tial *Hin*dIII digest, enabling the subcloning to be carried out with an *Eco*RI-*Hin*dIII fragment. The adjacent *Hin*dIII-*Eco*RI fragment of the mutant allele was cloned in-frame with the wild-type fragment in the *Eco*RI site of pUC18. The resulting *Eco*RI fragment was inserted in pAMF112 (see above) to construct pWM115 (Fig. 4C).

Triparental mating, plant transformation and screening of hairy roots. Binary plasmid transfer to *A. rhizogenes* and subsequent stem segment transformation were carried out as previously described (van der Leij et al. 1991). In each experiment a minimum of 30 starch-containing kanamycin-resistant hairy roots were screened for the presence of amylose by staining with a fresh mixture of chloral hydrate and Lugol's solution.

Sequencing of polymerase chain reaction (PCR) products. Direct sequencing of PCR fragments was carried out with each of the primers used for the PCR, after removal of the excess of both primers with the Geneclean II kit of Bio101. The primers used were 5′-CGTGTCGACTGAAACCTGCTACA-3′ and 5′-TTGGTCTCGGATCCGGATGCCAT-3′, which anneal at positions −340 to −318 and 177 to 200, respectively, in the wild-type sequence. A detailed description of the PCR procedure will be given elsewhere (van der Leij et al., manuscript in preparation).

Computer analysis. The PCGene software package (Intelligenetics, Geneva) version 6.26 and the alignment programs Fasta (Lipman and Pearson 1985) and Clustal (Higgins and Sharp 1989) were used for sequence analyses. The Swiss Prot database release 15 (August 1990) was used for protein sub-sequence comparison.

Results and discussion

Promoter sequence of the wild-type allele

The DNA sequence of the potato GBSS gene was determined from positions −1073 to 3590 relative to the first nucleotide of the start site of translation (Figs. 1, 4A, B). The promoter region of the wild-type allele from AM79.7322 has features of each of the promoters of two wild-type alleles, G1 and G28, from the tetraploid cv. Granola (Rohde et al. 1990). The promoter part of the sequence shown here is of about the same length as the shorter of the two Granola sequences, i.e. the 1.2 kb *Hin*dIII fragment of allele G1. The CAAT box of the two Granola promoters is in our sequence represented by CTAT at the same position, 32 nucleotides upstream of the putative promoter box TACAAAT. An imperfect inverted repeat of 29–31 nucleotides is positioned between −846 and −738 (Fig. 1). The sixfold direct repeat TCAC around the putative start site of transcription found in the Granola sequences (Rohde et al. 1990) is not so extensive in our sequence, but is present as a ninefold A/TC repeat (positions −298 to −280). The distance between this repeat and the TACAAAT box is different in all three alleles.

Between the CTAT and TACAAAT boxes the palindrome TGACACGTGTCA occurs; it probably carries the recognition sequence for a transcription factor since it shares seven out of eight nucleotides with the transcription factor target sequence, CACGTGGC, known as the abscisic acid (ABA) response element (Guiltinan et al. 1990) and UV-boxII or G-box (Schulze-Lefert et al. 1989). This sequence is found in regulatory regions of many differently expressed plant genes, including *rbcS*, patatin and wound-inducible genes of potato (Schulze-Lefert et al. 1989; Guiltinan et al. 1990) and is often surrounded by degenerate sequences. Two degenerate sequences are present in the GBSS gene as imperfect direct repeats starting 50 bp upstream of the G-box palindrome.

Scanning of the GBSS sequence with some other known regulatory sequences from plants (Buzby et al. 1990; Gilmartin et al. 1990; Jefferson et al. 1990; Liu et al. 1990; Maas et al. 1990; Springer et al. 1990; Takahashi et al. 1990) did not reveal regions with high similarity. It should be noted that sequences with low similarities (25–50% of the maximum values of a weight matrix scan) were found on searching with the two parts of the patatin repeat which is responsible for the sucrose response (Jefferson et al. 1990). These regions are located between positions −613 and −548 (three overlapping matches), −370 and −338 (overlapping the G-box palindrome) and −188 and −155 (in the first intron).

Analysis of the transcribed region

The cDNA used for the isolation of the genomic sequences contained one unspliced intron in the coding part (Hergersberg 1988) and also had an unspliced intron in the leader, as inferred from comparisons with the genes from maize and barley (Rohde et al. 1990). We decided to isolate more cDNAs in order to verify the existence of the latter intron. One cDNA clone with an almost complete leader lacked a part of the sequence present in the genome, showing that the potato GBSS gene indeed contains an intron in the leader sequence, with the intron/exon junction 36 nucleotides upstream of the translational start site. This is similar to the sequences of maize and barley (Klösgen et al. 1986; Rohde et al. 1988). The first introns of many plant genes, including genes encoding enzymes involved in starch metabolism like the sucrose synthase gene *Shrunken-1* of maize (Vasil et al. 1989), are reported to play a regulatory role in gene expression. Genomic sequence information on potato starch metabolism genes in the literature is for the moment restricted to the phosphorylase gene (Camirand et al. 1990), which has been reported not to carry an intron in the untranslated leader. The first intron of the potato GBSS gene shows many short direct repeats, among them an almost perfect 11-fold CTT stretch (positions −106 to −73). The presence of the first two introns in the cDNA of Hergersberg (1988) might indicate regulation at the level of splicing. The



Fig. 1. Complete genomic nucleotide sequence of the potato granule-bound starch synthase (GBSS) gene with the translational start at nucleotide +1. The stop codon at position 2961 is shown in *bold*. Introns are shown in *small italics*, bordered by *bold* type. The putative promoter sequences and polyadenylation signals are *underlined*, poly (A) addition sites are marked with *asterisks*. The start of the largest cDNA is marked with a *black dot*; the sequence around the start of transcription is *underlined* with a *dashed line*. G-box-like sequences are *overlined* with *arrows*, large imperfect inverted repeats with *dashed arrows*

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Fig. 2. Alignment of four deduced amino acid sequences of precursors of granule-bound starch synthases from plants, with the similarity shown in the lower row. *Asterisks* are identical residues and *dots* are conservative substitutions. Potato sequences are as deduced from the DNA sequence (1) and as determined by automatic Edman degradation (2). The upper sequence is a translation of the *Escherichia coli glgA* gene which was aligned with all four mature sequences of the starch synthases using the program Clustal. Similarity and identity between the *glgA* sequence and the four plant sequences are given as described above. Gaps in the alignment are indicated by *dashes*; *question marks* are residues which could not be unambigiously determined by Edman degradation. Conserved boxes are indicated

first exon and the first nucleotides of the first intron are missing in the cDNA sequence of Hergersberg (1988), which probably accounts for the negative outcome of attempts to complement the *amf* mutation with constructs containing this cDNA under the control of the CaMV promoter (our unpublished results). The second intron shows only two substitutions when compared with the genomic sequence (data not shown) and the latter was shown to be functionally active in complementation experiments (van der Leij et al. 1991).

The positions of the introns were determined by alignment of the genomic sequence with the cDNA sequences. As in the maize *Waxy* gene (Klösgen et al. 1986), the total number of intervening sequences in the potato GBSS gene is 13. The same number has been reported for the rice gene (Wang et al. 1990) without, however, an intron in the leader. Given the well-conserved intron position in the leader of the genes from potato, maize and barley, it would not be surprising if a 14th intron were present in the leader of the rice gene. The barley gene (Rohde et al. 1988) carries only 11 introns.

All introns in the potato GBSS gene follow the universal GT/AG rule and coincide with AT-rich regions. As is found for other dicot plant genes (Camirand et al. 1990; Hanley and Schuler 1988) and indeed plant genes in general (Brown 1986), the positions near the splice junctions are less fixed, although, in the potato GBSS gene, the third nucleotide of all splice donor sites is an A.

Putative polyadenylation signals have already been found 21 and 11–13 nucleotides upstream of the polyadenylation sites of three 3′ cDNA sequences (Hergersberg 1988).

After processing of the GBSS primary transcript a mature messenger RNA of about 2.4 kb remains, which encodes the precursor protein, using the first AUG sequence as the start codon. The codon usage in the potato GBSS gene complies with that in dicots (Campbell and Gowri 1990) with frequencies of 25% and 34% for A and U as third nucleotide, respectively, where monocots show much lower values for these nucleotides. For example, the maize *Waxy* sequence shows the respective nucleotides in the third position in only 4.4% and 2.8% of the codons used.

Protein sequences

The potato GBSS gene encodes a 607 amino acid preprotein with a calculated molecular weight of 66575 daltons. The amino acid sequence of the N-terminus of the mature GBSS protein, as determined by automated Edman degradation, enabled us to locate the transit peptide processing site (Fig. 2). A transit peptide of 77 amino acids, needed for the routing of the enzyme, is cleaved from the preprotein during transport into plastids. The mature form of potato GBSS, as deduced from the DNA sequence, is a 540 amino acid protein of 58243 kDa. Vos-Scheperkeuter et al. (1986) determined a M_r (rela-

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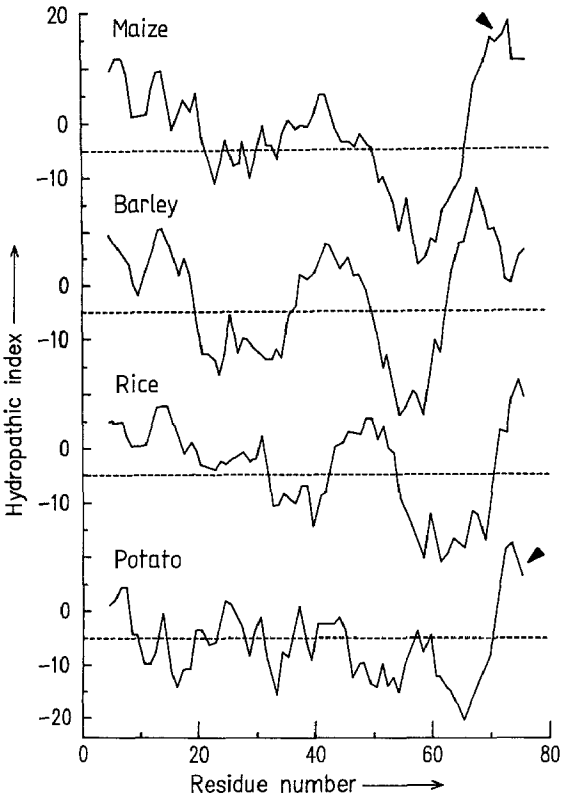


Fig. 3. Hydrophobicity plots according to Kyte and Doolittle (1982) of the 80 N-terminal residues of the four plant GBSS precursors containing the transit peptides. The cleavage sites, which are only known for the maize and the potato protein, are indicated by an *arrowhead*. The interval used for the calculation of the indices was nine amino acid residues

tive molecular weight) of 60 kDa by SDS-polyacrylamide gel electrophoresis.

A comparison of the amino acid sequence of the potato pre-GBSS with the sequences of the three waxy preproteins of the monocots maize, barley and rice reveals a high degree of similarity between the mature forms of all four enzymes, but not between the transit peptides (Fig. 2). Of the 540 amino acid residues of the mature potato GBSS, 336 (62%) are identical and 472 (87%) are similar (identical amino acids plus conservative substitutions) in all four enzymes. Alignment of the plant enzymes with the *E. coli glgA* gene product glycogen synthase (Kumar et al. 1986) shows 26% identity and 63% similarity of the glycogen synthase with the starch synthases and reveals at least three homologous boxes. The conservation of the ADP-glucose binding site, KTGGL (Furukawa et al. 1990), at residues 15–19 of the glycogen synthase has already been discussed by Preiss (1990) in a comparison of the *glgA* sequence with two monocot starch synthases and the N-terminus of the potato GBSS. A second conserved sequence is located at residues 372–384 of the glycogen synthase (478–490 of the potato sequence). Within this box, the sequence VPSRFEPCGL is identical between the potato, rice and *E. coli* sequences and shares one conservative substitution compared with the maize and barley sequences (Fig. 2). A scan of the Swiss Prot databank with this sequence did not reveal similarities with other known proteins.

A third box is separated from box II by 12 amino acids, with a proline 4–5 residues before box III. The sequence TGGLAD in the *E. coli* protein resembles the sequence of the six *E. coli* residues immediately beyond the ADP-glucose-binding lysine residue in box I. In glycogen synthase, the lysine in box III is replaced by an arginine and in the starch synthases by a serine, which makes it unlikely that box III can have the same function as box I. The major parts of the three boxes mentioned here are also present in the deduced protein sequence of the *Bacillus subtilis glgA* gene (J. Kiel, personal communication).

The well-conserved primary structures of the mature proteins of the four plants compared here do not show any similarity with proposed starch granule binding domains of bacterial and fungal amylases, branching enzymes and cyclodextrin glycosyl transferases (Svensson et al. 1989).

The primary structures of the transit peptides of the four plant preproteins, although highly diverged, share the common features of transit peptides (Keegstra et al. 1989) in containing a relatively high amount of alanine, serine and threonine residues, starting with a hydrophobic region followed by one or more hydrophilic parts and ending with a dramatic increase in hydrophobicity near the (putative) processing sites. Plots of the hydropathic indices are shown in Fig. 3; the pattern mentioned above is less pronounced in the case of the potato GBSS transit peptide.

Sequence of the amf *allele*

Since we had indications that the GBSS gene isolated earlier from 86040 was derived from a chimeric plant, with mutant and either wild-type or revertant tissues, we decided not to use clones from the monoploid 86040 for further analysis. The isolation of a genomic clone from a diploid amf plant was done by probing with parts of DNA surrounding the structural GBSS gene. Despite this approach of isolating only those clones which carry upstream as well as downstream sequences, the only complete clone obtained did not carry the distal *Bgl*II site. This did not interfere with our aim to reduce the part of the gene to be sequenced, since a wild-type subclone with even fewer potato sequences at the 3′ end (pWAM150, van der Leij et al. 1991, see also Fig. 4C) was shown to be able to complement the amf mutant.

