kaempferol and caffeoylquinic acid production in potato", Plant Biotech J. 6:870-886.	☐
/R.T.B/	6	Rommens et al. (2008) "Low-acrylamide French fries and potato chips", Plant Biotech. J. 6:843-853.	☐
/R.T.B/	7	Rommens et al. (2006) "Improving potato storage and processing characteristics through all-native DNA transformation-J", Agric. Food Chem 54:9882-9887.	☐
/R.T.B/	8	Roupe 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.	☐
/R.T.B/	9	Roupe 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.	☐
/R.T.B/	10	Roupe van der Voort et al. (1999) "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", Euphytica 106: 187-195.	☐
/R.T.B/	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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Examiner Name	Russell T. Boggs
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/R.T.B/	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.	☐
/R.T.B/	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.	☐
/R.T.B/	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.	☐
/R.T.B/	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.	☐
/R.T.B/	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).	☐
/R.T.B/	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.	☐
/R.T.B/	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.	☐
/R.T.B/	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.	☐
/R.T.B/	20	Thygesen et al. (1995) "Polyphenol oxidase in potato. A multigene family that exhibits differential expression patterns", Pl. Physiol. 109:525-531.	☐
/R.T.B/	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.	☐
/R.T.B/	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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Art Unit	1663
Examiner Name	Russell T. Boggs
Attorney Docket Number	VONL:004USC1

/R.T.B/	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).	☐
/R.T.B/	24	USPTO: Final Office Action dated December 15, 2016, regarding U.S. Application No. 14/112,747 (Atty. Dkt. No. VONL:006US).	☐
/R.T.B/	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).	☐
/R.T.B/	26	USPTO: Non-Final Office Action dated December 22, 2017, regarding U.S. Application No. 14/112,747 (Atty. Dkt. No. VONL:006US).	☐
/R.T.B/	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).	☐
/R.T.B/	28	USPTO: Final Office Action dated August 7, 2018, regarding U.S. Application No. 14/112,747 (Atty. Dkt. No. VONL:006US).	☐
/R.T.B/	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).	☐
/R.T.B/	30	USPTO: Final Office Action dated October 27, 2020, regarding U.S. Application No. 14/112,747 (Atty. Dkt. No. VONL:006US).	☐
/R.T.B/	31	van der Leij et al. (1991) "Sequence of the structural gene for granule-bound starch synthase of potato (<i>Solanum tuberosum</i> L.) and evidence for a single point deletion in the amf allele", Mol. Gen. Genet. 228:240-248.	☐
/R.T.B/	32	van der Vossen et al. (2000) "Homologues of a single resistance-gene cluster in potato confer resistance to distinct pathogens a virus and a nematode", Plant J. 23:567-576.	☐
/R.T.B/	33	van der Vossen et al., "An ancient R gene from the wild potato species <i>Solanum bulbocastanum</i> confers broad - spectrum resistance to <i>Phytophthora infestans</i> in cultivated potato and tomato," The Plant Journal 36:867-882, 2003.	☐

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Art Unit	1663	
Examiner Name	Russell T. Boggs	
Attorney Docket Number	VONL:004USC1	

/R. T. B./	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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Examiner Signature	/RUSSELL T BOGGS/	Date Considered	06/26/2021
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☐ See attached certification statement.

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Signature	/Amy Dunstan/	Date (YYYY-MM-DD)	2021-03-04
Name/Print	Amy Dunstan	Registration Number	61,069

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From: Walker, Sheila B.
To: Boggs, Russell
Subject: RE: SN 16/541,071 claim amendments discussed 7/13/21
Date: Wednesday, July 14, 2021 11:24:17 AM
Attachments: image001.png

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Hi Examiner Boggs,

You are correct. I have amended claim 45 below to reflect how the claim should read. We believe the client agrees with the amendments so you may proceed with an examiner's amendment as we discussed. We understand that the double patenting rejection over the '436 patent still remains from the Office Action and a Terminal Disclaimer will need to be filed to overcome this. Can we file a Telephonic Interview Summary along with a TD to address this? If that is acceptable, will you let us know as soon as possible if any other issues remain?

Thanks,
Sheila

45. (Currently Amended) A method of producing a uniform diploid hybrid *Solanum tuberosum* potato seed, which seed, when sown produces a diploid, fertile, and 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 *Sl* gene, said method comprising....



Sheila B. Walker
Patent Agent

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From: Boggs, Russell <Russell.Boggs@uspto.gov>
Sent: Wednesday, July 14, 2021 9:11 AM
To: Walker, Sheila B. <sheila.walker@dentons.com>
Cc: Boggs, Russell <Russell.Boggs@uspto.gov>
Subject: RE: SN 16/541,071 claim amendments discussed 7/13/21

[WARNING: EXTERNAL SENDER]

Good morning,
One minor point -- in claim 45 you deleted the word "and" and it needs to be re-inserted after "fertile." Right?
Please see if your client agrees with the changes.
The application would also need a terminal disclaimer with respect to US Patent No. 10,524,436 B2.
And I haven't looked at any other related patent applications.
---Russ Boggs
Patent Examiner AU 1663

From: Walker, Sheila B. <sheila.walker@dentons.com>
Sent: Tuesday, July 13, 2021 2:43 PM
To: Boggs, Russell <Russell.Boggs@uspto.gov>
Subject: SN 16/541,071 claim amendments discussed 7/13/21

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Hi Examiner Boggs,

Please find below the claim amendments we discussed earlier today for application serial no. 16/541,071. I understand that you will be able to enter these via an examiner's amendment. If there are any other issues, please feel free to contact me.

Kind regards,
Sheila Walker

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

44. (Currently Amended) A potato plant produced by the method of claim 41, wherein said potato plant is diploid, fertile, self-compatible, produces at least 200 grams fresh weight of tubers per plant, wherein at least 75% of the genomic loci in said potato plant are homozygous, and wherein said self-compatibility in said potato plant is conferred by the *Sli* gene.

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....(no

amendments needed to steps (a)-(d))

48. (Currently Amended) A uniform hybrid E₁ potato seed produced according to the method of claim
45.



Sheila B. Walker
Patent Agent

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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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/R.T.B/	12	De Jong, H., (1987) "Inheritance of pigmented tuber flesh in cultivated diploid potatoes", Amer. Potato J. 64:337-343.	☐
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/R.T.B/	31	Kim et al., "Broad spectrum late blight resistance in potato differential set plants MaR8 and MaR9 is conferred by multiple stacked R genes," Theor. Appl. Genet. 124:923-935, 2012.	☐
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Examiner Name	Russell T. Boggs	
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EXAMINER SIGNATURE

Examiner Signature	/RUSSELL T BOGGS/	Date Considered	06/26/2021
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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

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

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

OR

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

☐ See attached certification statement.

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

☒ A certification statement is not submitted herewith.

SIGNATURE

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

Signature	/Amy Dunstan/	Date (YYYY-MM-DD)	2021-03-04
Name/Print	Amy Dunstan	Registration Number	61,069

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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In re Application of:

Willem Hendrik Lindhout *et al.*

Serial No.: 16/541,071

Filed: August 14, 2019

For: Hybrid Seed Potato Breeding

Group Art Unit: 1663

Examiner: Russell T. Boggs

Attorney Docket No.: VONL:004USC1

Confirmation No. 4348

TELEPHONIC INTERVIEW SUMMARY

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

Commissioner:

On July 13, 2021, Applicants' undersigned representatives, Robert E. Hanson, Reg. No. 42,628, and Sheila Walker, Reg. No. 71,087, spoke telephonically with Examiner Boggs regarding potential allowance of the claims in view of a proposed Examiner's amendment. On July 14, 2021, the parties further communicated regarding the proposed amendments and the remaining double patenting rejection over U.S. Patent No. 10,524,436 and reached an agreement on overcoming all remaining rejections, including addressing the double patenting rejection by the submission of a Terminal Disclaimer over the cited '436 patent. In particular, the parties agreed to the following amendments:

1-34. (Cancelled)

35. (Previously Presented) 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 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, 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. (Previously Presented) The potato plant of claim 35, wherein said plant 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. (Previously Presented) The potato plant of claim 35, wherein said potato plant has a fresh weight of the foliage and shoots of at least 500 grams per plant at maturity.