Transformation experiments using the chimeric constructs pWM114 and pMW104 revealed that the mutation was located in the part of the gene upstream of the central *Eco*RI site (Fig. 4C). Sequencing of the region between the *Nsi*I site in the first intron and the central *Eco*RI site revealed the deletion of one of the three adenines at positions 46–48 of the GBSS sequence. The part of the gene between the *Hin*dIII site and the central *Eco*RI site showed no alterations in a range of single direction sequencing reactions. The absence of a mutation in that part was confirmed by the complemen-

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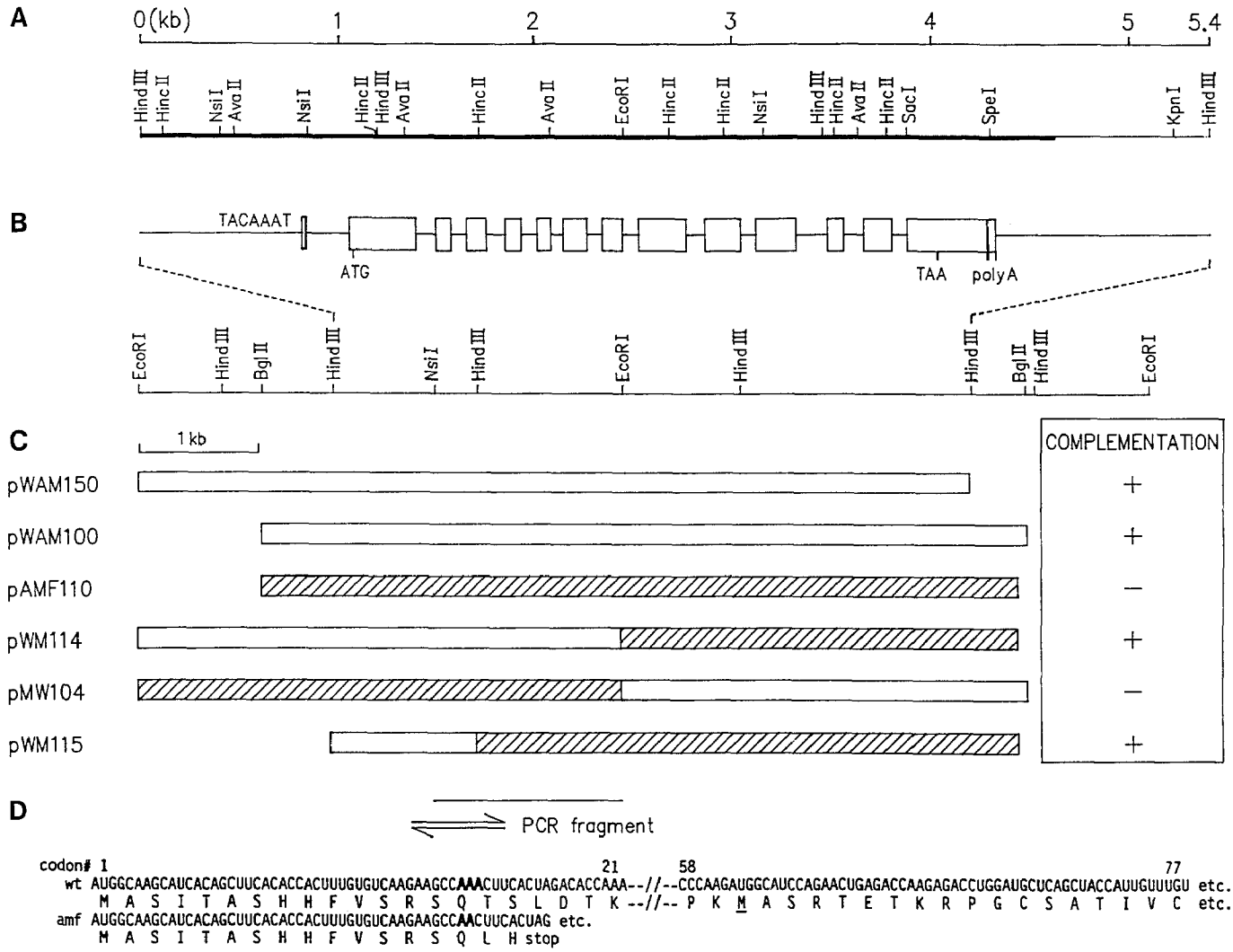


Fig. 4A–D. Schematic overview of the potato GBSS gene and derivatives and the sequence of the *amf* allele. **A** Representation of the sequenced parts and restriction map of the wild-type allele. The *thick line* represents the part of the three *Hin*dIII fragments which has been sequenced in both directions. The sequence of this part is shown in Fig. 1. **B** The GBSS gene as part of two *Eco*RI fragments with the promoter, translational start and stop, and poly(A) sites assigned. *Boxes* represent exons. **C** Chimeric constructs used to localize the *amf* mutation with the corresponding complementation results. *Open boxes* (wild type), *hatched boxes* (mutant). **D** Part of the *amf* allele which has been sequenced in one direction (upper) and both directions (polymerase chain reaction fragment). The relevant parts of the sequences of wild type and mutant are shown below as RNA sequences, together with the deduced protein sequences. The adenine residues in the wild type from which one has been deleted in the mutant are shown in *bold*. The *underlined* methionine is the possible start of (re-)initiation of translation in the mutant. The numbering of codons is indicated

tation of the amf mutant with construct pWM115 (Fig. 4C), which only carries the first *Hin*dIII fragment from the wild-type allele, the rest of the construct being derived from the amf clone G1107. To exclude the possibility of sequence alterations in the transcribed part upstream of the *Nsi*I site, a polymerase chain reaction (PCR) was performed with primers upstream of the promoter TACAAAT box and downstream of the *Hin*dIII site mentioned above. Sequencing of both strands of PCR fragments derived from G1107 and from plant DNA of 1031-29 and the original mutant 86040 confirmed that the only difference between the wild-type and the *amf* allele is the deletion of one of the three adenines of codons 15 and 16 (Fig. 4D). Point deletions, next to larger gene alterations, have been reported for mutant genes which were obtained after ionizing radiation (Grosovsky et al. 1988). However, the mutation may also have arisen spontaneously.

The consequence of this single base deletion is a frameshift and the subsequent termination of translation at the position of codon number 18, an amber (UAG) stop codon, of the *amf* sequence. Therefore, the amf mutant is expected to synthesize only a short peptide of 17 amino acids. However, in Fig. 5 we show the presence of a GBSS-like protein in a membrane fraction from tubers from the amf mutant. The M_r of this protein turned out to be 4 ± 2 kDa larger than a membrane-bound GBSS-like protein from the wild type, the latter having the same M_r (60 kDa) as the wild-type mature GBSS. It seems logical that the mutant GBSS-like protein is the product of the *amf* GBSS sequence. This phenomenon can be explained if it is assumed that (re-)initi-

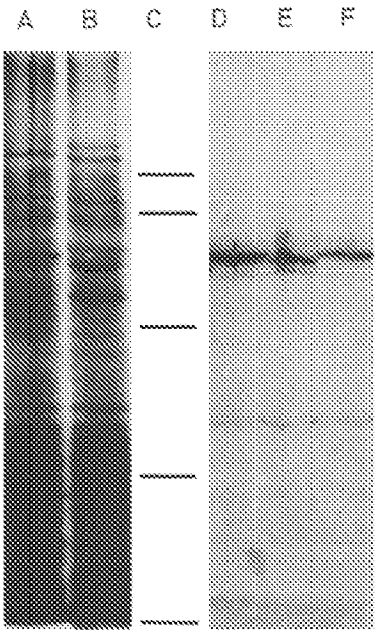


Fig. 5. Proteins cross-reacting with anti-GBSS serum. Membrane-bound proteins were separated in a 10% SDS-polyacrylamide gel either stained with Coomassie Brilliant Blue (lanes A, B) or blotted and immunoprecipitated (lanes D–F). Tuber membrane fractions obtained from the amf mutant (lanes A, D, F) and a wild-type potato (lanes B, E) show the presence of immunoreacting proteins of about 60 kDa, the mutant protein being apparently larger than the band from the wild-type preparation. Lane C represents the molecular weight markers with proteins of 78, 68, 45, 30 and 17 kDa, respectively, from the top downwards

ation of translation in the mutant takes place at internal AUG codons. Two of these AUG codons are out of phase but initiation at codon 60 of the wild-type sequence (Fig. 4D) would result in a protein which is about 2 kDa larger than the mature wild-type form. This protein carries only 17 amino acids of the transit peptide, starting with the amino acid sequence MASRT. Except for the hydrophilic arginine residue, this sequence forms a perfect start for a transit peptide which, however, will probably be too short to allow transport of the precursor across the plastid membranes. The shortest transit peptide known to be functionally active contains 29 amino acids (Keegstra et al. 1989). The amf mutant does not have GBSS attached to starch grains inside plastids (Hovenkamp-Hermelink et al. 1987). Binding to the plastid membrane, but without transport and the accompanying splitting off of the transit peptide, would result in the observed presence of a membrane-bound protein with M_r 62 kDa. Vancanneyt et al. (1990) showed in an analysis of transgenic tobacco plants that steady-state plant mRNA levels dramatically decrease when artificial stop codons prevent further translation. In contrast with their results, slightly increased amounts of GBSS mRNA were found in the amf mutant (Visser et al. 1989). This discrepancy implies either a difference in stability of the mRNAs, which might be related to the translatibility of the part of the gene beyond the stop codon, or a compensating feedback mechanism at the transcriptional level in the amf mutant.

Although the general rule is that the first start codon is used, initiation of translation at internal AUG codons has been shown to occur in plant cells (Putterill and Gardner 1989) and in plant viruses (Tacke et al. 1990).

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Note added in proof. The sequence data will appear in the EMBL/Gen Bank/DBBJ Nucleotide Sequence Data Bases under accession number X58453

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Homologues of a single resistance-gene cluster in potato confer resistance to distinct pathogens: a virus and a nematode

Edwin A.G. van der Vossen[1,*], Jeroen N.A.M. Rouppe van der Voort[2,†], Konstantin Kanyuka[3,‡], Abdel Bendahmane[3,¶], Hans Sandbrink[1], David C. Baulcombe[3], Jaap Bakker[2], Willem J. Stiekema[1] and Rene M. Klein-Lankhorst[1]

[1]*Plant Research International, Business unit Genomics, PO Box 16, 6700 AA, Wageningen, The Netherlands,*

[2]*Graduate School of Experimental Plant Sciences, Wageningen Agricultural University, Department of Nematology, PO Box 8123, 6700 ES Wageningen, The Netherlands, and*

[3]*The Sainsbury Laboratory, Norwich Research Park, Colney, Norwich NR4 7UH, UK*

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[*]For correspondence (fax +31 317 418094; e-mail e.a.g.vandervossen@plant.wag-ur.nl).

[†]Present address: Keygene NV, Agrobusiness Park 90, Wageningen, The Netherlands.

[‡]Present address: Department of Agricultural Sciences, University of Bristol, Long Ashton, Bristol BS41 9AF, UK

[¶]Present address: INRA-URGV, 2 Rue Gaston Crémieux CP 5708, 91057 Evry Cedex, France.

Summary

The isolation of the nematode-resistance gene *Gpa2* in potato is described, and it is demonstrated that highly homologous resistance genes of a single resistance-gene cluster can confer resistance to distinct pathogen species. Molecular analysis of the *Gpa2* locus resulted in the identification of an *R*-gene cluster of four highly homologous genes in a region of approximately 115 kb. At least two of these genes are active: one corresponds to the previously isolated *Rx1* gene that confers resistance to potato virus X, while the other corresponds to the *Gpa2* gene that confers resistance to the potato cyst nematode *Globodera pallida*. The proteins encoded by the *Gpa2* and the *Rx1* genes share an overall homology of over 88% (amino-acid identity) and belong to the leucine-zipper, nucleotide-binding site, leucine-rich repeat (LZ-NBS-LRR)-containing class of plant resistance genes. From the sequence conservation between Gpa2 and Rx1 it is clear that there is a direct evolutionary relationship between the two proteins. Sequence diversity is concentrated in the LRR region and in the C-terminus. The putative effector domains are more conserved suggesting that, at least in this case, nematode and virus resistance cascades could share common components. These findings underline the potential of protein breeding for engineering new resistance specificities against plant pathogens *in vitro*.

Keywords: disease resistance, resistance gene, gene-for-gene, potato cyst nematode, potato, map-based cloning.

Introduction

In their constant battle against challenging pathogen populations, plants have evolved defense recognition mechanisms that are mediated through gene-for-gene interactions (Flor, 1971). Classes of highly variable plant genes, so-called resistance (*R*) genes, play a key role in this process and are thought to encode specialized receptors that recognize corresponding avirulence (*Avr*) gene-dependent elicitors produced by pathogens (Crute, 1994; Keen, 1990). Whenever the *R* gene or the corresponding *Avr* gene is not functional, recognition does not occur and the plant is susceptible to pathogen attack. To survive, it is crucial that the host plant is able to respond to loss of *R* gene function by generating novel recognition capacities. The cloning and molecular characterization of numerous *R* genes from diverse plant species, conferring disease resistance to bacteria, fungi, viruses, nematodes, and insects (Baker *et al.*, 1997; Milligan *et al.*, 1998; Rossi *et al.*, 1998; Vos *et al.*, 1998) has revealed features characteristic of *R* genes that may form the basis of specificity in *R*-gene function and rapid generation of new *R* gene alleles. The

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majority of *R* genes are members of tightly linked multigene families. This is thought to facilitate the generation of novel *R*-gene specificities through unequal recombination between cluster members resulting in duplication, partial deletion or formation of new chimeric genes. Comparative sequence analysis of several *R* genes supports this idea (Botella *et al.*, 1998; Collins *et al.*, 1999; Dixon *et al.*, 1998; McDowell *et al.*, 1998; Meyers *et al.*, 1998; Noël *et al.*, 1999; Parniske *et al.*, 1997; Thomas *et al.*, 1997). All *R* genes characterized so far, with the exception of *Pto* (Martin *et al.*, 1993), encode leucine-rich repeats (LRRs) (Baker *et al.*, 1997; Hammond-Kosack and Jones, 1997), structures shown to be involved in protein–protein interactions (Kobe and Deisenhofer, 1994). LRR-containing R proteins can be divided into two classes based on the presence of a putative tripartite nucleotide binding site (NBS) (Bent, 1996; Staskawicz *et al.*, 1995). R proteins of the NBS-LRR class comprise motifs that are shared with animal apoptosis regulatory proteins (Aravind *et al.*, 1999; van der Biezen and Jones, 1998). The second class of R proteins encompasses proteins with a predicted hydrophobic membrane-anchoring domain with a predicted extracellular N-terminal LRR motif (Bent, 1996; Jones *et al.*, 1994; Song *et al.*, 1995). The common occurrence of LRRs in R proteins, along with the hypervariable character of predicted solvent-exposed amino acids within these LRRs (reviewed by Michelmore and Meyers, 1998), have implicated LRRs in the binding of pathogen-produced elicitors (Baker *et al.*, 1997; Jones and Jones, 1997). Although analysis of *R*-gene products has correlated variability within LRRs with differential pathogen recognition (Botella *et al.*, 1998; Collins *et al.*, 1999; Dixon *et al.*, 1998; Ellis *et al.*, 1999; McDowell *et al.*, 1998; Meyers *et al.*, 1998; Noël *et al.*, 1999; Parniske *et al.*, 1997; Thomas *et al.*, 1997), it is still unclear how Avr proteins actually interact with R proteins. It is, however, becoming apparent that R-protein specificity and physical interactions between LRR domain and Avr protein are influenced by intramolecular interactions between the LRR domain and other parts of the protein, presumably the N-terminal part (Bryan *et al.*, 2000; Ellis *et al.*, 1999).