39. (Cancelled)

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

41. (Previously Presented) 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 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 hybrid offspring seeds;
- (d) growing said hybrid offspring seeds to provide 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 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 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. (Currently Amended) A potato plant produced by the method of claim 41, wherein said potato plant is diploid, fertile, self-compatible, produces at least 200 grams fresh weight of tubers per plant, wherein at least 75% of the genomic loci in said potato plant are homozygous, and wherein said self-compatibility in said potato plant is conferred by the *Sli* gene.

45. (Currently Amended) A method of producing a uniform diploid hybrid *Solanum tuberosum* potato seed, which seed, when sown produces a diploid, fertile, and 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 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 *Sli* gene, 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 75% of the genomic loci in said second potato line are homozygous, wherein said self-compatibility in said second potato line is conferred by *Sli* gene, 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 and allowing said potato plant to produce berries with seeds; and

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

46. (Cancelled)

47. (Previously Presented) The method according to claim 45, wherein at least one of said first and said second potato plant 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. (Currently Amended) A uniform hybrid E₁ potato seed produced according to the method of claim 45.

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

DENTONS US LLP
2000 McKinney Avenue, Suite 1900
Dallas, Texas 75201
214-259-0931 (telephone)
214-259-0910 (fax)

Respectfully submitted,

/Sheila Walker/

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

Date: July 28, 2021

Electronic Acknowledgement Receipt

EFS ID:	43362874
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
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Attorney Docket Number:	VONL:004USC1
Receipt Date:	28-JUL-2021
Filing Date:	14-AUG-2019
Time Stamp:	16:46:24
Application Type:	Utility under 35 USC 111(a)

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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. <u>New Applications Under 35 U.S.C. 111</u> 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. <u>National Stage of an International Application under 35 U.S.C. 371</u> 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. <u>New International Application Filed with the USPTO as a Receiving Office</u> 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.	

Doc Code: DIST.E.FILE Document Description: Electronic Terminal Disclaimer - Filed		PTO/SB/26 U.S. Patent and Trademark Office Department of Commerce
Electronic Petition Request	TERMINAL DISCLAIMER TO OBIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT	
Application Number	16541071	
Filing Date	14-Aug-2019	
First Named Inventor	Willem Lindhout	
Attorney Docket Number	VONL:004USC1	
Title of Invention	Hybrid Seed Potato Breeding	
☒ Filing of terminal disclaimer does not obviate requirement for response under 37 CFR 1.111 to outstanding Office Action ☒ This electronic Terminal Disclaimer is not being used for a Joint Research Agreement.		
Owner	Percent Interest	
Agventure B.V.	100%	
The owner(s) with percent interest listed above in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of prior patent number(s) 10524436 as the term of said prior patent is presently shortened by any terminal disclaimer. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the prior patent are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns. In making the above disclaimer, the owner does not disclaim the terminal part of the term of any patent granted on the instant application that would extend to the expiration date of the full statutory term of the prior patent, "as the term of said prior patent is presently shortened by any terminal disclaimer," in the event that said prior patent later: - expires for failure to pay a maintenance fee; - is held unenforceable; - is found invalid by a court of competent jurisdiction; - is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321; - has all claims canceled by a reexamination certificate; - is reissued; or - is in any manner terminated prior to the expiration of its full statutory term as presently shortened by any terminal disclaimer. ☒ Terminal disclaimer fee under 37 CFR 1.20(d) is included with Electronic Terminal Disclaimer request.		

☐ I certify, in accordance with 37 CFR 1.4(d)(4), that the terminal disclaimer fee under 37 CFR 1.20(d) required for this terminal disclaimer has already been paid in the above-identified application.	
Applicant claims the following fee status: ☒ Small Entity ☐ Micro Entity ☐ Regular Undiscounted	
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.	
THIS PORTION MUST BE COMPLETED BY THE SIGNATORY OR SIGNATORIES I certify, in accordance with 37 CFR 1.4(d)(4) that I am: ☒ An attorney or agent registered to practice before the Patent and Trademark Office who is of record in this application Registration Number <u>71087</u> ☐ A sole inventor ☐ A joint inventor; I certify that I am authorized to sign this submission on behalf of all of the inventors as evidenced by the power of attorney in the application ☐ A joint inventor; all of whom are signing this request	
Signature	/Sheila Walker/
Name	Sheila Walker

*Statement under 37 CFR 3.73(b) is required if terminal disclaimer is signed by the assignee (owner).
 Form PTO/SB/96 may be used for making this certification. See MPEP § 324.

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:				
STATUTORY OR TERMINAL DISCLAIMER	2814	1	170	170
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Extension-of-Time:				
Miscellaneous:				
Total in USD (\$)				170

Doc Code: DISQ.E.FILE

Document Description: Electronic Terminal Disclaimer – Approved

Application No.: 16541071

Filing Date: 14-Aug-2019

Applicant/Patent under Reexamination: Lindhout

Electronic Terminal Disclaimer filed on July 28, 2021

☒ APPROVED

This patent is subject to a terminal disclaimer

☐ DISAPPROVED

Approved/Disapproved by: Electronic Terminal Disclaimer automatically approved by EFS-Web

U.S. Patent and Trademark Office

Electronic Acknowledgement Receipt

EFS ID:	43363542
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:	28-JUL-2021
Filing Date:	14-AUG-2019
Time Stamp:	16:58:14
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	\$ 170
RAM confirmation Number	E20217RG58090121
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	Terminal Disclaimer-Filed (Electronic)	eTerminal-Disclaimer.pdf	41275	no	2
			df74e4bc4a3be5623dcbcf7958cd593c7fd0e041		
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	38001	no	2
			aa18983d47bad7688c9ebf9a510a179d3378bb047		
Warnings:					
Information:					
Total Files Size (in bytes):			79276		
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. <u>New Applications Under 35 U.S.C. 111</u> 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. <u>National Stage of an International Application under 35 U.S.C. 371</u> 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. <u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.					



UNITED STATES PATENT AND TRADEMARK OFFICE

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

NOTICE OF ALLOWANCE AND FEE(S) DUE

125771 7590 08/06/2021
DENTONS US LLP
P.O. Box 061080
Chicago, IL 60606

EXAMINER	
BOGGS, RUSSELL T	
ART UNIT	PAPER NUMBER

1663

DATE MAILED: 08/06/2021

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

TITLE OF INVENTION: Hybrid Seed Potato Breeding

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	SMALL	\$600	\$0.00	\$0.00	\$600	11/08/2021

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Maintenance fees are due in utility patents issuing on applications filed on or after Dec. 12, 1980. It is patentee's responsibility to ensure timely payment of maintenance fees when due. More information is available at www.uspto.gov/PatentMaintenanceFees.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

125771 7590 08/06/2021
DENTONS US LLP
P.O. Box 061080
Chicago, IL 60606

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)
(Signature)
(Date)

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

TITLE OF INVENTION: Hybrid Seed Potato Breeding

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	SMALL	\$600	\$0.00	\$0.00	\$600	11/08/2021

EXAMINER	ART UNIT	CLASS-SUBCLASS
BOGGS, RUSSELL T	1663	800-265000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,

1 _____

(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

2 _____

3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ☐ Individual ☐ Corporation or other private group entity ☐ Government

4a. Fees submitted: ☐ Issue Fee ☐ Publication Fee (if required) ☐ Advance Order - # of Copies _____

4b. Method of Payment: (Please first reapply any previously paid fee shown above)

☐ Electronic Payment via EFS-Web ☐ Enclosed check ☐ Non-electronic payment by credit card (Attach form PTO-2038)

☐ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. _____

5. Change in Entity Status (from status indicated above)

☐ Applicant certifying micro entity status. See 37 CFR 1.29

☐ Applicant asserting small entity status. See 37 CFR 1.27

☐ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____

Date _____

Typed or printed name _____

Registration No. _____



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/541,071	08/14/2019	Willem Hendrik Lindhout	VONL:004USC1	4348
125771	7590	08/06/2021	EXAMINER	
DENTONS US LLP			BOGGS, RUSSELL T	
P.O. Box 061080			ART UNIT	
Chicago, IL 60606			PAPER NUMBER	
			1663	
DATE MAILED: 08/06/2021				

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b) (Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. 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 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	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--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to See Continuation Sheet.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ 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.