Although most *R*-gene clusters characterized to date have currently been assigned single-recognition specificities, the picture that is emerging from molecular analysis of *R*-gene loci is that most contain an array of related sequences, of which several can be active *R* genes. The Arabidopsis *RPP1* complex resistance locus, for instance, contains four tightly linked genes, three of which encode functional *R* genes that recognize distinct *Peronospora parasitica* avirulence determinants (Botella *et al.*, 1998). Recently Takken *et al.* (1999) identified a second active gene at the tomato *Cf-4* locus conferring resistance to *Cladosporium fulvum* through the recognition of a novel avirulence determinant. In most cases where only a single recognition specificity is assigned to an *R* gene cluster, additional cluster members are transcriptionally active (*Dm3*, Meyers *et al.*, 1998; *I2*, Ori *et al.*, 1997; Simons *et al.*, 1998; *Mi*, Milligan *et al.*, 1998; *Rp1*, Collins *et al.*, 1999; *Xa21*, Song *et al.*, 1995). Transcriptional activity of these cluster members suggests functionality, and the screening of these haplotypes with an exhaustive pathogen collection would probably identify these members as functional *R* genes. Interestingly, in those cases where two or more members within a single haplotype are functional *R* genes, resistance is directed to different avirulence determinants of the same pathogen species (Botella *et al.*, 1998; Takken *et al.*, 1999). The only locus described so far with specificity to distinct pathogen species is the *Mi* locus in tomato. However, in this case it is the same gene that confers dual specificity, as the *Mi* gene confers resistance to both root-knot nematodes and potato aphids (Rossi *et al.*, 1998; Vos *et al.*, 1998).

In potato the *Gpa2* locus, which confers specific resistance to a small set of populations of the potato cyst nematode *Globodera pallida* (Rouppe van der Voort *et al.*, 1997), was previously shown to be tightly linked to the *Rx1* gene, an NBS-LRR gene responsible for resistance to potato virus X (Bendahmane *et al.*, 1999; Rouppe van der Voort *et al.*, 1999). Both genes were localized to an approximately 200 kb region on chromosome 12 of potato. Here we describe the further molecular analysis of this region, which contains four highly homologous *R* gene-like sequences. One of these corresponds to *Rx1* (Bendahmane *et al.*, 1999) and another to the nematode-resistance gene *Gpa2*. This finding demonstrates that separate members of an *R*-gene cluster can evolve to confer resistance to unrelated pathogen species.

Results

Identification of the Gpa2 *gene and complementation of function*

The nematode-resistance locus *Gpa2* was previously localized to the genetic interval IPM4c-111R on chromosome 12 of potato (Rouppe van der Voort *et al.*, 1999), a region physically spanned by the four overlapping bacterial artificial chromosome (BAC) clones SHBAC43, BAC45, BAC221 and BAC111 (Figure 1a). Sequencing of the four BAC clones resulted in the identification four highly homologous *R*-gene candidates (RGC) of the NBS-LRR class of plant genes in a region of approximately 115 kb (Figure 1b). Three of these genes, *RGC1*, *RGC2* and *RGC3*, were considered as *Gpa2* candidates. The fourth RGC, identified on BAC clone SHBAC43, contained marker IPM4c and therefore corresponds to the recently cloned virus resistance gene *Rx1* (Bendahmane *et al.*, 1999) (Figure 1). For *Gpa2* complementation analysis, binary

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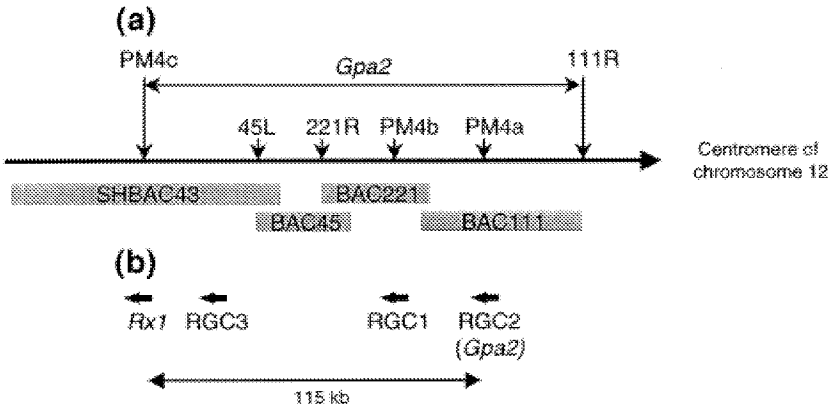


Figure 1. Physical map of the *Gpa2/Rx1* locus.
(a) Genetic and physical map of the potato genomic region containing *Gpa2*. Vertical arrows indicate the positions of markers linked to *Rx1* and *Gpa2* (Rouppe van der Voort *et al.*, 1999). Shaded rectangles represent bacterial artificial chromosome (BAC) clones
(b) Relative positions of three *Gpa2* candidate genes (*RGC1–3*) and *Rx1*. The size of the resistance-gene cluster is indicated.

plasmids (pBINPLUS; van Engelen *et al.*, 1995) containing restriction fragments of approximately 11, 10.3, 5.5 and 6.5 kb, spanning *RGC1*, *RGC2*, *RGC3* and *Rx1*, respectively, were transferred to the nematode-susceptible potato line V using *Agrobacterium*-mediated transformation. The different genetic constructs (pBINRGC1–3 and pBINRx1, respectively) retained at least 2 kb upstream and 0.5 kb downstream of the putative coding regions of each RGC. DNA gel-blot analysis indicated that transgene copy numbers in the transgenic plants obtained varied between one and ten (data not shown).

Initial evidence that *RGC2* represents the *Gpa2* gene was obtained from *in vitro* complementation assays in which *in vitro*-grown primary transformants harboring *RGC1*, *RGC2*, *RGC3*, or the empty binary vector pBINPLUS were inoculated with sterilized second-stage juveniles of *G. pallida* population Pa_2-D383. Resistant (R) and susceptible (S) progeny plants derived from the F_1 (SH × RH) mapping population used to map the *Gpa2* gene (Rouppe van der Voort *et al.*, 1999) were included in the assay as controls. The development of female nematodes on the roots of R_0(RGC1), R_0(RGC3) and R_0(BINPLUS) plants was similar to that observed on the roots of the susceptible control plants (Table 1). In contrast, the R_0(RGC2) plants showed the same incompatible interaction with *G. pallida* population Pa_2-D383 as the resistant control plants. Firstly, the number of females able to develop on the roots of R_0(RGC2) plants was equivalent to the number of females able to develop on the roots of resistant control plants (Table 1). Secondly, the majority of the females that did develop on the roots of R_0(RGC2) and resistant control plants remained small and translucent (Figure 2). Thirdly, while only adult females were recovered on susceptible plants, R_0(RGC2) plants showed a high number of males (data not shown). Such a change in sex ratio for developing PCN juveniles has also been reported for the incompatible interaction between *G. rostochiensis* and the *H1* resistance gene (Bakker *et al.*, 1993). These results demonstrate that the 10.3 kb DNA insert harboring *RGC2* is able to complement the susceptible phenotype in transgenic plants.

Final proof that the 10.3 kb DNA insert contained *Gpa2* was obtained by testing 24 independent primary transformants of each RGC for resistance to *G. pallida* population Pa_2-D383 in assays carried out in the greenhouse. Of the 24 *RGC2* transformants tested, 21 were resistant to *G. pallida* population Pa_2-D383 (Table 1). In contrast, all *RGC1*, *RGC3* and *Rx1* transformants were susceptible to this *G. pallida* population.

Structure of Gpa2 *gene and corresponding protein*

The size and structure of the *Gpa2* gene was determined by comparing the genomic sequence derived from the insert of BAC111 with cDNA fragments generated by 5′ and 3′ rapid amplification of cDNA ends. RACE identified 5′ and 3′ *Gpa2*-specific cDNA fragments of a single species, respectively, suggesting that the genomic clone encodes a single *Gpa2*-specific transcript. The putative *Gpa2* transcript is estimated to be 3127 nucleotides (nt) and contains a 30 and 358 nt 5′- and 3′-untranslated region (UTR), respectively. Although *Gpa2* contains exactly the same two introns as *Rx1* (Bendahmane *et al.*, 1999) (Figure 3a), the stop codon of Gpa2, in contrast to that of *Rx1*, is present in the second exon. This change in frame, as compared to that of *Rx1*, is the result of an 11 bp insertion immediately downstream of the first intron splice site of *Gpa2*. The second intron sequence of 112 bp is located within the 3′-UTR of the mature mRNA of *Gpa2*.

The deduced open reading frame of the *Gpa2* gene encodes a predicted polypeptide of 912 amino acids with a molecular weight of 104.5 kDa (Figure 3b). Analysis using CLUSTALW showed that *Gpa2* shares over 88% amino-acid sequence identity with *Rx1* (Table 2). Like *Rx1*, several putative functional motifs present in *R* genes of the NBS-LRR type are apparent in the encoded protein, which can be subdivided into six domains (A–F; Figure 3b). The N-terminal part of the protein contains a potential five-heptad leucine zipper (residues 10–45; domain A). Domain B harbors the NBS and other motifs that constitute the NB-ARC domain (ARC for Apaf-1, R protein, and CED-4) of R

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Table 1. Nematode-resistance assays

Genotype[a]	*In vitro* assay: Average no. cysts[b]	Greenhouse assay: R plants/ plants tested[c]	No. cysts ±SD on R plants	No. cysts ±SD on S plants
R_0(RGC1)	39	0/24	–	300 ± 101
R_0(RGC2)	2	21/24	4 ± 9	251 ± 91
R_0(RGC3)	40	0/25	–	270 ± 106
R_0(Rx1)	nt	0/25	–	312 ± 81
R_0(vector)	33	0/4	–	162 ± 71
Line V	nt	0/4	–	525 ± 88
F_1 (S)	42	nt	–	–
F_1 (R)	5	nt	–	–

[a]R_0(RGC1–3) and R_0(Rx) are primary transformants containing T-DNA with the *Gpa2* gene candidates *RGC1*, *RGC2*, *RGC3* and *Rx1*, respectively. R_0(vector) are control primary transformants containing T-DNA with the empty binary vector pBINPLUS. Line V is the nematode susceptible potato genotype used for *Agrobacterium*-mediated transformation. F_1(S) and F_1(R) are susceptible and resistant progeny plants, respectively, derived from the F_1(SH × RH) mapping population used to map the *Gpa2* gene (Rouppe van der Voort *et al.*, 1997).

[b]Three plants from four independent primary transformants generated for each *RGC* were assayed *in vitro*. Each data point is the average number of females per Petri dish. Three plants of each primary transformant were grown per Petri dish, and each dish was inoculated with 45 sterile juveniles.

[c]Plants were scored as resistant (R) if the number of cysts found on the roots of the plants was <20.

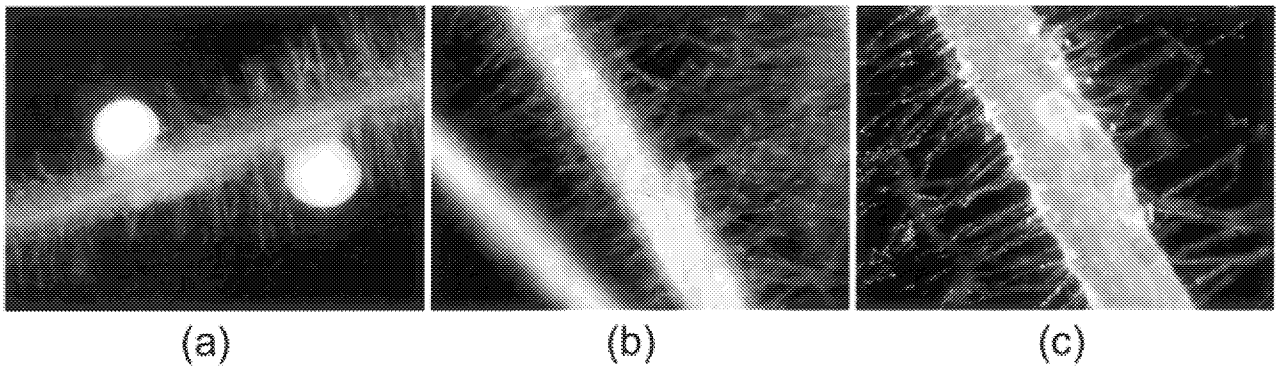


Figure 2. Genetic complementation for nematode resistance.
Characteristic differences in female nematode development 5 weeks post-inoculation on the roots of susceptible (a) and resistant (b) control plants, and transgenic potato plants harboring the *Gpa2* candidate gene *RGC2* (c). The development of stagnated and translucent female nematodes is characteristic for an incompatible reaction. Photographs at (b) twice and (c) three times the magnification of (a), 25×.

proteins and cell death regulators in animals (van der Biezen and Jones, 1998). This domain includes the Ap–ATPase motifs present in proteins of eukaryotic and prokaryotic origin (Aravind *et al.*, 1999). The C-terminal half of *Gpa2* comprises a series of 16 irregular LRRs (Figure 3b). The LRRs could be aligned according to the consensus sequence axxLxxLxLxxC/Nxxa, where 'x' designates any residue and 'a' designates the positions of aliphatic amino acids, followed by a region of varying length. This repeat format approximates the consensus for cytoplasmic LRRs (Jones and Jones, 1997). The first 11 LRRs in domain C are separated from those in domain E by a 17 amino-acid region with a poor match to the above LRR consensus sequence (domain D). This region contains a relatively high amount of proline residues, suggesting that it may act as a molecular hinge between the two LRR domains. Domain F encompasses the C-terminus of *Gpa2*.