3. ☒ The allowed claim(s) is/are See Continuation Sheet. As a result of the allowed claim(s), 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**.

4. ☒ 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)).

* Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892) 2. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____. 3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____. 4. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____. /RUSSELL T BOGGS/ Examiner, Art Unit 1663	5. ☒ Examiner's Amendment/Comment 6. ☐ Examiner's Statement of Reasons for Allowance 7. ☐ Other _____.
---	--

Continuation of 1. This communication is responsive to: the response filed on 9 February 2021 and the authorization for an examiner's amendment on 28 July.

Continuation of 3. The allowed claim(s) is/are: 30,35,37-38,41,43-45 and 47-48

Notice of Pre-AIA or AIA Status

The present application, filed on or after March 16, 2013, is being examined under the first-inventor-to-file provisions of the AIA.

EXAMINER'S AMENDMENT

An Examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this amendment was given via the interview summary filed 28 July 2021 by Sheila Walker.

The application is amended as follows:

Claim 40. A seed or tuber that produces[[of]] the plant of claim 35.

Claim 44. A potato plant produced by the method of claim 41, wherein said potato plant is diploid, fertile, self-compatible, produces at least 200 grams fresh weight of tubers per plant, wherein at least 75% of the genomic loci in said potato plant are homozygous, and wherein said self-compatibility in said potato plant is conferred by the *Sli* gene.

Claim 45. A method of producing a uniform diploid hybrid *Solanum tuberosum* potato seed, which seed, when sown produces a diploid, fertile, and 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 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 *Sli* gene, 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, wherein said second potato line is diploid fertile, self-compatible and wherein at least 75% of the genomic loci in said second potato line are homozygous, wherein said self-compatibility in said second potato line is conferred by *Sli* gene, 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 and allowing said potato plant to produce berries with seeds; and
- (d) collecting said seeds from said berries to provide hybrid potato seeds.

Claim 48. A uniform hybrid F1 potato seed produced according to the method of claim 45.

EXAMINER'S STATEMENT OF REASONS FOR ALLOWANCE

The following is an examiner's statement of reasons for allowance.

Applicant's amendments overcame the objections of record.

Two of the remaining rejections were under 35 USC 112(a), written description and enablement (*ex Parte Jackson*). Those rejections were overcome by Applicant's arguments and especially the declarations by Dr. Jones (3 February 2021) and Dr. Koornneef (22 January 2021). These declarations also were persuasive in not making a new rejection under 35 USC 103.

The rejection under USC 112(a) enablement (deposit) on pages 20-22 in the Office action mailed on 9 November 2020 is withdrawn in view of Applicant's statement on pages 11-12 in the response filed on 9 February 2021.

The rejection under 35 USC 112(d) is withdrawn in view of Applicant's amendments to claim 37.

The rejections under 35 USC 103 of record are withdrawn in view of the Examiner's amendment.

The double patenting rejection is withdrawn in view of Applicant's filing a terminal disclosure.

Finally, in the Examiner's Interview Summary filed 23 July 2021, the line that reads in part "composition claims, 40, 45 and 48" should have read "composition claims, 40, 44 and 48."

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

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, Amjad Abraham can be reached on 571-270-0708. 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 <https://ppair-my.uspto.gov/pair/PrivatePair>. 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.

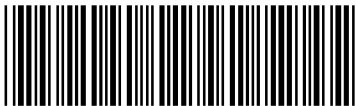
/RUSSELL T BOGGS/
Examiner, Art Unit 1663

<i>Issue Classification</i> 	Application/Control No. 16/541,071	Applicant(s)/Patent Under Reexamination Lindhout et al.
	Examiner RUSSELL T BOGGS	Art Unit 1663

CPC						
Symbol					Type	Version
A01H	/	5	/	04	F	2013-01-01
A01H	/	6	/	827	I	2018-05-01
A01H	/	1	/	02	I	2013-01-01

CPC Combination Sets					
Symbol				Type	Version
	/		/		

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	10	
/RUSSELL T BOGGS/ Examiner, Art Unit 1663	30 July 2021	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	none

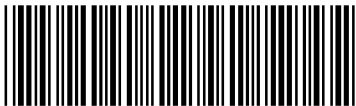
<i>Issue Classification</i> 	Application/Control No. 16/541,071	Applicant(s)/Patent Under Reexamination Lindhout et al.
	Examiner RUSSELL T BOGGS	Art Unit 1663

INTERNATIONAL CLASSIFICATION			
CLAIMED			
A01H5/04	/	5	/ 04
A01H6/82	/	6	/ 82
A01H1/02	/	1	/ 02
NON-CLAIMED			
	/		/

US ORIGINAL CLASSIFICATION	
CLASS	SUBCLASS

CROSS REFERENCES(S)					
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)				

NONE (Assistant Examiner) _____ (Date) _____		Total Claims Allowed: 10	
/RUSSELL T BOGGS/ Examiner, Art Unit 1663 (Primary Examiner) _____ (Date) _____		30 July 2021 O.G. Print Claim(s) 1	O.G. Print Figure none

<i>Issue Classification</i> 	Application/Control No. 16/541,071	Applicant(s)/Patent Under Reexamination Lindhout et al.
	Examiner RUSSELL T BOGGS	Art Unit 1663

☒ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47															
CLAIMS															
Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
	1		10		19		28	2	37		46				
	2		11		20		29	3	38	9	47				
	3		12		21		30		39	10	48				
	4		13		22		31	4	40						
	5		14		23		32	5	41						
	6		15		24		33		42						
	7		16		25		34	6	43						
	8		17		26	1	35	7	44						
	9		18		27		36	8	45						

NONE (Assistant Examiner) _____ (Date) _____		Total Claims Allowed: 10	
/RUSSELL T BOGGS/ Examiner, Art Unit 1663 (Primary Examiner) _____ (Date) 30 July 2021		O.G. Print Claim(s) 1	O.G. Print Figure none

<i>Search Notes</i> 	Application/Control No. 16/541,071	Applicant(s)/Patent Under Reexamination Lindhout et al.
	Examiner RUSSELL T BOGGS	Art Unit 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
inventor search in PALM	07/17/2021	RTB

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

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner
A01H	6/827	07/30/2021	RTB
A01H	1/02	07/30/2021	RTB

/RUSSELL T BOGGS/ Examiner, Art Unit 1663	
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EAST Search History

EAST Search History (Prior Art)

<This search history is empty>

EAST Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	876	A01H1/00.cpc.	US-PGPUB; USPAT	ADJ	ON	2021/07/30 10:18
L2	3,926	A01H1/02.cpc.	US-PGPUB; USPAT	ADJ	ON	2021/07/30 10:18
L3	410	L1 and potato	US-PGPUB; USPAT	ADJ	ON	2021/07/30 10:18
L4	2,127	L2 and potato	US-PGPUB; USPAT	ADJ	ON	2021/07/30 10:19
L5	0	L3 and (sli gene)	US-PGPUB; USPAT	ADJ	ON	2021/07/30 10:19
L6	5	L4 and (sli gene)	US-PGPUB; USPAT	ADJ	ON	2021/07/30 10:19
L7	729	L4 and heterosis	US-PGPUB; USPAT	ADJ	ON	2021/07/30 10:20
L8	235	L7 NOT corn.ti.	US-PGPUB; USPAT	ADJ	ON	2021/07/30 10:24
L9	229	L8 NOT pepper.ti.	US-PGPUB; USPAT	ADJ	ON	2021/07/30 10:25
L10	214	L9 NOT canola.ti.	US-PGPUB; USPAT	ADJ	ON	2021/07/30 10:25
L11	122	L10 NOT maize.ti.	US-PGPUB; USPAT	ADJ	ON	2021/07/30 10:25
L12	103	L11 NOT (cotton or rice or tomato or broccoli).ti.	US-PGPUB; USPAT	ADJ	ON	2021/07/30 10:26
L13	424	a01H5/04.cpc.	US-PGPUB; USPAT	ADJ	ON	2021/07/30 10:33
L14	25	L13 and ((Sli gene) or heterosis)	US-PGPUB; USPAT	ADJ	ON	2021/07/30 10:33

7/30/2021 10:35:59 AM

C:\Users\rboggs\Documents\EAST\Workspaces\16541071.wsp

Bibliographic Data

Application No: 16/541,071

Foreign Priority claimed: ☒ Yes ☐ No

35 USC 119 (a-d) conditions met: ☒ Yes ☐ No ☐ Met After Allowance

Verified and Acknowledged: /RUSSELL T BOGGS/

Examiner's Signature

Initials

Title: Hybrid Seed Potato Breeding

FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
08/14/2019	800	1663	VONL:004USC1
RULE			

APPLICANTS

Agventure B.V., Diepenveen, NETHERLANDS

INVENTORS

Willem Hendrik Lindhout, Wageningen, NETHERLANDS

Teunis Philippus Schotte, Hazerswoude-Dorp, NETHERLANDS

Richard Gerardus Franciscus Visser, Bennekom, NETHERLANDS

Herman Johannes van Eck, Wageningen, NETHERLANDS

Ronaldus Cornelis Bernardus Hutten, Rhenen, NETHERLANDS

CONTINUING DATA

This application is a CON of 13504156 07/10/2012 PAT 10524436

13504156 is a 371 of PCT/NL10/50716 10/26/2010

PCT/NL10/50716 has PRO of 61254980 10/26/2009

/R.T.B/

FOREIGN APPLICATIONS

EPO EP09013474.3 10/26/2009

IF REQUIRED, FOREIGN LICENSE GRANTED**

09/05/2019

**** SMALL ENTITY ****

STATE OR COUNTRY

NETHERLANDS

ADDRESS

DENTONS US LLP

P.O. Box 061080

Chicago, IL 60606

UNITED STATES

FILING FEE RECEIVED

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To: patents.us@dentons.com,,
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Subject: Private PAIR Correspondence Notification for Customer Number 125771

Aug 06, 2021 03:31:11 AM

Dear PAIR Customer:

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UNITED STATES

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

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Application	Document	Mailroom Date	Attorney Docket No.
16541071	NOA	08/06/2021	VONL:004USC1

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PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

PRINTER RUSH
(PTO ASSISTANCE)

Application: 16541071

Examiner: Boggs

GAU: 1663

From:

Location: IDC

Creation Date: 08/12/2021

Week Date: 8/2/2021

<u>DOC CODE</u>	<u>DOC DATE</u>	<u>MISCELLANEOUS</u>
☒ 1449	<u>07/23/2021</u>	☐ Continuing Data
☐ IDS		☐ Foreign Priority
☒ CLM	<u>02/09/2021</u>	☐ Document Legibility
☐ IIFW/FWCLM		☐ Fees
☐ SRFW		☐ Petition (TC)
☐ DRW		☐ Other
☐ OATH		
☐ 312		
☐ SPEC		

[RUSH] Message:

1. Please initial/line through citations on (1449 07/23/2021, page 1 of 7). Each page must be individually stamped as per MPEP 609.08.
2. Original claim 40 is not numbered on PTOL-37. Please verify allowance of claim and renumber PTOL-37 accordingly.
3. Original claim 30 has been canceled per (CLM 02/09/2021), but is shown on PTOL-37 as allowed.
Thank You

[XRUSH] Response:

Initials: _____

Examiner: PUBS contact - James Leija, (703) 756-1197

NOTE: This form will be included as part of the official USPTO record with the response document coded as XRUSH.

REV: AUG 16

Ardevo Exhibit 1010
Page 2704

Page 1/1



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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	08/25/2021	EXAMINER	
DENTONS US LLP			BOGGS, RUSSELL T	
P.O. Box 061080			ART UNIT	PAPER NUMBER
Chicago, IL 60606			1663	
			NOTIFICATION DATE	DELIVERY MODE
			08/25/2021	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

Corrected Notice of Allowability	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--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to See Continuation Sheet.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ 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.

3. ☒ The allowed claim(s) is/are See Continuation Sheet. As a result of the allowed claim(s), 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**.

4. ☐ 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)).

* Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)	5. ☐ Examiner's Amendment/Comment
2. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____	6. ☐ Examiner's Statement of Reasons for Allowance
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____	7. ☐ Other _____
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____	

/RUSSELL T BOGGS/ Examiner, Art Unit 1663	
--	--

Continuation of 1. This communication is responsive to: the response filed on 9 February 2021 and the authorization for an examiner's amendmend on 28 July 2021

Continuation of 3. The allowed claim(s) is/are: 35,37-38,40-41 and 43-45

Doc code: IDS

Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)

Approved for use through 11/30/2020. OMB 0651-0031

U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

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

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

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

U.S.PATENT APPLICATION PUBLICATIONS						
Examiner Initial*	Cite No	Publication Number	Kind Code ¹	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1					

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

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

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

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

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /R.T.B/

**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

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

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

EXAMINER SIGNATURE

Examiner Signature	/RUSSELL T BOGGS/	Date Considered	06/26/2021
--------------------	-------------------	-----------------	------------

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

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

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

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

CERTIFICATION STATEMENT

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

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

OR

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

☐ See attached certification statement.

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

☒ A certification statement is not submitted herewith.

SIGNATURE

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

Signature	/Sheila Walker/	Date (YYYY-MM-DD)	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.**

Doc code: IDS

Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)

Approved for use through 11/30/2020. OMB 0651-0031

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Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

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

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

/R.T.B/	1	Uitdewilligen et al., PLoS ONE 8(5), e62355 (2013)	☐
/R.T.B/	2	Ye et al., Nature Plants 4(9): 651-654 (2018)	☐

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Examiner Signature	/RUSSELL T BOGGS/	Date Considered	06/26/2021
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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.

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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.

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

Signature	/Amy Dunstan/	Date (YYYY-MM-DD)	2021-03-29
Name/Print	Amy Dunstan	Registration Number	61,069

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.**

Doc code: IDS

Doc description: Information Disclosure Statement (IDS) Filed

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/R.T.B/	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.	☐
/R.T.B/	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.	☐
/R.T.B/	3	Ritte et al. (2002) "The starch-related R1 protein is an α -glucan, water dikinase", Proc. Nat. Acad. Sci. USA. 99:7166-7171.	☐
/R.T.B/	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.	☐
/R.T.B/	5	Rommens et al. (2008) "Engineered native pathways for high kaempferol and caffeoylquinic acid production in potato", Plant Biotech J. 6:870-886.	☐
/R.T.B/	6	Rommens et al. (2008) "Low-acrylamide French fries and potato chips", Plant Biotech. J. 6:843-853.	☐
/R.T.B/	7	Rommens et al. (2006) "Improving potato storage and processing characteristics through all-native DNA transformation-J", Agric. Food Chem 54:9882-9887.	☐
/R.T.B/	8	Roupe 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.	☐
/R.T.B/	9	Roupe 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.	☐
/R.T.B/	10	Roupe van der Voort et al. (1999) "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", Euphytica 106: 187-195.	☐
/R.T.B/	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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/R.T.B/	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.	☐
/R.T.B/	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.	☐
/R.T.B/	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.	☐
/R.T.B/	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.	☐
/R.T.B/	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).	☐
/R.T.B/	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.	☐
/R.T.B/	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.	☐
/R.T.B/	19	Tan et al. (2009) "GpaXI lta 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.	☐
/R.T.B/	20	Thygesen et al. (1995) "Polyphenol oxidase in potato. A multigene family that exhibits differential expression patterns", Pl. Physiol. 109:525-531.	☐
/R.T.B/	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.	☐
/R.T.B/	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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/R.T.B/	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).	☐
/R.T.B/	24	USPTO: Final Office Action dated December 15, 2016, regarding U.S. Application No. 14/112,747 (Atty. Dkt. No. VONL:006US).	☐
/R.T.B/	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).	☐
/R.T.B/	26	USPTO: Non-Final Office Action dated December 22, 2017, regarding U.S. Application No. 14/112,747 (Atty. Dkt. No. VONL:006US).	☐
/R.T.B/	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).	☐
/R.T.B/	28	USPTO: Final Office Action dated August 7, 2018, regarding U.S. Application No. 14/112,747 (Atty. Dkt. No. VONL:006US).	☐
/R.T.B/	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).	☐
/R.T.B/	30	USPTO: Final Office Action dated October 27, 2020, regarding U.S. Application No. 14/112,747 (Atty. Dkt. No. VONL:006US).	☐
/R.T.B/	31	van der Leij et al. (1991) "Sequence of the structural gene for granule-bound starch synthase of potato (<i>Solanum tuberosum</i> L.) and evidence for a single point deletion in the amf allele", Mol. Gen. Genet. 228:240-248.	☐
/R.T.B/	32	van der Vossen et al. (2000) "Homologues of a single resistance-gene cluster in potato confer resistance to distinct pathogens a virus and a nematode", Plant J. 23:567-576.	☐
/R.T.B/	33	van der Vossen et al., "An ancient R gene from the wild potato species <i>Solanum bulbocastanum</i> confers broad - spectrum resistance to <i>Phytophthora infestans</i> in cultivated potato and tomato," The Plant Journal 36:867-882, 2003.	☐