Amino-acid sequence homology and nucleotide substitution patterns in different regions of Gpa2 *and* Rx1

Alignment of the deduced amino-acid sequences of *Gpa2* and *Rx1* shows that the N-terminal halves of the proteins,

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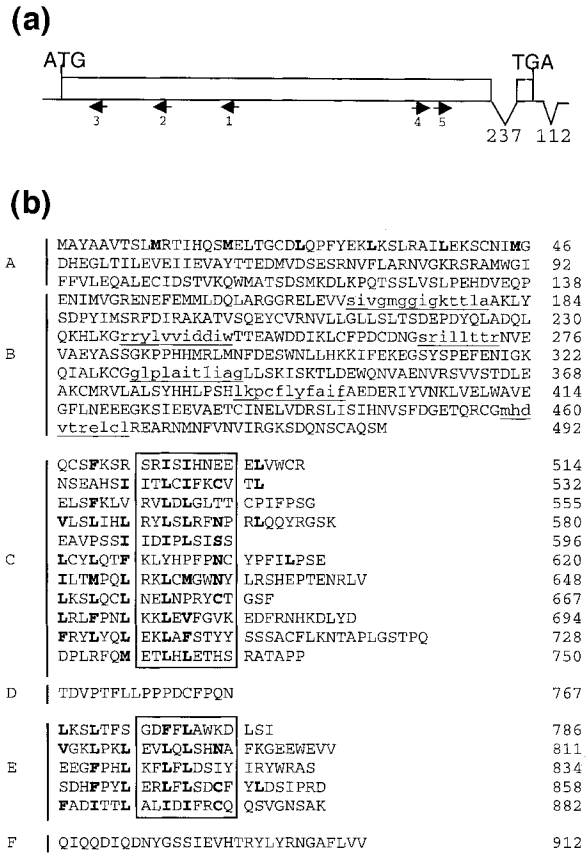


Figure 3. Structure of the *Gpa2* gene.
(a) Schematic diagram of the *Gpa2* transcript (3127 nucleotides). Exons are indicated by horizontal lines. Open boxes indicate coding sequence. Lines angled downwards indicate intron positions (numbers 237 and 112 represent intron sizes). Arrows indicate the relative positions of primers used for 5′-RACE (arrows 3–5) and 3′-RACE (arrows 1 and 2)
(b) Deduced Gpa2 protein sequence. The amino-acid sequence deduced from the DNA sequence of *Gpa2* is divided into six domains (A–F) as described in the text. The leucine or aliphatic residues in domain A that make up the potential five-heptad leucine zipper (residues 10–45) are indicated in bold. Conserved motifs in R proteins are underlined and written in lower case in domain B. Residues matching the consensus of the cytoplasmic LRR are indicated in bold (domains C and E). The LRR subdomain xxLxLxxxx is framed.

harboring the putative effector domains, share a higher degree of sequence homology (92% amino-acid sequence identity) than the C-terminal halves of the proteins, which are thought to contain the putative recognition domains (84% amino-acid sequence identity; Table 2). The most striking difference between Gpa2 and Rx1 is that Gpa2 lacks the acidic tail which is present in Rx1 (Bendahmane *et al.*, 1999) (Figure 4a). Further comparison between the two protein sequences showed that much of the remaining amino-acid sequence divergence between Gpa2 and Rx1 is concentrated in the subdomains of the LRRs which are predicted to be solvent-exposed (Jones and Jones, 1997; Figure 4). Sequence divergence of the amino-acid residues positioned around the two conserved leucine residues in the consensus xxLxLxxxx (framed residues in Figure 3b)

Table 2. Comparison of synonymous and non-synonymous nucleotide substitutions and nucleotide and amino-acid homology in different regions of Gpa2 and Rx1

	Gpa2 versus Rx1		
	Homology[b]		
Analysed	nt	aa	K_a/K_s[c]
Complete gene	93	88	1.46
N-terminal half[a]	95	92	0.82
C-terminal-half[a]	93	84	2.5***
LRR domain			
xx(L)x(L)xxx			3***
Non-framed			1.35

[a]The regions analysed are defined in Figures 3 and 4. Separate comparisons were made between the xxLxLxxxx region of the LRRs, omitting the codons for the conserved aliphatic positions, and the regions between the xxLxLxxxx motifs (non-framed).
[b]Percentage nucleotide (nt) and amino acid (aa) identity.
[c]Rates of non-synonymous (K_a) and synonymous (K_s) nucleotide substitutions per non-synonymous/synonymous site were calculated using the DIVERGE program in the GCG software package.
***Significant at the 0.1% level.

was three times higher (29.5%) than that observed in the rest of the LRR domain (9.6%; Figure 4b).

To determine the type of selection pressure operating on different parts of the genes, ratios of synonymous (K_s) and non-synonymous (K_a) substitution rates per synonymous/non-synonymous site were calculated for different regions of the open reading frames of *Gpa2* and *Rx1*. A K_a/K_s ratio of 1 suggests random mutation, i.e. that there is no selection pressure operating either towards sequence diversification ($K_a/K_s > 1$) or sequence conservation ($K_a/K_s < 1$) (Hughes and Nei, 1988). When the N-terminal halves of the open reading frames of *Gpa2* and *Rx1* are compared, K_s exceeds K_a, suggesting sequence conservation within this part of the genes. Calculations of K_a and K_s for the C-terminal halves of the proteins supported the hypothesis that diversifying selection is operating on those parts of the proteins that encode the putative recognition domains. The K_a/K_s ratio calculated separately for the nucleotides encoding the xxLxLxxxx region of the LRRs, omitting the codons for the conserved aliphatic positions, was significantly >1, whereas the K_a/K_s ratio of the LRR-encoding sequence between the xxLxLxxxx motifs did not significantly deviate from 1, suggesting an alternating pattern of variability in the LRRs of Gpa2 and Rx1 (Table 2).

Discussion

Mapping experiments carried out in different potato genotypes have shown that genes for resistance to distinct

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(a)

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Rx1                                                          V    60
Gpa2  MAYAAVTSLMRTIHQSMELTGCDLQPFYEKLKSLRAILEKSCNIMGDHEGLTILEVEIIE  60

Rx1                     Q LEE      E                              120
Gpa2  VAYTTEDMVDSESRNVFLARNVGKRSRAMWGIFFVLEQALECIDSTVKQWMATSDSMKDL  120

Rx1                                                              180
Gpa2  KPQTSSLVSLPEHDVEQPENIMVGRENEFEMMLDQLARGGRELEVVSivgmggigkttla  180

Rx1   T      C                                  D    R           240
Gpa2  AKLYSDPYIMSRFDIRAKATVSQEYCVRNVLLGLLSLTSDEPDYQLADQLQKHLKGrry1  240

Rx1                          Y                                   300
Gpa2  vviddiwTTEAWDDIKLCFPDCDNGsrillttrNVEVAEYASSGKPPHHMRLMNFDESWN  300

Rx1                                      v       MGQR     RIG   S 360
Gpa2  LLHKKIFEKEGSYSPEFENIGKQIALKCGglplaitliagLLSKISKTLDEWQNVAENVR  360

Rx1         P  Q                          T   Q S  E     P       420
Gpa2  SVVSTDLEAKCMRVLALSYHHLPSHlkpcflyfaifAEDERIYVNKLVELWAVEGFLNEE  420

Rx1              T      I     F   F  R TIES                        480
Gpa2  EGKSIEEVAETCINELVDRSLISIHNVSFDGETQRCGmhdvtrelclREARNMNFVNVIR  480
                                                  N-terminal  C-terminal

Rx1                    R         R  KV   A              M GG E     540
Gpa2  GKSDQNSCAQSMQCSFKSRSRISLIHNEEELVWCRNSEAHSIITLCIFKCVTLELSFKLVR  540

Rx1          N W                          C    Q                  600
Gpa2  VLDLGLTTCPIFPSGVLSLIHLRYLSLRFNPRLQQYRGSKEAVPSSIIDIPLSISSLCYL  600

Rx1          NL   SY                 T                    N     Q   660
Gpa2  QTFKLYHPFPNCYPFILPSEILTMPQLRKLCMGWNYLRSHEPTENRLVLKSLQCLNELNP  660

Rx1            F          Q    P     SQ             E T RL   PYA    720
Gpa2  RYCTGSFLRLFPNLKKLEVFGVKEDFRNHKDLYDFRYLYQLEKLAFSTYYSSSACFLKNT  720

Rx1     S   -        T I   K IDFGG     ---- L       A           R E SV  775
Gpa2  APLGSTPQDPLRFQMETLHLETHSRATAPPTDVPTFLLPPPDCFPQNLKSLTFSGDFFLA  780

Rx1                     I  W    I K                        DV        835
Gpa2  WKDLSIVGKLPKLEVLQLSHNAFKGEEWEVVEEGFPHLKFLFLDSIYIRYWRASSDHFPY  840

Rx1       VI R   RN                      DY      V                  895
Gpa2  LERLFLSDCFYLDSIPRDFADITTLALIDIFRCQQSVGNSAKQIQQDIQDNYGSSIEVHT  900

Rx1     H FIPKSVTTVEDDDDSVTTDEDDDDDDFEKEVASCRNNVE*                  937
Gpa2  RYLYRNGAFLVV*                                                  912
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(b)

Amino acid differences in the LRR domain

	Gpa2 vs *Rx1*
xx(L)x(L)xxxx	33/112 (29.5%)
non-framed	22/228 (9.6%)

Figure 4. Sequence comparison between *Gpa2* and *Rx1*.
(a) Alignment of the deduced protein products encoded by *Gpa2* and *Rx1*. The complete amino-acid sequence of Gpa2 is shown, along with amino-acid residues from Rx1 that differ from the corresponding residue in Gpa2. Dashes indicate gaps inserted to maintain optimal alignment. Amino-acid residues that are specific for Gpa2, when compared to those at corresponding positions in Rx1 and Rx2 (Bendahmane *et al.*, 2000), are highlighted in bold italic. The division between N-terminal and C-terminal half is indicated by arrows. LRR subdomains xxLxLxxxx are underlined; NB-ARC motifs (van der Biezen and Jones, 1998) are indicated in lower case.
(b) Amino-acid differences within the LRR domains between Gpa2 and Rx1. The number of different amino acids, the number of amino acids used, and the percentage of difference are indicated.

pathogen species can occupy similar genetic positions. For example, complex loci have been identified on chromosomes 5 and 11 of potato. The cluster on chromosome 5 comprises at least five *R* loci: *R1*, conferring resistance to *Phytophthora infestans* (Leonards-Schippers *et al.*, 1992); *Nb* and *Rx2*, loci that determine potato virus X resistance (De Jong *et al.*, 1997; Ritter *et al.*, 1991); and *Gpa* and *Grp1* which bestow resistance to the potato cyst nematode (Kreike *et al.*, 1994; Rouppe van der Voort *et al.*, 1998). The distal end of chromosome 11 contains *R* loci for resistance to potato virus Y (Ry_{adg}, Hämäläinen *et al.*, 1997; Ry_{sto}, Brignetti *et al.*, 1997); potato virus A (Ra_{adg}, Hämäläinen *et al.*, 1998); potato wart disease (*Sen1*, Hehl *et al.*, 1999); and the potato cyst nematode (P. Wolters, personal communication). Although these loci originate from different genetic backgrounds (haplotypes), the existence of these complex loci suggests that genes for resistance to distinct pathogens might be structurally similar. The isolation of the *Gpa2* gene of potato unequivocally demonstrates that within a single haplotype, members of the same *R*-gene cluster can evolve to confer resistance to distinct pathogen species. Molecular analysis of the *Gpa2* locus resulted in the identification of an *R*-gene cluster of four highly homologous genes that span a region of approximately 115 kb. Two of these *R*-gene homologues are active *R* genes; the one most distal to the centromere corresponds to the previously isolated *Rx1* gene that confers resistance to potato virus X (Bendahmane *et al.*, 1999), whereas that most proximal to the centromere corresponds to the *Gpa2* gene conferring resistance to the potato cyst nematode *G. pallida*.

Based on its structural features, the Gpa2 protein belongs to the NBS-LRR class of R proteins (Hammond-Kosack and Jones, 1997). To date, two other nematode *R* genes have been cloned: the *Mi-1* gene from tomato (Milligan *et al.*, 1998), and the $Hs1^{pro1}$ gene from sugar beet (Cai *et al.*, 1997). The Mi-1 protein, like Gpa2, belongs to the LZ-NBS-LRR class of R proteins (Milligan *et al.*, 1998). Alignment of the putative amino-acid sequences encoded by *Gpa2* and *Mi-1* to those encoded by *RPS2* (Mindrinos *et al.*, 1994), *RPM1* (Grant *et al.*, 1995), *I2* (Simons *et al.*, 1998) and *Prf* (Salmeron *et al.*, 1996), proteins which do not confer resistance to nematodes but all belong to the LZ-NBS-LRR class of R proteins, did not reveal any nematode R protein-specific features. Gpa2 and Mi-1 lack a signal sequence, which suggests that recognition of the nematodes by the host plant, if indeed mediated by these R proteins, would occur in the cytoplasm. The other cloned nematode-resistance gene, $Hs1^{pro1}$, appears to represent a different class of resistance gene, as the predicted protein encoded by $Hs1^{pro1}$ deviates strongly from all other known classes of resistance genes.

The Gpa2 protein is also highly homologous (88.7% amino-acid sequence identity) to the Rx1-related Rx2 protein encoded by the *Rx2* locus on potato chromosome 5 (Bendahmane *et al.*, 2000). From the sequence conservation in Gpa2, Rx1 and Rx2, it is clear that there is a direct evolutionary relationship between these proteins. This suggests that, at least in this case, virus- and nematode-resistance pathways may share mutual components. Clearly, different elicitor molecules initiate the resistance cascades. In the case of potato cyst nematodes, infectious juveniles penetrate the root and migrate to a site near the

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vascular cylinder, where they initiate elaborate developmental and morphological changes in host root cells leading to the formation of a feeding cell. Secretory proteins derived from the nematode's esophageal glands are thought to trigger this process (Lambert *et al.*, 1999; Smant *et al.*, 1998). It is likely that injection by the nematodes of secretions intended to initiate feeding-cell development also elicit the resistance response. In the case of *Rx* genes, the resistance response is triggered by the coat protein of PVX (Bendahmane *et al.*, 1995; Querci *et al.*, 1995). It is likely that the sequences involved in direct or indirect interaction with these distinct elicitors are, for the most part, situated in the C-terminal half of the proteins. Out of the 36 amino acids that differ between Gpa2 and Rx1 in the N-terminal half of the protein (including the NBS), only 14 are Gpa2-specific; the other 22 are identical to the corresponding residues in Rx2 (Bendahmane *et al.*, 2000). In contrast, 68 of the 70 amino acids that differ between Gpa2 and Rx1 in the C-terminal half (including the LRR domain) are specific to Gpa2. Another observation that implicates the C-terminal half of the molecules in differential elicitor recognition is the hypervariability of the predicted solvent-exposed residues of the parallel β-sheets within the LRR domains of Gpa2 and Rx1. These are also subject to diversifying selection. Nevertheless, the most striking difference in the C-terminal domains of Gpa2 and the Rx proteins is the absence of the 'acidic tail' in the Gpa2 molecule (Bendahmane *et al.*, 1999; Bendahmane *et al.*, 2000). Experiments involving the exchange of this domain between Gpa2 and Rx1 should provide information on the significance of the acidic tail difference in elicitor recognition or signal transduction.

Although the mechanism of *Gpa2*-mediated resistance has not yet been thoroughly investigated, preliminary results suggest that it is not associated with a localized necrosis response (unpublished data). This is in contrast to the response mediated by the nematode-resistance genes *Mi-1* and *H1* from tomato and potato (Rice *et al.*, 1985). The natural *Rx*-mediated responses are also not associated with localized necrosis at the site of infection (Bendahmane *et al.*, 1999). Interestingly, the degree of amino-acid sequence divergence between the N-terminal halves of Rx1 and Rx2 is similar to that found between the N-terminal halves of Gpa2 and both Rx proteins (Bendahmane *et al.*, 2000). As the sequence diversion between Rx1 and Rx2 in this region does not appear to influence the induced resistance responses, we speculate that binding of the virus- or nematode-specific ligands to the C-terminal halves of the Gpa2 and Rx molecules, respectively, may lead to conformational changes in the proteins which expose the more conserved N-terminal effector regions to trigger similar defense responses. Determination of whether this model is true requires additional experiments. However, the finding that highly homologous genes can confer resistance to such unrelated pathogen species as viruses and nematodes underlines the potential of protein breeding *in vitro* for engineering new resistance specificities against plant pathogens.

Experimental procedures

Plasmid constructs for complementation analysis

Binary plasmids pBIN(RGC1), pBIN(RGC2) and pBIN(Rx1) were made by digestion of bacterial artificial chromosome clones BAC111, BAC221 and SHBAC43, respectively, with *Xba*I and ligation of the 11, 10.3 and 6.5 kb *RGC*-containing fragments, respectively, into the *Xba*I site of the plant binary vector pBINPLUS (van Engelen *et al.*, 1995). The plasmid pBIN(RGC3) was made by digestion of SHBAC43 with *Xba*I and *Sst*I, and ligation of the 5.5 kb *RGC* harboring fragment into a pBINPLUS vector digested with *Xba*I and *Sst*I.