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/R. T. B./	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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If you wish to add additional non-patent literature document citation information please click the Add button

EXAMINER SIGNATURE

Examiner Signature	/RUSSELL T BOGGS/	Date Considered	06/26/2021
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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.

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

☒ A certification statement is not submitted herewith.

SIGNATURE

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

Signature	/Amy Dunstan/	Date (YYYY-MM-DD)	2021-03-04
Name/Print	Amy Dunstan	Registration Number	61,069

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Doc code: IDS

Doc description: Information Disclosure Statement (IDS) Filed

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/R. T. B./	1	20210029956		2021-02-04	Lindhout et al.	
/R. T. B./	2	20210137041		2021-05-13	de Vries et al.	

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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
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Signature	/Kristi D. Wilson/	Date (YYYY-MM-DD)	2021-06-28
Name/Print	Kristi D. Wilson	Registration Number	77,672

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PRINTER RUSH
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Application: 16541071

Examiner: Boggs

GAU: 1663

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Location: IDC

Creation Date: 08/12/2021

Week Date: 8/2/2021

<u>DOC CODE</u>	<u>DOC DATE</u>	<u>MISCELLANEOUS</u>
☒ 1449	<u>07/23/2021</u>	☐ Continuing Data
☐ IDS		☐ Foreign Priority
☒ CLM	<u>02/09/2021</u>	☐ Document Legibility
☐ IIFW/FWCLM		☐ Fees
☐ SRFW		☐ Petition (TC)
☐ DRW		☐ Other
☐ OATH		
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☐ SPEC		

[RUSH] Message:

1. Please initial/line through citations on (1449 07/23/2021, page 1 of 7). Each page must be individually stamped as per MPEP 609.08.
2. Original claim 40 is not numbered on PTOL-37. Please verify allowance of claim and renumber PTOL-37 accordingly.
3. Original claim 30 has been canceled per (CLM 02/09/2021), but is shown on PTOL-37 as allowed.
Thank You

[XRUSH] Response:

The accompanying Corrected PTOL-37 with the 1449s should address the above issues.
Thank you

/R.T.B/
Initials: _____

Document Description: Issue Fee Payment (PTO-85B)

Issue Fee Transmittal Form

Application Number	Filing Date	First Named Inventor	Atty. Docket No.	Confirmation No.
16541071	14-Aug-2019	Willem Hendrik Lindhout	VONL:004USC1	4348

TITLE OF INVENTION :

Hybrid Seed Potato Breeding

Entity Status	Application Type	Art Unit	Class - Subclass	EXAMINER
Small	Utility under 35 USC 111(a)	1663	265000	
Issue Fee Due	Publication Due	Total Fee(s) Due	Date Due	Prev. Paid Fee
\$600	\$0	\$600	08-Nov-2021	\$0

1.Change of Correspondence Address and/or Indication Of Fee Address (37 CFR 1.33 & 1.363)

Current Correspondence Address:	Current Indicated Fee Address :
125771 DENTONS US LLP P.O. Box 061080 Chicago IL 60606 UNITED STATES 312-876-7925 ~~patents.us@dentons.com~~	
☐ Change of correspondence address requested, system generated AIA/122-EFS form attached	☐ Fee Address indication requested, system generated SB/47-EFS form attached

2.Entity Status**Change in Entity Status**

Applicant certifying micro entity status; system generated Micro Entity certification form attached. See 37 CFR 1.29.

Note: Absent a valid certification of micro entity status, issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment. If this box is checked, you will be prompted to choose a micro entity status on the gross income basis (37 CFR 1.29(a)) or the institution of higher education basis (37 CFR 1.29(d)), and make the applicable certification online.



Applicant asserting small entity status. See 37 CFR 1.27.

Note: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.



Applicant changing to regular undiscounted fee status.

Note: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.



Document Description: Issue Fee Payment (PTO-85B)

3.The Following Fee(s) Are Submitted:

☒ Issue Fee

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4.Firm and/or Attorney Names To Be Printed

NOTE: If no name is listed, no name will be printed
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1. Dentons US LLP
2.
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5.Assignee Name(s) and Residence Data To Be Printed

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Agventure B.V.	Diepenveen		NETHERLANDS	corporation

6.Signature

I certify, in accordance with 37 CFR 1.4(d)(4) that I am an attorney or agent registered to practice before the Patent and Trademark Office who has filed and has been granted power of attorney in this application. I also certify that this Fee(s) Transmittal form is being transmitted to the USPTO via EFS-WEB on the date indicated below.

Signature	/Robert E. Hanson/	Date	08-24-2021
Name	Robert E. Hanson	Registration Number	42628

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:	Robert E. Hanson			
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:				
UTILITY APPL ISSUE FEE	2501	1	600	600
PUBL. FEE- EARLY, VOLUNTARY, OR NORMAL	1504	1	0	0
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
Total in USD (\$)				600

Electronic Acknowledgement Receipt

EFS ID:	43589116
Application Number:	16541071
International Application Number:	
Confirmation Number:	4348
Title of Invention:	Hybrid Seed Potato Breeding
First Named Inventor/Applicant Name:	Willem Hendrik Lindhout
Customer Number:	125771
Filer:	Robert E. Hanson
Filer Authorized By:	
Attorney Docket Number:	VONL:004USC1
Receipt Date:	24-AUG-2021
Filing Date:	14-AUG-2019
Time Stamp:	19:10:38
Application Type:	Utility under 35 USC 111(a)

Payment information:

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Payment Type	CARD
Payment was successfully received in RAM	\$ 600
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Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Issue Fee Payment (PTO-85B)	Web85b.pdf	62481	no	2
			2c.cfb6bbc483d6372ac48b8f50b4eb6c3a7546989		
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	40087	no	2
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Total Files Size (in bytes):			102568		
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To: patents.us@dentons.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 125771

Aug 25, 2021 03:46:15 AM

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DENTONS US LLP
P.O. Box 061080
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UNITED STATES

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Application	Document	Mailroom Date	Attorney Docket No.
16541071	NOA	08/25/2021	VONL:004USC1
	1449	08/25/2021	VONL:004USC1
	1449	08/25/2021	VONL:004USC1
	1449	08/25/2021	VONL:004USC1
	1449	08/25/2021	VONL:004USC1

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PRINTER RUSH
(PTO ASSISTANCE)

Application: 16541071

Examiner: Boggs

GAU: 1663

From:

Location: IDC

Creation Date: 08/26/2021

Week Date: 8/2/2021

<u>DOC CODE</u>	<u>DOC DATE</u>	<u>MISCELLANEOUS</u>
☒ 1449	<u>07/23/2021</u>	☐ Continuing Data
☐ IDS		☐ Foreign Priority
☒ CLM	<u>02/09/2021</u>	☐ Document Legibility
☐ IIFW/FWCLM		☐ Fees
☐ SRFW		☐ Petition (TC)
☐ DRW		☒ Other
☐ OATH		
☐ 312		
☐ SPEC		

[RUSH] Message:

2nd QUERY

1. Please initial/line through citations on page 1 of 7 (1449 07/23/2021).

* Each page must be individually stamped as per MPEP 609.08.

2. In the FORM PTOL-37 (NOA 08/25/2021)

--- CLAIMS 47 and 48 are not listed as allowed.

Please resolve.

[XRUSH] Response:

Initials: _____



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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	09/01/2021	EXAMINER	
DENTONS US LLP			BOGGS, RUSSELL T	
P.O. Box 061080				
Chicago, IL 60606				
			ART UNIT	PAPER NUMBER
			1663	
			NOTIFICATION DATE	DELIVERY MODE
			09/01/2021	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):

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**2nd Corrected
Notice of Allowability**

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--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to See Continuation Sheet.

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

2. ☐ 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.

3. ☒ The allowed claim(s) is/are See Continuation Sheet. As a result of the allowed claim(s), 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**.

4. ☐ 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)).

* Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.

THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.

☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)

2. ☒ Information Disclosure Statements (PTO/SB/08),
Paper No./Mail Date _____.

3. ☐ Examiner's Comment Regarding Requirement for Deposit
of Biological Material _____.

4. ☐ Interview Summary (PTO-413),
Paper No./Mail Date _____.

5. ☐ Examiner's Amendment/Comment

6. ☐ Examiner's Statement of Reasons for Allowance

7. ☐ Other _____.

/RUSSELL T BOGGS/
Examiner, Art Unit 1663

Continuation of 1. This communication is responsive to: the response filed on 9 February 2021 and the authorization for an examiner's amendment on 28 July 2021

Continuation of 3. The allowed claim(s) is/are: 30,35,37-38,41,43-45 and 47-48

Doc code: IDS

Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)

Approved for use through 11/30/2020. OMB 0651-0031

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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	10524436		2020-01-07	Lindhout et al.	

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

U.S.PATENT APPLICATION PUBLICATIONS						
Examiner Initial*	Cite No	Publication Number	Kind Code ¹	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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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

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /R.T.B/

**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

/R.T.B/	1	Bakker et al. (2003) "Genetic and physical mapping of homologues of the virus resistance Rx1 and the cyst nematode resistance gene Gpa2 in potato", Theor. Appl. Genet 106:1524-1531.	☐
/R.T.B/	2	Balivora et al. (2002) "The R1 gene for potato resistance to late blight (Phytophthora infestans) belongs to the leucine zipper/NBS/LRR class of plant resistance genes", Plant J, 30:361-371.	☐
/R.T.B/	3	Bendahmane et al. (1999) "The RX gene from potato controls separate virus resistance and cell death responses", Plant Cell 11:781-792.	☐
/R.T.B/	4	Black et al. (1953) "A proposal for an international nomenclature of races of Phytophthora infestans and of genes controlling immunity in Solanum demissum derivatives", Euphytica 2: 173-179.	☐
/R.T.B/	5	Bonierbale et al. (1988) "RFLP maps based on a common set of clones reveal modes of chromosomal evolution in potato and tomato", Genetics 120:1095-1103.	☐
/R.T.B/	6	Bradshaw et al.(1998) "Identification of AFLP and SSR marker associated with quantitative resistance to Globodera pallida (Stone) in tetraploid potato (Solanum tuberosum subsp. tuberosum) with a view to marker-assisted selection", Theor. Appl. Genet. 97:202-210.	☐
/R.T.B/	7	Brown et al. (2006) "Segregation of total carotenoid in high level potato germplasm and its relationship to beta-carotene hydroxylase polymorphism", Amer. J. Potato Res 83:365-372.	☐
/R.T.B/	8	Brown et al. (2003) "Characteristics of resistance to Colombia root-knot nematode Introgressed from several Mexican and North American wild potato species", Acta Hort. 619: 117-125.	☐
/R.T.B/	9	Brown et al. (1996) "RFLP analysis of resistance to Columbia root-knot nematode derived from Solanum bulbocastanum is a BC2 population", Theor. Appl. Genet. 92:572-576.	☐
/R.T.B/	10	Brummell et al. (2011) "Induction of vascular invertase inhibitor mRNA in potato tubers contributors to cold-induced sweetening resistance and includes spliced hybrid mRNA variants", J. Exper. Bot., Vol., 62, No 10, pp. 3519-3534.	☐
/R.T.B/	11	Champouret, N. (2010) "Functional genomics of Phytophthora infestans effectors and Solanum resistance genes", PhD thesis, Wageningen University.	☐

**INFORMATION DISCLOSURE
STATEMENT BY APPLICANT**
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Application Number	16541071
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First Named Inventor	Willem Hendrik Lindhout
Art Unit	1663
Examiner Name	Russell T. Boggs
Attorney Docket Number	VONL:004USC1

/R.T.B/	12	De Jong, H., (1987) "Inheritance of pigmented tuber flesh in cultivated diploid potatoes", Amer. Potato J. 64:337-343.	☐
/R.T.B/	13	De Jong et al. (1997) "A potato hypersensitive resistance gene against potato virus X maps to a resistance gene cluster on chromosome 5", Theor. Appl. Genet. 95:246-252.	☐
/R.T.B/	14	De Jong et al. (2003) "An allele of dihydroflavonol 4-reductase associated with the ability to produce red anthocyanin pigments in potato (Solanum tuberosum L.)", Theor. Appl. Genet. 107:1375-1333.	☐
/R.T.B/	15	Draaistra, J, (2008) "Genetic analysis of root-knot nematode resistance in potato", PhD thesis Wageningen University The Netherlands.	☐
/R.T.B/	16	El-Kharbotly et al.(1994) "Segregation analysis and RFLP mapping of the R1 and R3 alleles conferring race-specific resistance to Phytophthora infestans in progeny of dihaploid potato ", Mol. Gen. Genet. 242: 749-754.	☐
/R.T.B/	17	El-Kharbotly et al. (1996) "R6 and R7 alleles of potato conferring race-specific resistance to Phytophthora infestans (Mont.) de Bary identified genetic loci clustering with the R3 locus on chromosome XI", Theor. Appl. Genet 92:880-884.	☐
/R.T.B/	18	Ewing et al., "Genetic mapping from field tests of qualitative and quantitative resistance to Phytophthora infestans in population derived from Solanum tuberosum and Solanum berthaultii," Molecular Breeding 6:25-36, 2000.	☐
/R.T.B/	19	Fan et al., "Molecular Cloning and Expression of a FT Homologous Gene from Solanum tuberosum", Agricultural Sciences in China, 9:1133-1139 (2010).	☐
/R.T.B/	20	Foster et al., "Rpi-vnt1.1, a Tm-22 Homolog from Solanum venturii, Confers Resistance to Potato Late Blight," Mol. Pt. Microb. Interact. 22: 589-600, 2009.	☐
/R.T.B/	21	Gebhardt et al. (1993) "Identification of RFLP markers closely linked to the H1 gene conferring resistance to Globodera rostochiensis in potato", Theor. Appl. Genet. 85: 541-544.	☐
/R.T.B/	22	Hämäläinen et al. (1997) "Mapping and marker-assisted selection for a gene for extreme resistance to potato virus y", Theor. Appl. Genet. 94:192-197.	☐

**INFORMATION DISCLOSURE
STATEMENT BY APPLICANT**
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First Named Inventor	Willem Hendrik Lindhout
Art Unit	1663
Examiner Name	Russell T. Boggs
Attorney Docket Number	VONL:004USC1