Complementation analysis

The above *RGC*-carrying binary plasmids were introduced in *Agrobacterium tumefaciens* strain AGLO (Lazo *et al.*, 1991) by electroporation. Stem explants of 5-week-old tissue culture plants of the susceptible potato line V were transformed with the various *RGC*-carrying *Agrobacterium* strains (Visser, 1991). Individual transformants of every *RGC* binary plasmid were tested for complementation for resistance to *Globodera pallida* Pa_2-D383 in an *in vitro* and/or greenhouse disease assay. Complementation assays were carried out in triplicate. Plants containing the binary vector pBINPLUS without insert were included as a control.

Disease assays

The *in vitro* resistance assay was carried out on roots of sterile tissue-cultured plants. Three plants from four independent primary transformants accommodating the four different T-DNA constructs were assayed. Two or three nodia from *in vitro*-grown primary transformants were grown on solidified B5 medium with 0.5% Phytagel (Sigma, St Louis, MO, USA) and 2% sucrose for 1 week (25°C, 16 h light regime). Each new root tip (on average two per nodium) was then inoculated with 15 sterilized second-stage juveniles. Preparation of inoculum was essentially as described (Heungens *et al.*, 1996). Petri dishes with the inoculated root tips were incubated in the dark at 20°C. After 5–6 weeks the level of infection was determined by counting the number of female nematodes formed on the roots. The greenhouse resistance assay was carried out as described (Rouppe van der Voort *et al.*, 1997, 1999).

DNA sequencing and computer analysis

The sequence of the BAC clones SHBAC43, BAC45, BAC111 and BAC221 was determined using a shotgun cloning strategy. Sequencing reactions were performed using a dye terminator cycle sequencing reaction kit (Perkin-Elmer, Pt Biosystems, Warrington, UK), M13 universal forward and reverse primers, and an ABI377 automated sequencer (Applied Biosystems, La Jolla, CA, USA). Sequence contigs were assembled using the STADEN sequence and analysis program (Dear and Staden, 1991).

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Homology searches were carried out using the BLAST program (Altschul *et al.*, 1990). Positions of putative genes were predicted using GENSCAN (Burge and Karlin, 1997). DNAstar (Lasergene, Madison, WI, USA) and Genetics Computer Group (Madison, WI, USA) software packages were used for sequence alignments and sequence comparisons. Nucleotide substitution rates were calculated using the DIVERGE program in the Genetics Computer Group software package. A 2×2 contingency table *G*-test was used to test for significance of differences in synonymous and non-synonymous substitution rates (Nei and Gojobori, 1986).

Rapid amplification of cDNA ends

The 5′ and 3′ ends of the *Gpa2* cDNA were determined by rapid amplification of cDNA ends (RACE) using the Marathon cDNA amplification kit (Clontech Laboratories, Palo Alto, CA, USA) essentially as described previously (Bendahmane *et al.*, 1999). 3′ RACE was carried out with the oligonucleotides 1 (5′-CTCGAGGTCCTTCAACTATCACAC-3′) and 2 (5′-CTTGAAGTTCT-TGTTTTTGGATAGCA-3′) in combination with the adapter primers (AP1 and AP2) of the kit (Figure 3a). 5′ RACE was carried out on cDNA synthesized with primer 3 (5′-GCATTCCACATCTCTGT-GTTTCC-3′) using oligonucleotides 4 (5′-CAGGAGTATTCTGCTT-CCATTATCG-3′) and 5 (5′-GCTTCTTTTCCCCACATTCC-3′) in combination with AP1 and AP2, respectively (Figure 3a).

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An ancient *R* gene from the wild potato species *Solanum bulbocastanum* confers broad-spectrum resistance to *Phytophthora infestans* in cultivated potato and tomato

Edwin van der Vossen[1,*], Anne Sikkema[2], Bas te Lintel Hekkert[1], Jack Gros[2], Patricia Stevens[2], Marielle Muskens[2], Doret Wouters[1], Andy Pereira[1], Willem Stiekema[1,†] and Sjefke Allefs[2]

[1]*Plant Research International BV, PO Box 16, 6700 AA Wageningen, the Netherlands, and*

[2]*Agrico Research, PO Box 40, 8300 AA Emmeloord, the Netherlands*

Received 19 June 2003; revised 29 September 2003; accepted 30 September 2003.

*For correspondence (fax +31 317 418094; e-mail edwin.vandervossen@wur.nl).

†Present address: Centre for Biosystems Genomics, PO Box 98, 6700 AA Wageningen, the Netherlands.

Summary

Late blight, caused by the oomycete pathogen *Phytophthora infestans*, is the most devastating disease for potato cultivation. Here, we describe the positional cloning of the *Rpi-blb1* gene from the wild potato species *Solanum bulbocastanum* known for its high levels of resistance to late blight. The *Rpi-blb1* locus, which confers full resistance to complex isolates of *P. infestans* and for which race specificity has not yet been demonstrated, was mapped in an intraspecific *S. bulbocastanum* population on chromosome 8, 0.3 cM from marker CT88. Molecular analysis of a bacterial artificial chromosome (BAC) clone spanning the *Rpi-blb1* locus identified a cluster of four candidate resistance gene analogues of the coiled coil, nucleotide-binding site, leucine-rich repeat (CC-NBS-LRR) class of plant resistance (*R*) genes. One of these candidate genes, designated the *Rpi-blb1* gene, was able to complement the susceptible phenotype in a *S. tuberosum* and tomato background, demonstrating the potential of interspecific transfer of broad-spectrum late blight resistance to cultivated *Solanaceae* from sexually incompatible host species. Paired comparisons of synonymous and non-synonymous nucleotide substitutions between different regions of *Rpi-blb1* paralogues revealed high levels of synonymous divergence, also in the LRR region. Although amino acid diversity between Rpi-blb1 homologues is centred on the putative solvent exposed residues of the LRRs, the majority of nucleotide differences in this region have not resulted in an amino acid change, suggesting conservation of function. These data suggest that *Rpi-blb1* is relatively old and may be subject to balancing selection.

Keywords: *Rpi-blb1*, disease resistance, *Phytophthora infestans*, *Solanum bulbocastanum*, potato, tomato.

Introduction

Despite the notorious Irish potato famine of the mid-19th century, late blight, caused by the oomycete pathogen *Phytophthora infestans*, still continues to be one of the most devastating of all plant diseases (Duncan, 1999). Although chemicals targeted against *P. infestans* provide reasonable levels of disease control, world-wide losses because of late blight and costs of control measures are estimated to still exceed $3 billion annually. *P. infestans* is a specialised pathogen, primarily causing disease on the foliage and fruits of a range of Solanaceae species (Erwin and Ribeiro, 1996), especially potato and tomato (Fry and Goodwin, 1997). The necessity of developing crops that possess durable resistance increases as more virulent, crop-specialised and pesticide-resistant strains of the pathogen are rapidly emerging. The relatively recent introduction of both mating types to major potato-growing areas of the world has enabled the pathogen to reproduce sexually (Goodwin *et al.*, 1995; Smart and Fry, 2001; Spielman *et al.*, 1991). This allows the recombination of traits in the sexual cycle, thereby generating new possibilities for quick adaptation.

To obtain late blight resistance, breeders have in the past focussed on the introgression of dominant resistance (*R*) genes from *Solanum demissum*, a wild potato species indigenous to Mexico (Malcomson and Black, 1966; Wastie, 1991). Eleven such *R* genes have been identified, several of which have been mapped to specific loci on the genetic map of potato (reviewed by Gebhardt and Valkonen, 2001).

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Unfortunately, the resistance caused by these *R* genes, although nearly complete to specific races of the pathogen, appeared not to be durable. Once newly bred potato cultivars are grown on larger scale in commercial fields, new virulences emerge in *P. infestans*, which render the pathogen able to overcome the introgressed resistance (Wastie, 1991). More durable field resistance to late blight, often quantitative in nature and presumed to be race non-specific, can be found in several Mexican and Middle and South American *Solanum* species (Ross, 1986). However, this type of resistance is difficult to transfer into potato cultivars through crossing and phenotypic selection. The ultimate goal of breeders, therefore, is to introduce into potato varieties major *R* genes that confer broad-spectrum resistance to late blight through recognition of pathogen factors that are essential for pathogen fitness.

Diploid *S. bulbocastanum* from Mexico and Guatemala is one of the tuber-bearing species that is known for its high levels of resistance to late blight (Niederhauser and Mills, 1953). Unfortunately, classic transfer of resistance from wild *Solanum* species to cultivated potato is not easy because of differences in ploidy and Endosperm Balance Number (Johnston *et al.*, 1980). Despite these problems, introgression of the *S. bulbocastanum* resistance trait has been successful. Ploidy manipulations and a series of tedious bridge crosses resulted in *S. bulbocastanum*-derived, *P. infestans*-resistant germplasm (Hermsen and De Boer, 1971; Hermsen and Ramanna, 1969, 1973; Ramanna and Hermsen, 1971). However, 40 years of intense and continuous breeding efforts with this germplasm has still not resulted in market introduction of resistant cultivars. More recently, somatic hybrids of *S. bulbocastanum* and *S. tuberosum* and backcrossed germplasm were found to be highly resistant to late blight, even under extreme disease pressure (Helgeson *et al.*, 1998). However, suppression of recombination within this material could form a potential obstacle for reconstitution of the cultivated potato germplasm by recurrent crosses, to a level that could meet the standards for newly bred potato cultivars (Helgeson *et al.*, 1998). Isolation of the genes that code for the resistance traits found in wild sources and subsequent transformation of existing potato cultivars with these genes could be a much quicker means of exploiting potentially durable late blight resistance in wild *Solanum* species.

According to the gene-for-gene hypothesis (Flor, 1971), disease resistance starts with recognition of pathogen avirulence (Avr) factors by plant R proteins, followed by signal transduction leading to a hypersensitive response (HR) and resistance (Dangl *et al.*, 1996). The cloning and molecular characterisation of over 30 plant *R* genes conferring resistance to bacteria, fungi, oomycetes, viruses, nematodes or insects has allowed their classification in structural classes regardless of pathogen specificity (reviewed by Dangl and Jones, 2001; Hulbert *et al.*, 2001). The most abundant class of characterised *R* genes, comprising approximately 0.5% of the genes predicted in the *Arabidopsis* genome (Meyers *et al.*, 2002), is predicted to encode intracellular proteins that carry leucine-rich repeat (LRR) and nucleotide-binding site (NBS) domains, motifs also found in other receptor and signal transduction proteins. In common with other receptors, it is generally considered that NBS-LRR R proteins have a modular structure with separate recognition and signalling domains, whereby the LRR is the candidate recognition domain and the N-terminal region including the NBS, the major signalling domain. Functional analysis of recombinant R proteins indicates that recognition specificity indeed resides in the LRR (Dodds *et al.*, 2001; Ellis *et al.*, 1999). Moreover, the LRR is the most variable region in closely related NBS-LRR proteins and is under selection to diverge (Meyers *et al.*, 1998; Noël *et al.*, 1999; Van der Vossen *et al.*, 2000). However, evidence is accumulating that LRRs also contribute to signalling through negative regulation involving putative intramolecular interactions (Bendahmane *et al.*, 2002; Hwang *et al.*, 2000; Moffet *et al.*, 2002).

Currently, five *R* genes have been cloned from potato, including the *S. demissum*- and *S. bulbocastanum*-derived late blight resistance genes *R1* and *RB*, respectively (Ballvora *et al.*, 2002; Bendahmane *et al.*, 1999, 2000; Song *et al.*, 2003; Van der Vossen *et al.*, 2000). Here, we report the positional cloning of *Rpi-blb1*, a CC-NBS-LRR plant *R* gene derived from *S. bulbocastanum*. *Rpi-blb1*, when expressed in potato or tomato, confers full resistance to a range of *P. infestans* isolates carrying multiple virulence factors. Race specificity has not yet been found. The potential significance of single *R* genes from the wider gene pool for cultivated *Solanaceae* is demonstrated.

Results

Evaluation of late blight resistance in S. bulbocastanum

A first and crucial step in determining the genetic basis of late blight resistance in *S. bulbocastanum* was the identification of susceptible individuals for the generation of intraspecific mapping populations. Five *S. bulbocastanum* accessions, originating from different areas in Mexico, were screened for *P. infestans* susceptibility in detached leaf assays using a complex isolate (IPO655-2A). Although the accessions predominantly produced resistant individuals, fully susceptible individuals were encountered at a low frequency in three of the five accessions tested. A series of intraspecific crosses were subsequently made between resistant individuals from each accession with a susceptible genotype from BGRC accession 8006, and segregation of the resistance was analysed. Resistance in the F_1 population resulting from a cross between a resistant plant from

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Table 1 Characteristics of *P. infestans* isolates for which the Blb8005-8 clone showed a typical HR in detached leaf assays

Isolate ID	Country of origin	Isolation year	Host	Mating type	RACE[a]
IPO98014	the Netherlands	1998	Potato	A1	1.2.3.4.7.11
IPO428-2	the Netherlands	1992	Potato	A2	1.2.3.4.5.6.7.8.9.10.11
IPO82001	Belgium	1982	Potato	A2	1.2.3.4.5.6.7.10.11
IPO655-2A	the Netherlands	1992	Potato	A2	1.2.3.4.5.6.7.8.9.10.11
PIC96001	Mexico	1996	Potato	A1	n.d.
PIC96002	Mexico	1996	Potato	A2	n.d.
IPO01900	the Netherlands	2001	*S. sisymbriifolium*	A1	1.2.3.7.8.11
GNVM48-2	the Netherlands	2000	Potato	A1	n.d.
GNVM49-1	the Netherlands	2000	Potato	A2	n.d.
GNVM62-1	the Netherlands	2000	Potato	A1	n.d.

[a]Numbers indicate *R*-gene differentials for which isolates showed compatible interactions in detached leaf assays.

BGRC accession 8005 (individual 8005-8) and the susceptible plant from BGRC accession 8006 (individual 8006-9) segregated in a ratio not significantly different from 1 : 1. Of the 42 F_1 genotypes screened, 22 were resistant, 16 were susceptible and 4 initially displayed unclear phenotypes. However, these data indicated that the resistance in 8005-8 inherited as a dominant allele at a single locus. In the expectation that additional late blight resistance loci will be found in *S. bulbocastanum*, the locus identified in 8005-8 was named *Rpi-blb1*.

To determine the specificity of the *Rpi-blb1* locus, we screened the resistant genotype 8005-8 with 10 different *P. infestans* field isolates with varying complexity and aggressiveness (Table 1). Included in this panel of isolates were some of the highly aggressive isolates that can nowadays be collected in Dutch potato fields. the Netherlands forms a region with a high level of disease pressure because of the presence of both mating types A1 and A2 (Flier and Turkensteen, 1999; Flier *et al.*, 2003). *Rpi-blb1* conferred full resistance to all the tested isolates thereby inducing a typical HR reaction, suggesting the involvement of an *R* gene that currently has an unmatched specificity.