/R.T.B/	23	Huang et al. (2005) "Comparative genomics enabled the isolation of the R3a late blight resistance gene in potato", Plant J. 42:251-261.	☐
/R.T.B/	24	Huang, "Discovery and characterization of the major late blight resistance complex in potato genomic structure, diversity, and implications." PhD dissertation, Wageningen University, The Netherlands. 2005.	☐
/R.T.B/	25	Huang et al. (2004) "The R3 resistance to Phytophthora Infestans in potato is conferred by two closely linked R genes with distinct specialties", Mol. Pt. Microbe Interact. 17:428-435.	☐
/R.T.B/	26	International Preliminary Report on Patentability issued October 31, 2013 in PCT/NL2012/050269.	☐
/R.T.B/	27	Jung et al., "The potato P locus codes for flavonoid 3',5'-hydroxylase", Theor. Appl. Genet 110:269-275, (2005).	☐
/R.T.B/	28	Jung et al. (2009) "The potato developer (D) locus encodes in R2R3 MYB transcription factor that regulates expression of multiple anthocyanin structural genes in tuber skin", Theor. Appl. Genet. 120:45-57.	☐
/R.T.B/	29	Kanyuka et al. (1999) "Mapping of intra-locus duplications and introgressed DNA: aids to map-based cloning of genes from complex genomes illustrated by physical analysis of the Rx locus in tetraploid potato", Theor, Appl. Genet. 98:679-689.	☐
/R.T.B/	30	Kasai et al.(2000) "Development of SCAR markers to the PVY resistance gene Ryadg based on a common feature of plant disease resistance genes", Genome 43: 1-8.	☐
/R.T.B/	31	Kim et al., "Broad spectrum late blight resistance in potato differential set plants MaR8 and MaR9 is conferred by multiple stacked R genes," Theor. Appl. Genet. 124:923-935, 2012.	☐
/R.T.B/	32	Kim et al. (2000) "Regulation of starch contents in potato (Solanum tuberosum L) by manipulation of sucrose synthase gene.", J. Jap. Soc. Hort. Sci. 69:243-249.	☐
/R.T.B/	33	Kreike et al. (1994) "Quantitatively inherited resistance to Globodera pallida is dominated by one major locus in Solanum spegazzinii", Theor. Appl. Genet. 88: 764-769.	☐

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)

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First Named Inventor	Willem Hendrik Lindhout
Art Unit	1663
Examiner Name	Russell T. Boggs
Attorney Docket Number	VONL:004USC1

/R.T.B/	34	Kuhl et al. (2001) "Characterization and mapping of Rpi1, a late-blight resistance locus from diploid (1EBN) Mexican Solanum pinnatisectum", Mol. Genetics and Genomics 265:977-985.	☐
/R.T.B/	35	Lebecka et al., "Host—pathogen interaction between Phytophthora infestans and Solanum tuberosum following to short and long daylight hours," Acta. Physiol. Plant. 35:1131-1139, 2013.	☐
/R.T.B/	36	Leonards-Schippers et al., "Quantitative resistance to Phytophthora infestans in potato: a case study for QTL mapping in an allogamous plant species", Genetics 137:67-77, (1994).	☐
/R.T.B/	37	Li et al. (2008) "Natural DNA variation at candidate loci is associated with potato chip color, tuber starch content, yield and starch yield", Theor. Appl. Genet. 116:1167-1181.	☐
/R.T.B/	38	Lokossou, "Dissection of the major late blight resistance cluster on potato linkage group IV." PhD dissertation, Wageningen University, The Netherlands. 2010.	☐
/R.T.B/	39	Lokossou et al., "Exploiting Knowledge of R/Avr Genes to Rapidly Clone a New LZ-NBS-LRR Family Late Blight Resistance Genes from Potato Linkage Group IV," MPMI 22(6):630-641, 2009.	☐
/R.T.B/	40	Malcomson et al., "New R Genes in Solanum Demissum Lindl. and Their Complementary Races of Phytophthora (Mont.) De Bary," Euphytica 15:199-203, 1966.	☐
/R.T.B/	41	Marano et al. (2002) "High-resolution genetic map of Nb, a gene that confers hypersensitive resistance to potato virus X In Solanum tuberosum", Theor. Appl. Genet. 105:192-200.	☐
/R.T.B/	42	Marczewski et al. (2002) "The Potato virus S resistance gene Ns maps to potato chromosome Vill", Theor. Appl. Genet. 105:564-567.	☐
/R.T.B/	43	Moloney et al. (2010) "Development of diagnostic markers for use in breeding potatoes resistant to Globodera pallida pathotype Pa2/3 using germplasm derived from Solanum tuberosum ssp. andigena CPC 2802", Theor. Appl. Genet. 120:679-689.	☐
/R.T.B/	44	Oosumi et al., "Gene Rpi-bt1 from Solanum bulbocastanum Confers Resistance to Late Blight in Transgenic Am. J. Pot. Res. 86:456-465, 2009.	☐

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)

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

/R.T.B/	45	Paal et al. (2004) "Molecular cloning of the potato Gro1-4 gene conferring resistance to pathotype Ro1 of the root cyst nematode Globodera rostochiensis, based on a candidate gene approach", Plant J. 38:285-297.	☐
/R.T.B/	46	Park et al., "Characterization and high-resolution mapping of a late blight resistance locus similar to R2 in potato," Appl. Genet. 111:591-597, 2005.	☐
/R.T.B/	47	Park et al., "High-resolution mapping and analysis of the resistance locus Rpi-abpt against Phytophthora infestans in Molecular Breeding 16:33-43, 2005.	☐
/R.T.B/	48	Park et al., "Two distinct potato late blight resistance genes from Solanum berthaultii are located on chromosome Eyphtica 165:269-278, 2009.	☐
/R.T.B/	49	Pel et al. (2009) "Mapping and cloning of late blight resistance genes tom Solanum venturii using an interspecific candidate gene approach", Mol. Plant Microb. Interact. 22:601-615.	☐
/R.T.B/	50	Pineda et al. (1993) "Identification of RFLP markers linked to the H1 gene conferring resistance to the potato cyst nematode Globodera rostochiensis", Genome 36:152-156.	☐

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

EXAMINER SIGNATURE

Examiner Signature	/RUSSELL T BOGGS/	Date Considered	06/26/2021
--------------------	-------------------	-----------------	------------

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

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

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

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

CERTIFICATION STATEMENT

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

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

OR

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

☐ See attached certification statement.

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

☒ A certification statement is not submitted herewith.

SIGNATURE

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

Signature	/Amy Dunstan/	Date (YYYY-MM-DD)	2021-03-04
Name/Print	Amy Dunstan	Registration Number	61,069

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.**

PRINTER RUSH
(PTO ASSISTANCE)

Application: 16541071

Examiner: Boggs

GAU: 1663

From:

Location: IDC

Creation Date: 08/26/2021

Week Date: 8/2/2021

<u>DOC CODE</u>	<u>DOC DATE</u>	<u>MISCELLANEOUS</u>
☒ 1449	<u>07/23/2021</u>	☐ Continuing Data
☐ IDS		☐ Foreign Priority
☒ CLM	<u>02/09/2021</u>	☐ Document Legibility
☐ IIFW/FWCLM		☐ Fees
☐ SRFW		☐ Petition (TC)
☐ DRW		☒ Other
☐ OATH		
☐ 312		
☐ SPEC		

[RUSH] Message:

2nd QUERY

1. Please initial/line through citations on page 1 of 7 (1449 07/23/2021).

* Each page must be individually stamped as per MPEP 609.08.

2. In the FORM PTOL-37 (NOA 08/25/2021)

--- CLAIMS 47 and 48 are not listed as allowed.

Please resolve.

[XRUSH] Response:

Please refer to the IDS by its EFS date or some means of uniquely identifying an IDS. I believe I have supplied the one in question herewith.

The current PTOL-37 lists claims 47-48.