Genetic and physical mapping of the Rpi-blb1 *locus*

Screening of the original 42 F_1 progeny plants, derived from the intraspecific cross between *S. bulbocastanum* genotypes 8005-8 and 8006-9, with the chromosome 8 marker CT88 (Tanksley *et al.*, 1992) indicated that *Rpi-blb1* mapped to the same genetic region on chromosome 8 as the *S. bulbocastanum*-derived *Phytophthora* resistance locus *RB* (Naess *et al.*, 2000). To investigate this further, restriction fragment length polymorphism (RFLP) markers flanking CT88 (TG513 and CT64, respectively; Tanksley *et al.*, 1992) were developed into cleaved amplified polymorphic sequence (CAPS) markers (Table 2) and tested on 512 F_1

Table 2 Overview of markers and primers used for mapping Rpi-blb1

Marker	F/R[a]	Primer sequence[b]	Annealing temperature (°C)	Enzyme[c]
CT88	F	GGCAGAAGAGCTAGGAAGAG	57	*Mbo*I
	R	ATGGCGTGATACAATCCGAG		
	F	TTCAAGAGCTTGAAGACATAACA	60	a.s.
	R	ATGGCGTGATACAATCCGAG		
TG513	F	CGTAAACGCACCAAAAGCAG	58	a.s.
	R	GATTCAAGCCAGGAACCGAG		
CT64	F	ACTAGAGGATAGATTCTTGG	56	*Cfo*I
	R	CTGGATGCCTTTCTCTATGT		
B139R	F	GATCAGAAGTGCCTTGAACC	56	TaqI
	R	CAAGGAGCTTGGTCAGCAG		
SPB33L	F	ATTGCACAGGAGCAGATCTG	59	*Hin*fI
	R	TGTAAGAGAGCAAGAGGCAC		
SPB42L	F	AGAGCAGTCTTGAAGGTTGG	58	*Cfo*I
	R	GATGGTAACTAAGCCTCAGG		
B149R	F	GACAGATTTCTCATAAACCTGC	58	*Mse*I/*Xba*I
	R	AATCGTGCATCACTAGAGCG		
RGA1–4	F	TGTGGAGTAAGAGAGGAAGG	62	*Ssp*I/*Mse*I
	R	TCAGCTGAGCAGTGTGTGG		

[a]Orientation of the primer: F, forward; R, reverse.
[b]Primer sequences according to IUB codes.
[c]a.s., allele specific.

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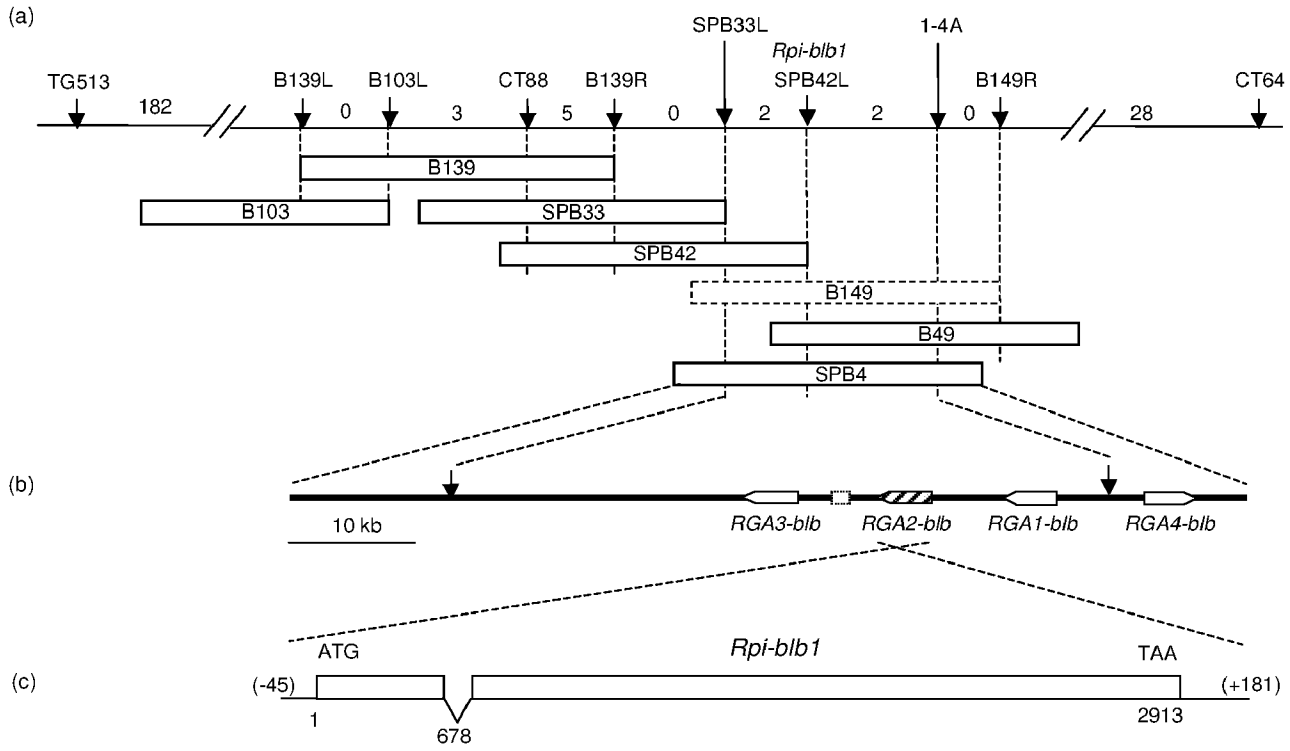


Figure 1. Genetic and physical map of the *Rpi-blb1* locus.
(a) Genetic and physical map of the *S. bulbocastanum* genomic region containing *Rpi-blb1*. Vertical arrows indicate the relative positions of markers linked to resistance. Numbers above the horizontal line indicate the number of recombinants identified between the flanking markers in 2109 progeny plants. Rectangles represent BAC clones. The dotted rectangle represents a BAC clone derived from the recessive *rpi-blb1* haplotype.
(b Relative positions of candidate genes for late blight resistance on BAC SPB4.
(c) Schematic representation of the *Rpi-blb1* gene structure. Horizontal lines indicate exons. Open boxes represent coding sequence. Lines angled downwards indicate the position of a 678-nt long intron sequence. Numbers in-between brackets indicate the length of the 5′- and 3′-UTRs of the putative *Rpi-blb1* transcript.

individuals. In this screen, 5 CT64-CT88 recombinant genotypes and 41 CT88-TG513 recombinant genotypes were identified. A single recombination event was found between the *Rpi-blb1* locus and marker CT88.

For further fine mapping of the *Rpi-blb1* locus, a total of 2109 F_1 individuals were screened for recombination between markers TG513 and CT64. The recombinant genotypes (219/2109) were subsequently screened with CAPS markers derived from left (L) and right (R) border sequences of bacterial artificial chromosome (BAC) clones isolated from a BAC library prepared from the resistant *S. bulbocastanum* parent genotype 8005-8 (Table 2). The BAC library was initially screened with marker CT88. BAC clones identified with this marker were used as seed BACs for a subsequent chromosome walk to the *Rpi-blb1* locus. These data together with the disease resistance data of each recombinant, obtained through detached leaf assays, positioned the *Rpi-blb1* locus within a 0.1-cM genetic interval between markers SPB33L and B149R (4/2109 recombinants), an interval physically spanned by the overlapping BAC clones SPB4 and B49 (Figure 1a). Within this interval, resistance co-segregated with the BAC end marker SPB42L, the sequence of which was highly homologous to partial NBS fragments (Q97, Q137, Q152 and Q153) from tomato (Pan *et al.*, 2000). Southern analyses of BAC clones spanning the SPB33L–B149R interval using a ^{32}P-labelled PCR fragment of marker SPB42L as a probe revealed the presence of at least four copies of this NBS sequence within the *Rpi-blb1* interval. Moreover, all of these copies were present on BAC SPB4. Sequencing and annotation of the complete insert of this BAC clone identified four complete *R* gene analogues (RGA) of the NBS-LRR class of plant *R* genes and a remnant part of such a gene (Figure 1b). Although two recombinant genotypes indicated that *RGA4-blb* was not a candidate for *Rpi-blb1*, all four RGAs were selected for complementation analysis.

Complementation analysis

Genomic fragments of 7–10 kbp harbouring *RGA1-blb, RGA2-blb, RGA3-blb* or *RGA4-blb* were subcloned from BAC SPB4 into the binary plant transformation vector pBINPLUS (Van Engelen *et al.*, 1995) and transferred to a susceptible potato cultivar through *Agrobacterium*-mediated transformation. In view of clone instability and low transformation frequencies encountered with

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Table 3 Complementation of late blight susceptibility in potato and tomato

	AGL0[b]			LBA4404			UIA143		
Genotype[a]	T_0[c]	RGA[d]	R[e]	T_0	RGA	R	T_0	RGA	R
IMP (RGA1-blb)	17	15	0	–	–	–	9	8	0
IMP (RGA2-blb)	–	–	–	31	6	6	14	12	9
IMP (RGA3-blb)	–	–	–	6	0	–	5	5	0
IMP (RGA4-blb)	19	18	0	12	1	0	–	–	–
IMP (vector)	8	8	0	–	–	–	10	9	0
MM (RGA2-blb)	–	–	–	–	–	–	11	9	7

[a]Primary transformants obtained from transformation of the susceptible potato and tomato genotypes Impala (IMP) and Moneymaker (MM), respectively, with T-DNA constructs containing the *Rpi-blb1* gene candidates *RGA1-blb*, *RGA2-blb*, *RGA3-blb* or *RGA4-blb*.
[b]*A. tumefaciens* strains used for transformation.
[c]Number of primary transformants resistant to kanamycin.
[d]Number of kanamycin-resistant primary transformants containing a complete gene construct.
[e]Number of primary transformants resistant to *P. infestans*. Resistance was tested in detached leaf assays using the complex isolates IPO655-2A and IPO428-2.

Agrobacterium tumefaciens strain LBA4404 (Hoekema *et al.*, 1983), second and third transformation experiments were carried out independently using the *A. tumefaciens* strains AGL0 (Lazo *et al.*, 1991) or UIA143 (Farrand *et al.*, 1989; Table 3). Primary transformants harbouring the transgenes of interest were tested for resistance to *P. infestans* in detached leaf assays. Only the genetic construct harbouring *RGA2-blb* was able to complement the susceptible phenotype; 15 out of 18 *RGA2-blb* containing primary transformants were resistant (Table 3) whereas all *RGA1-blb*, *RGA3-blb* and *RGA4-blb* containing primary transformants were susceptible to *P. infestans*. As the *RGA2-blb* transformants showed similar resistance phenotypes as the *S. bulbocastanum*-resistant parent (Figure 2), *RGA2-blb* was designated the *Rpi-blb1* gene.

Rpi-blb1 *is functional in tomato*

To investigate whether *Rpi-blb1* could also complement the susceptible phenotype in tomato, primary transformants of cultivar Moneymaker harbouring the *Rpi-blb1* gene construct were initially tested with the potato-derived *P. infestans* isolates IPO655-2A and IPO428. Seven out of nine primary transformants were resistant (Table 3). As the tested potato *P. infestans* isolates were less virulent on tomato than on potato, the primary transformants were also tested with a *P. infestans* isolate collected from late blight diseased plants of cultivar Moneymaker in a home garden. Even though this isolate was significantly more aggressive on Moneymaker than the isolates IPO655-2A and IPO428 (data not shown), all seven primary transformants remained resistant. These results illustrate the potential effectiveness of the *Rpi-blb1* gene not only against complex isolates derived from potato but also against those specialised on tomato.

Rpi-blb1 *gene structure and putative amino acid sequence*

The size and structure of the *Rpi-blb1* gene were determined by comparing the genomic sequence derived from the insert of pRGA2-blb with cDNA fragments generated by 5′ and 3′ rapid amplification of cDNA ends (RACE). RACE identified 5′ and 3′ *Rpi-blb1*-specific cDNA fragments

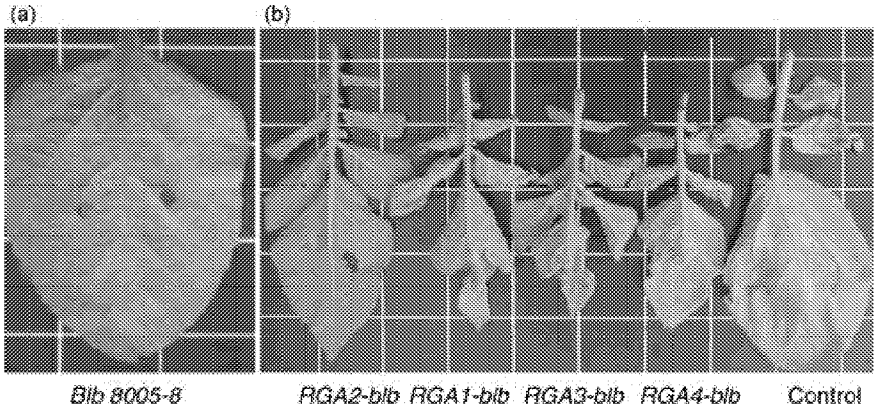


Figure 2. Detached leaf disease assays.
(a) Resistant phenotype of the *S. bulbocastanum* 8005-8 parent genotype 6 days post inoculation (dpi) with *P. infestans* sporangiospore droplets.
(b) Genetic complementation for late blight resistance in potato. Typical disease phenotypes of leaves derived from transgenic potato plants harbouring *RGA1-blb*, *RGA2-blb*, *RGA3-blb* or *RGA4-blb* and control plants 6 dpi with *P. infestans* sporangiospore droplets.

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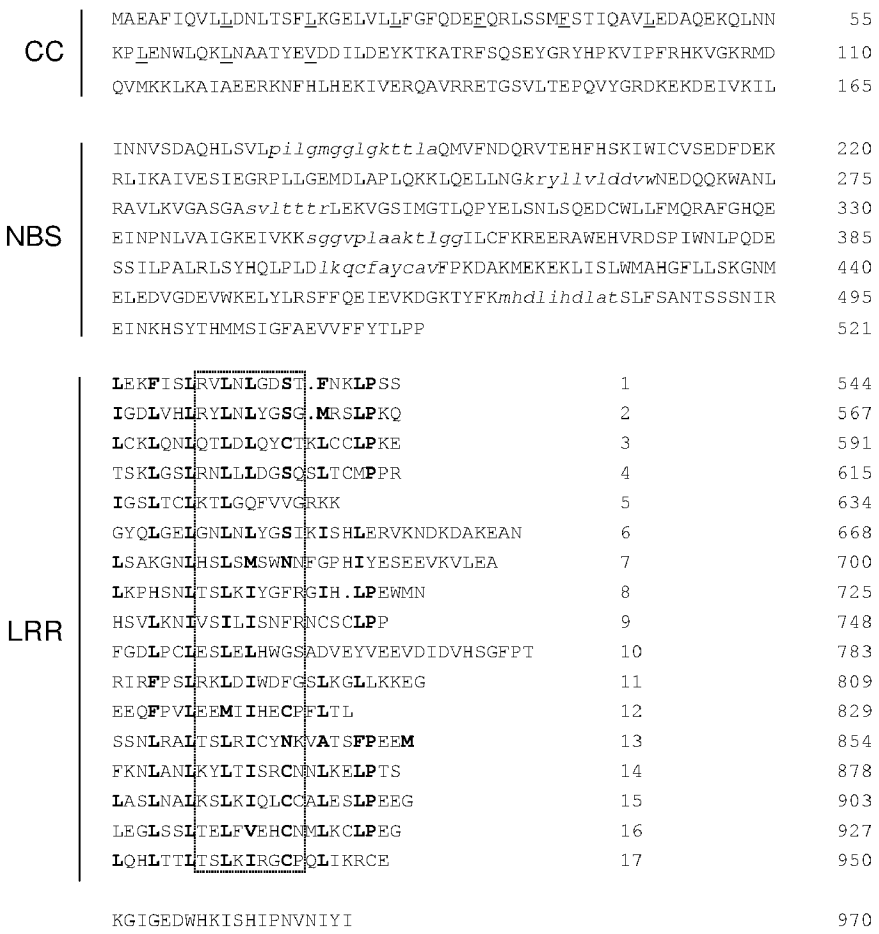


Figure 3. Deduced Rpi-blb1 protein sequence. The amino acid sequence deduced from the DNA sequence of *Rpi-blb1* is divided into three domains (CC, NBS and LRR). Hydrophobic residues in the CC domain that form the first and fourth residues of heptad repeats of potential coiled-coil domains are underlined. Conserved motifs in R proteins are written in lowercase and in italic in the NBS domain. Residues matching the consensus of the cytoplasmic LRR are indicated in bold in the LRR domain. Dots in the sequence have been introduced to align the sequence to the consensus LRR sequence of cytoplasmic LRRs. The LRR subdomain xxLxLxxxx is framed.

comprising 5′ and 3′ untranslated regions (UTRs) of 45 and 181 nucleotides (nt), respectively. The *Rpi-blb1* gene contains a single intron of 678 nt starting 428 nt downstream of the ATG start codon of the gene (Figure 1c). The coding sequence of the *Rpi-blb1* transcript is 2910 nt.

The deduced open-reading frame (ORF) of the *Rpi-blb1* gene encodes a predicted polypeptide of 970 amino acids with an estimated molecular weight of 110.3 kDa (Figure 3). Several functional motifs present in *R* genes of the NBS-LRR class of plant *R* genes are apparent in the encoded protein. As illustrated in Figure 3, the Rpi-blb1 protein belongs to the coiled-coil subset of NBS-LRR resistance proteins (Pan *et al.*, 2000). The N-terminal half of the Rpi-blb1 protein contains a potential coiled-coil region between amino acids 10 and 72 and six conserved motifs indicative of a nucleotide-binding site (Van der Biezen and Jones, 1998). The C-terminal half of Rpi-blb1 comprises a series of 17 LRRs of irregular size that can be aligned according to the consensus sequence LxxLxxLxxLxLxxC/N/Sx(x)LxxLPxx observed in other cytoplasmic R proteins (Jones and Jones, 1997). The third LRR contains the xLDL motif that is conserved in the third LRR of many NBS-LRR proteins (Axtell *et al.*, 2001; Banerjee *et al.*, 2001). PROSITE analysis (Hofmann *et al.*, 1999) identified 8 *N*-glycosylation, 1 cAMP- and cGMP-dependent protein kinase phosphorylation, 12 protein kinase C phosphorylation, 8 casein kinase II phosphorylation, 10 *N*-myristoylation and 3 amidation putative sites in the deduced Rpi-blb1 amino acid sequence.

To identify *in silico* homologues of the *Rpi-blb1* gene, BLAST searches (Altschul *et al.*, 1990) were carried out with the coding sequence of the *Rpi-blb1* gene. BLASTN searches identified a number of sequences with significant homology to short stretches of the *Rpi-blb1* gene. Nucleotides 549–1245 of the coding sequence of the *Rpi-blb1* gene share 81–90% sequence identity to partial NBS fragments from tomato (e.g. Q194, Q137, Q198 and Q199; Pan *et al.*, 2000). These sequences vary in length between 525 and 708 nt and are PCR fragments, which were identified by systematically scanning the tomato genome using (degenerate) primer pairs based on ubiquitous NBS motifs (Leister *et al.*, 1996; Pan *et al.*, 2000). Nucleotides 76–805 of the coding sequence of the *Rpi-blb1* gene share 91% sequence identity with an expressed sequenced tag (EST) from potato (EMBL database Accession no. BG890602). No hits were identified that share homology with *Rpi-blb1* gene sequences downstream of nucleotide 1245, comprising the LRR region. At

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Table 4 Sequence variability and nucleotide substitution rates in different regions of *Solanaceous R* gene paralogues

Comparison	Region[a]	Nucleotide substitutions[c]			
		aa[b]	K_a	K_s	K_a/K_s
Rpi-blb1 versus *RGA3-blb*	Gene	81	0.099	0.163	0.61
	CC-NBS	91	0.043	0.122	0.35
	LRR	71	0.169	0.207	0.81
	xxLxLxxxx	63	0.280	0.335	0.84
Rpi-blb1 versus *RGA1-blb*	Gene	76	0.132	0.270	0.49
	CC-NBS	79	0.112	0.345	0.33
	LRR	72	0.155	0.201	0.77
Rpi-blb1 versus *RGA4-blb*	Gene	70	0.171	0.297	0.58
	CC-NBS	75	0.142	0.359	0.36
	LRR	66	0.207	0.237	0.86
Mi-1.2 versus *Mi-1.1*	Gene	91	0.048	0.037	1.31
	CC-NBS		0.043	0.029	1.48
	LRR		0.064	0.057	1.11
I2 versus *I2C-2*	Gene	93	0.064	0.091	0.70
	CC-NBS		0.038	0.101	0.37
	LRR		0.085	0.083	1.02
Sw5B versus *Sw5A*	Gene	97	0.024	0.018	1.34
	CC-NBS		0.012	0.009	1.30
	LRR		0.056	0.040	1.41
Gpa2 versus *Rx1*	Gene	88	0.060	0.041	1.46
	CC-NBS		0.041	0.050	0.82
	LRR		0.080	0.032	2.50

[a]Regions analysed are defined in Figure 4.
[b]Percentage amino acid (aa) sequence identity.
[c]The rates of non-synonymous (K_a) and synonymous (K_s) nucleotide substitutions per non-synonymous/synonymous site were calculated using the DIVERGE program in the GCG software package.

the amino acid level, similarity with any known sequence did not exceed 36% sequence identity. The highest BLASTX score was obtained with an NBS-LRR gene derived from rice (*Oryza sativa*; 36.5% amino acid sequence identity). The most homologous NBS-LRR gene derived from *Solanaceae* species is a member of the *I2* locus in tomato, which confers resistance to race 2 of the soil-borne fungus *Fusarium oxysporum* f sp. *lycopersici* (*I2C-1*: 27% sequence identity; Ori *et al.*, 1997; Simons *et al.*, 1998).

Comparison of the protein sequences encoded by the four members of the *Rpi-blb1* gene cluster on BAC SPB4, revealed that amino acid sequence identity within the *Rpi-blb1* gene cluster varies between 70 and 81%. Within the cluster, Rpi-blb1 shares the highest overall homology with RGA3-blb (81% identity; Table 4). To further deduce sequence variation within the *Rpi-blb1* gene family, additional (susceptible) alleles of the *Rpi-blb1* gene cluster were isolated. The 8005-8-derived BAC clone B149 and two other existing BAC libraries, derived from *S. tuberosum* and *S. tarijense,* respectively, were screened with primers designed around or in the vicinity of the start and stop codons of the *Rpi-blb1* gene. BAC clones containing putative *Rpi-blb1* homologues were further screened with the *Rpi-blb1* flanking markers SPB33L and B149R (Figure 1) to identify those BAC clones that were derived from the same genetic interval as BAC clone SPB4. Putative *Rpi-blb1* homologous PCR fragments derived from BAC clone B149 or from *S. tuberosum* or *S. tarijense* BAC clones for which it was possible to generate a positive PCR signal of the correct size with the primers of markers SPB33L and/or B149R were subsequently cloned and sequenced. In this way, we were able to compare the deduced protein sequences of the *Rpi-blb1* haplotype with the deduced protein sequences of four additional *Rpi-blb1* homologues (RGA_B149-blb, RGA_SH10-tub, RGA_SH20-tub and RGA_T118-tar; Figure 4). Interestingly, two pairs of protein sequences shared a significantly higher degree of similarity than the paralogues of the *Rpi-blb1* haplotype. RGA1-blb and RGA_B149-blb are nearly identical to each other and RGA_SH20-tub and RGA_T118-tar are 96.5% identical, suggesting that they are orthologues and occupy allelic positions. Comparison of the eight sequences identified a total of 51 Rpi-blb1-specific amino acid residues (51/970; 5.25%; Figure 4). The majority are located in the LRR region (36/51; 70%) and are centred on the β-strand/β-turn motif xxLxLxxxx (framed region; Figure 3), which is predicted to encode the putative solvent exposed residues of the repeats (Figure 4). These data are compatible with the idea that the LRR region of R proteins mainly defines ligand specificity.

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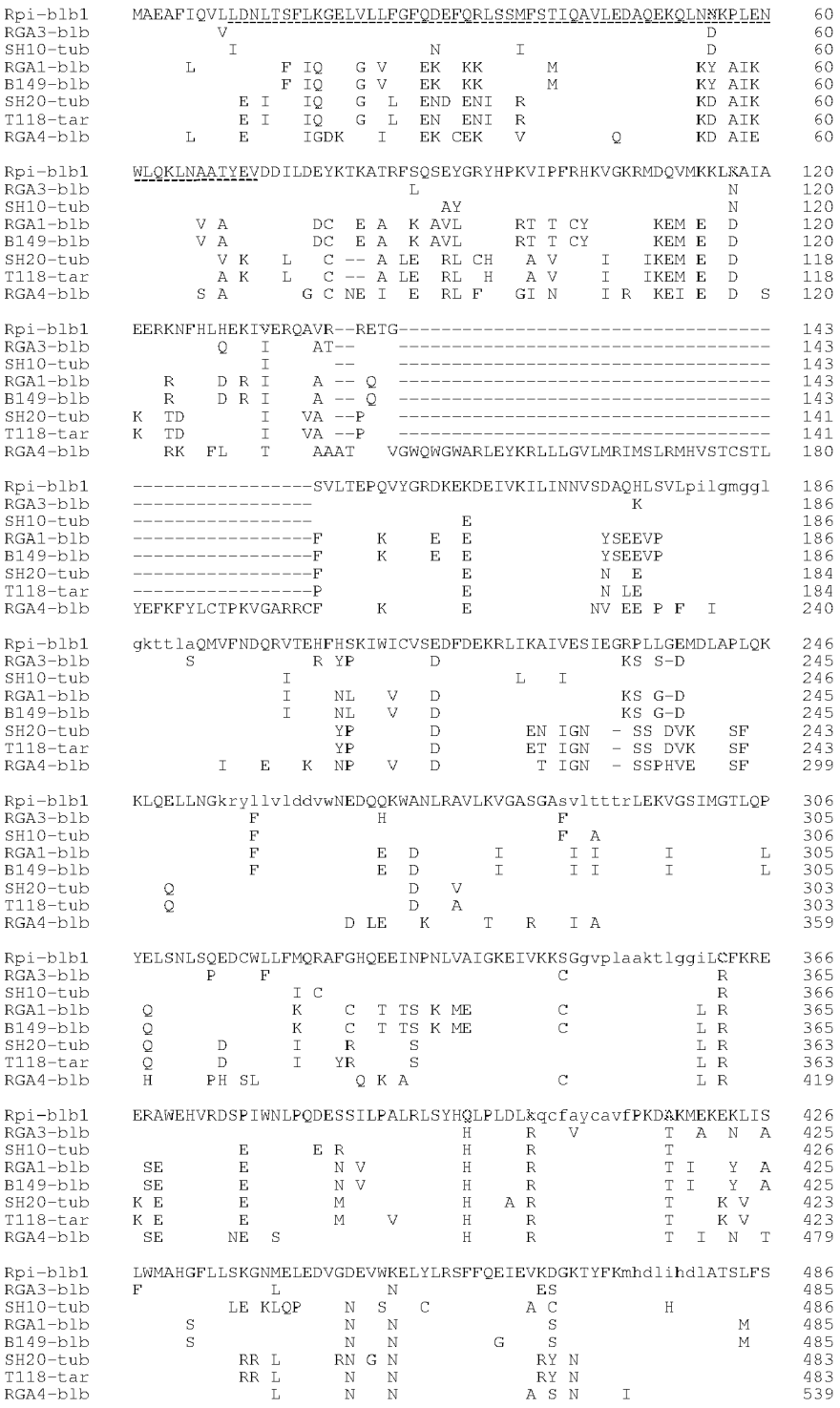


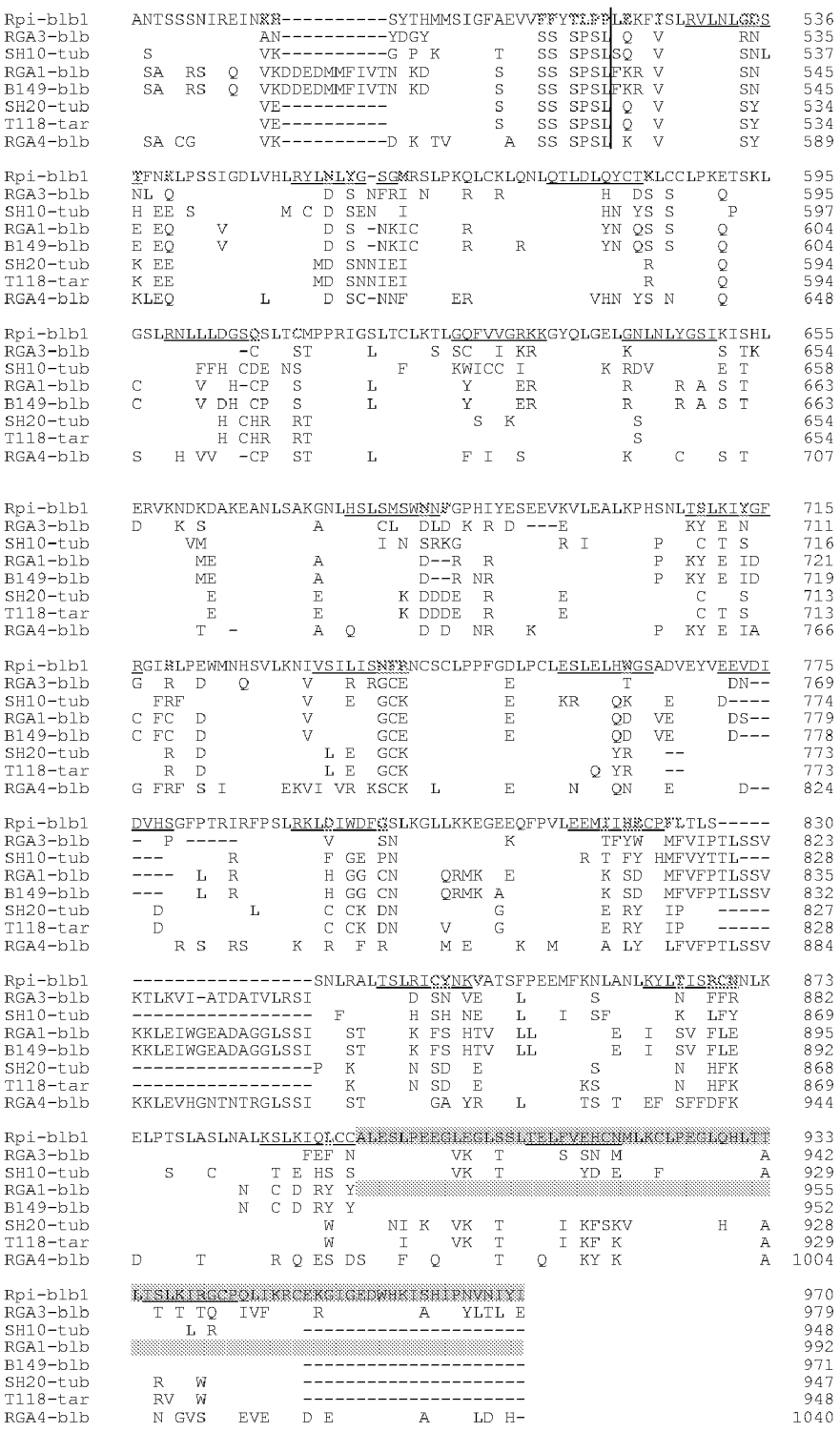
Figure 4. Alignment of the deduced protein products encoded by *Rpi-blb1*, *RGA1-blb*, *RGA3-blb*, *RGA4-blb*, *RGA_B149-blb*, *RGA_SH10-tub*, *RGA_SH20-tub* and RGA_*T118-tar*.
The complete amino acid sequence of Rpi-blb1 is shown and amino acid residues from RGA1-blb, RGA3-blb and RGA4-blb that differ from the corresponding residue in Rpi-blb1. Dashes indicate gaps inserted to maintain optimal alignment. Amino acid residues that are specific for Rpi-blb1, when compared to those at corresponding positions in RGA1-blb, RGA3-blb and RGA4-blb, RGA_B149-blb, RGA_SH10-tub, RGA_SH20-tub and RGA_T118-tar, are highlighted in red. The regions of the LRRs that correspond to the β-strand/β-turn motif xxLxLxxxx are underlined. Conserved motifs in the NBS domain are indicated in lowercase. A vertical line indicates the division between CC-NBS and LRR region. The shaded rectangle indicates the region at the C-termini of Rpi-blb1 and RGA1-blb that are identical.