Initials: /R.T.B/

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

Sep 01, 2021 03:57:53 AM

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

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Application	Document	Mailroom Date	Attorney Docket No.
16541071	NOA	09/01/2021	VONL:004USC1
	1449	09/01/2021	VONL:004USC1

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

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PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

PRINTER RUSH
(PTO ASSISTANCE)

Application: 16541071

Examiner: Boggs

GAU: 1663

From:

Location: IDC

Creation Date: 09/01/2021

Week Date: 8/2/2021

<u>DOC CODE</u>	<u>DOC DATE</u>	<u>MISCELLANEOUS</u>
☐ 1449		☐ Continuing Data
☐ IDS		☐ Foreign Priority
☐ CLM		☐ Document Legibility
☐ IIFW/FWCLM		☐ Fees
☐ SRFW		☐ Petition (TC)
☐ DRW		☒ Other
☐ OATH		
☐ 312		
☐ SPEC		

[RUSH] Message:

3rd request

NOA 09/01/2021 does not show the correct number of allowed claims. Please provide an NOA showing claims 35, 37-38, 40-41, 43-45, and 47-48 as allowed claims.

***Thank you**

[XRUSH] Response:

Initials: _____

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875				Application or Docket Number 16/541,071		Filing Date 08/14/2019		☐ To be Mailed			
ENTITY: ☐ LARGE ☒ SMALL ☐ MICRO											
APPLICATION AS FILED - PART I											
		(Column 1)		(Column 2)							
FOR		NUMBER FILED		NUMBER EXTRA		RATE (\$)		FEE (\$)			
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))		N/A		N/A		N/A					
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))		N/A		N/A		N/A					
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))		N/A		N/A		N/A					
TOTAL CLAIMS (37 CFR 1.16(i))		minus 20 =		*		x \$50 =					
INDEPENDENT CLAIMS (37 CFR 1.16(h))		minus 3 =		*		x \$230 =					
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))		If the specification and drawings exceed 100 sheets of paper, the application size fee due is \$310 (\$155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).									
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))											
* If the difference in column 1 is less than zero, enter "0" in column 2.						TOTAL					
APPLICATION AS AMENDED - PART II											
		(Column 1)		(Column 2)		(Column 3)					
AMENDMENT	07/28/2021	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 \$50 =		0		
	Independent (37 CFR 1.16(h))	* 2	Minus	*** 3	= 0		x \$240 =		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.								SPE			
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".								/MARY R HUNTER/			
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".											
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.											

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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	09/09/2021	EXAMINER	
DENTONS US LLP			BOGGS, RUSSELL T	
P.O. Box 061080				
Chicago, IL 60606				
			ART UNIT	PAPER NUMBER
			1663	
			NOTIFICATION DATE	DELIVERY MODE
			09/09/2021	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

3rd Notice of Allowability	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--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to See Continuation Sheet.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ 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.

3. ☒ The allowed claim(s) is/are See Continuation Sheet. As a result of the allowed claim(s), 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**.

4. ☐ 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)).

* Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892) 2. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____. 3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____. 4. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____. /RUSSELL T BOGGS/ Examiner, Art Unit 1663	5. ☐ Examiner's Amendment/Comment 6. ☐ Examiner's Statement of Reasons for Allowance 7. ☐ Other _____
---	--

Continuation of 1. This communication is responsive to: the response filed on 9 February 2021 and the authorization for an examiner's amendment on 28 July 2021.

Continuation of 3. The allowed claim(s) is/are: 35,37-38,40-41,43-45 and 47-48

PRINTER RUSH
(PTO ASSISTANCE)

Application: 16541071

Examiner: Boggs

GAU: 1663

From:

Location: IDC

Creation Date: 09/01/2021

Week Date: 8/2/2021

<u>DOC CODE</u>	<u>DOC DATE</u>	<u>MISCELLANEOUS</u>
☐ 1449		☐ Continuing Data
☐ IDS		☐ Foreign Priority
☐ CLM		☐ Document Legibility
☐ IIFW/FWCLM		☐ Fees
☐ SRFW		☐ Petition (TC)
☐ DRW		☒ Other
☐ OATH		
☐ 312		
☐ SPEC		

[RUSH] Message:

3rd request

NOA 09/01/2021 does not show the correct number of allowed claims. Please provide an NOA showing claims 35, 37-38, 40-41, 43-45, and 47-48 as allowed claims.

***Thank you**

[XRUSH] Response:

Done.
Thanks.

Initials: /R.T.B./

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

Sep 09, 2021 04:02:23 AM

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P.O. Box 061080
Chicago, IL 60606
UNITED STATES

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Application	Document	Mailroom Date	Attorney Docket No.
16541071	NOA	09/09/2021	VONL:004USC1

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

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

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE
PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

NCIMB, Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB219YA, Scotland under accession number NCIMB 41765 and under breeders reference AGVD17, which line represents an F3 population obtained by crossing a plant of parent IVP07-1001/4 as indicated above with a plant of parent D2 as indicated above, and backcrossing the resulting F1 offspring plants with the IVP07-1001/4 parent, followed by two steps of selfing. It is estimated that AGVD17 is > 80% homozygous for the loci for which the parents differ.

Change(s) applied
to document,
/R.K.C./
8/9/2021

(10) Please amend the paragraph in the specification at ~~page 37, lines 5-19~~^{page 32, line 31} as follows:

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

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

Table 5A. Sequences of 100 SNP markers in potato

Change(s) applied
to document,
/R.K.C./
8/9/2021

(7) Please amend the paragraph in the specification at ^{page 29, line 29}~~page 33, lines 23-31~~ as follows:

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

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

Plants according to the present invention also include the line with breeders reference AGVD3, representative seeds of said line having been deposited on 23 October 2009 with the NCIMB, Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB219YA, Scotland under accession number NCIMB 41665 and under breeders reference AGVD3, which line represents a BC1 population obtained by crossing a plant of parent IVP07-1001/4 as indicated above with a plant of parent D1 as indicated above, and backcrossing the resulting F1 offspring plants with the IVP07-1001/4 parent. It is estimated that AGVD3 is about 75% homozygous for the loci for which the parents differ.

(9) Please amend the paragraph in the specification at ^{page 30, line 11}~~page 34, lines 7-14~~ as follows:

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

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

Change(s) applied to document,
/R.K.C./
8/9/2021

(5) Please amend the paragraph in the specification at ~~page 52, lines 19-23~~ ^{page 46, line 10} as follows:

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

(6) Please amend the paragraph in the specification at ~~page 33, lines 11-22~~ ^{page 29, line 18} as follows:

Plants according to the present invention include the line with breeders reference AGVD1, representative seeds of said line having been deposited on 23 October 2009 with the NCIMB, Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB219YA, Scotland under accession number NCIMB 41663 and breeders reference AGVD1, which plant represents an F2 obtained by crossing a plant of parent IVP07-1001/4 (see Example 1), which is an essentially homozygous, diploid, fertile and self-compatible but non-vigorous potato line with a plant of parent D1, which is an elite diploid breeding clone that is essentially non-homozygous, and is not self-compatible, but which has the desirable agronomic trait of yellow flesh color and exhibits good cooking quality in that the flesh is not greying after cooking, and selfing the resulting F1 offspring plants. It is estimated that AGVD1 is about 50% homozygous, meaning that about 50% of the loci for which the parents differ are homozygous.

Change(s) applied
to document,
/R.K.C./
8/9/2021

SPECIFICATION AMENDMENT

(1) Please amend the paragraph in the specification at ^{page 8, line 9}~~page 9, lines 3-9~~ as follows:

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

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

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

(3) Please amend the paragraph in the specification at ^{page 45, line 11}~~page 51, lines 18-23~~ as follows:

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

(4) Please amend the paragraph in the specification at ^{page 45, line 28}~~page 52, lines 2-7~~ as follows:



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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/541,071	10/12/2021	11140841	VONL:004USC1	4348

125771 7590 09/22/2021
DENTONS US LLP
P.O. Box 061080
Chicago, IL 60606

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b) (application filed on or after May 29, 2000)

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (<http://pair.uspto.gov>).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site <http://pair.uspto.gov> for additional applicants):

Agventure B.V., Diepenveen, NETHERLANDS;

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

Sep 23, 2021 04:54:28 AM

Dear PAIR Customer:

DENTONS US LLP
P.O. Box 061080
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UNITED STATES

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Application	Document	Mailroom Date	Attorney Docket No.
16541071	ISSUE.NTF	09/22/2021	VONL:004USC1

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