Evolution of the Rpi-blb1 *locus*

Studies of plant *R* gene sequences involving the comparison of amino acid substitution rates (K_a) to synonymous (K_s) substitution rates has revealed that the LRR region of *R* genes and more specifically the β-strand/β-turn motif is subject to divergent selection (reviewed in Bergelson *et al.*, 2001). Under the assumption that synonymous changes are selectively neutral, values of the ratio K_a/K_s greater than 1 provide strong evidence for positive selection for amino acid substitution. Paired comparisons of synonymous and non-synonymous nucleotide substitutions between

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Figure 4. continued



different regions of Rpi-blb1 with those of RGA1-blb, RGA3-blb and RGA4-blb revealed that although rates of amino acid replacement changes are higher in the LRR region, K_a/K_s values for the LRR domain remain smaller than 1. Higher rates of amino acid replacement changes in the LRR region are masked by striking high levels of synonymous divergence (K_s for the entire LRR) among paralogues of 20.1–23.7% (Table 4). For comparison, synonymous divergence in the LRRs of the most related paralogues of the *Solanaceous R* gene clusters *Mi* (Milligan *et al.*, 1998), *I2* (Ori *et al.*, 1997; Simons *et al.*, 1998), *Sw5* (Spassova *et al.*, 2001) and *Gpa2/Rx* (Bendahmane *et al.*, 2000; Van der Vossen *et al.*,

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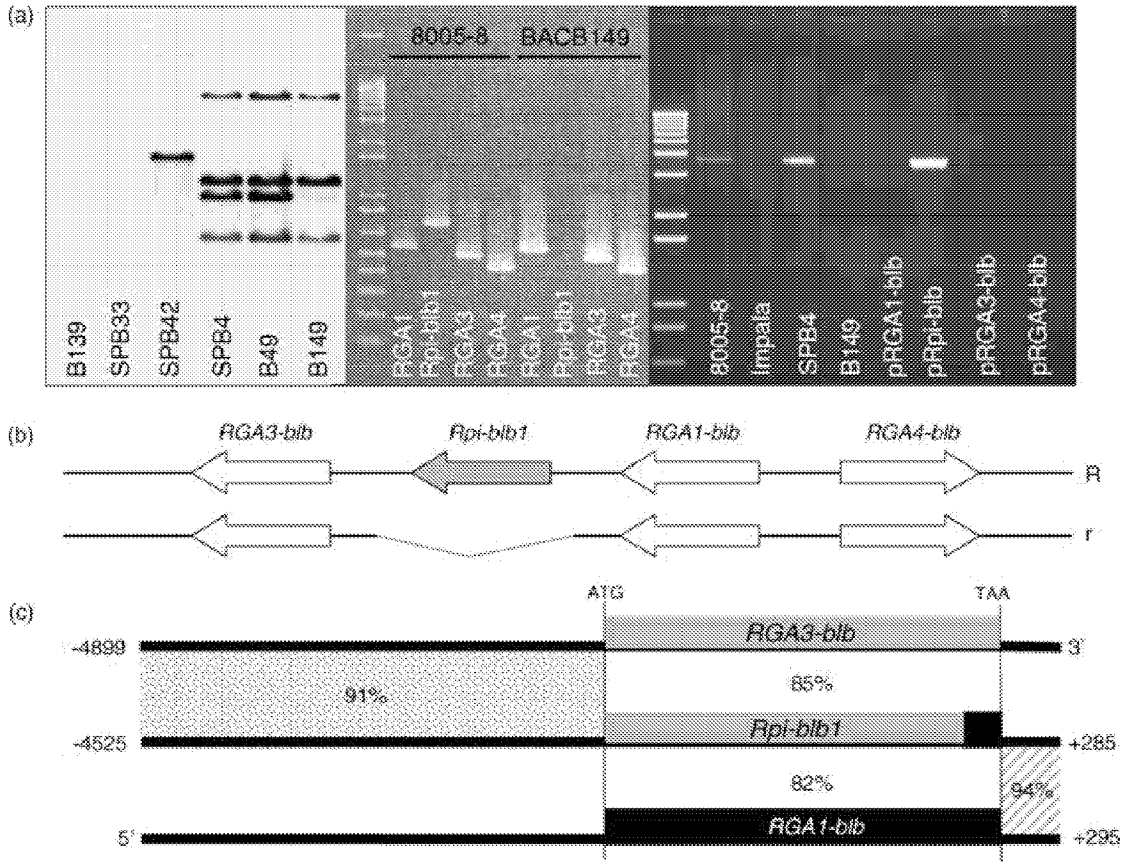


Figure 5. Genomic organisation of *Rpi-blb1* locus.
(a) Southern analysis of BAC DNA (left panel) and PCR analysis (central and right panel) of genomic DNA (8005-8, Impala), BAC DNA (B139, SPB33, SPB42, B49 and B149) or binary plasmid DNA (pRGA1-blb; pRpi-blb1, pRGA3-blb and pRGA4-blb) with gene-specific primer sets (RGA1, Rpi-blb1, RGA3 and RGA4).
(b) Schematic representation of the *Rpi-blb1* (R) and *rpi-blb1* (r) haplotype in the *S. bulbocastanum* genotype 8005-8.
(c) Sequence relationships between the coding and flanking sequences of *Rpi-blb1, RGA1-blb* and *RGA3-blb*. Homologous regions are shown in the same colour. Percentage nucleotide sequence identity is indicated. ATG and TAA indicate the relative positions of the start and stop codons of *Rpi-blb1*, respectively. The length of the homologous 5′ and 3′ flanking sequences are indicated.

2000) ranges between 3.2 and 8.3% (K_s for the LRR region; Table 4). The extraordinary high K_s values calculated for the *Rpi-blb1* gene cluster suggest that these genes are relatively old. Moreover, although amino acid diversity within the LRR region is centred on the xxLxLxxxx motif, the majority of nucleotide differences in this region have apparently not resulted in an amino acid change, leading to K_a/K_s ratio <1 (Table 4). Despite the relatively smaller genealogical antiquity of *Rpi-blb1* and *RGA3-blb*, exemplified by lower synonymous divergence in the CC-NBS region (12.2%) compared to *RGA1-blb* (34.5%) or *RGA4-blb* (35.9%), K_s values calculated for the LRR region are as high as those calculated for the paired comparisons between *Rpi-blb1* and *RGA1-blb* or *RGA4-blb* (Table 4). Altogether, these data suggest that the LRR region of *Rpi-blb1* may well be subject to purifying selection rather than to divergent selection.

Southern analyses of the BAC clones spanning the *Rpi-blb1* interval revealed that BAC SPB4 contained an extra copy of the SPB42L sequence compared to BAC B149 (Figure 5a). The latter BAC clone is derived from the homologous chromosome harbouring the putative susceptible *Rpi-blb1* allele. The finding that the RFLP was specific for BAC clones harbouring the *Rpi-blb1* gene and genetic constructs that complemented the susceptible phenotype in potato and tomato (data not shown), suggested that the *Rpi-blb1* gene was present as an extra copy on the *Rpi-blb1* bearing chromosome. To verify this, we designed several sets of *Rpi-blb1*-specific primers both within and flanking the coding region of *Rpi-blb1* and used these to screen BAC B149. These primer sets were able to amplify fragments of the expected size only from DNA templates containing the *Rpi-blb1* gene and not from B149 (Figure 5a), indicating that *Rpi-blb1* is present as an extra gene in the *Rpi-blb1* haplotype (Figure 5b).

Recombination and sequence exchange between paralogues at complex resistance loci may contribute to the evolution of novel resistance specificities by generating new combinations of polymorphic residues (Parniske *et al.*, 1997). We therefore examined the gene sequences for

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shared polymorphisms that may be indicative of sequence exchange. Although alignment of informative polymorphic sites for paralogues of the *Rpi-blb1* haplotype did not reveal a patchwork arrangement of sequence identities, it did reveal an apparent recombination break point at the C-terminus of *Rpi-blb1*. Consistent sequence affiliation was observed throughout the ORFs of *Rpi-blb1* and *RGA3-blb*, except for the C-terminal region of the gene corresponding to the last 75 amino acids. This region of the *Rpi-blb1* gene is identical to the corresponding region of *RGA1-blb* (Figures 4 and 5). Comparison of the 5′ and 3′ sequences flanking the paralogues of the *Rpi-blb1* haplotype reveals sequence affiliation at the 5′-end between *Rpi-blb1* and *RGA3-blb* and at the 3′-end between *Rpi-blb1* and *RGA1-blb* (Figure 5c). The 4522 bp preceding the translation start codon of *Rpi-blb1* are highly homologous (91%) to the 4899 bp upstream of *RGA3-blb*. Sequence homology at the 3′-end is retained between *Rpi-blb1* and *RGA1-blb* until 285 and 295 bp after the stop codon of *Rpi-blb1* and *RGA1-blb*, respectively. The *Rpi-blb1* gene has apparently evolved through intragenic recombination between the ancestral genes of *RGA3-blb* and *RGA1-blb*.

Discussion

Development of disease-resistant cultivars is an effective way to control diseases if sufficient genetic variation for resistance is available. When sources of resistance are limited, breeders look to the secondary gene pool for species that can serve as such and that can be hybridised with cultivated species. However, because of the heterozygous nature of potato, introgression of resistance from wild species through recurrent backcross programmes is very inefficient. Moreover, several wild *Solanum* species displaying high levels of late blight resistance, e.g. *S. bulbocastanum*, are sexually incompatible with cultivated potato. Isolation of the genes responsible for resistance could be a faster means of exploiting potentially durable late blight resistance in wild *Solanum* species. Several resistance genes from species in the Solanaceae, the *Pto* gene of tomato (Rommens *et al.*, 1995), the *N* gene of tobacco (Whitham *et al.*, 1994), the *Cf-9* gene of tomato (Hammond-Kosack *et al.*, 1998) and the *Bs2* gene of pepper (Tai *et al.*, 1999), have successfully been transferred as transgenes to other *Solanaceous* species. In cloning the *Rpi-blb1* gene, we demonstrate the efficient interspecific transfer of broad-spectrum late blight resistance from *S. bulbocastanum* to cultivated potato and tomato.

The *Rpi-blb1* resistance locus was mapped in an intraspecific *S. bulbocastanum* population to the same genetic location on chromosome 8 as the previously reported late blight resistance locus *RB* (Naess *et al.*, 2000). Physical mapping of the *Rpi-blb1* locus identified a BAC clone comprising a tightly linked cluster of four *R* gene homologous sequences of the CC-NBS-LRR class of plant *R* genes. Complementation analysis showed that one of these candidate genes, *RGA2-blb*, was able to facilitate a typical HR in the otherwise susceptible cultivar Impala in more than 80% of the primary transformants carrying an intact genetic construct. This *RGA* was therefore designated the *Rpi-blb1* gene. Interestingly, the coding sequence of *Rpi-blb1* is identical to that of the *RB* gene, which has also recently been cloned (Song *et al.*, 2003). This suggests that *Rpi-blb1* and *RB* are identical genes. However, when comparing flanking sequences and the intron of these two genes, single nucleotide polymorphisms or single nucleotide insertions can be found at a frequency ranging between 0.4 and 0.8%. We therefore conclude that *Rpi-blb1* and *RB* are allelic, although functionally equivalent.

Significant DNA homology (81–90%) between *Rpi-blb1* and partial NBS sequences from tomato, which have been mapped to loci on chromosomes 2, 8 and 9 of tomato (Pan *et al.*, 2000), indicates that *Rpi-blb1* is probably a member of a Solanaceae NBS-LRR gene family, which apparently is not restricted to the *Rpi-blb1* gene cluster on chromosome 8. Map positions of *Rpi-blb1* homologous sequences on chromosomes 2 and 9 of tomato correspond to the resistance loci *Tm-1* and *Tm-2*, respectively, that encode resistance to tomato mosaic virus (Levesque *et al.*, 1990; Pan *et al.*, 2000; Young *et al.*, 1988). This suggests that *Rpi-blb1* gene family members also function against taxa of plant pathogens other than oomycetes. Interestingly, quantitative trait loci (QTL) associated with resistance to *P. infestans* (Oberhagemann *et al.*, 1999) and *Erwinia carotovora* ssp. *atroseptica* (Zimnoch-Guzwska *et al.*, 2000) also correspond to the *Rpi-blb1* homologous gene cluster on chromosome 2.

As *R* genes of the NBS-LRR class lack predicted transmembrane segments or signal peptides, perception of cognate *Avr* products probably occurs in the cytoplasm. The most straightforward interpretation of *R* gene-mediated elicitor recognition invokes a direct intermolecular interaction. However, experimental evidence for direct binding is limited (Jia *et al.*, 2000). This suggests that either the interaction is transient or the elicitor interacts indirectly with the *R* gene product through a virulence target (Banerjee *et al.*, 2001; Dangl and Jones, 2001; Kooman-Gersmann *et al.*, 1996; Leister and Katagiri, 2000; Mackey *et al.*, 2002; Van der Biezen and Jones, 1998). According to the 'guard model' (Van der Biezen and Jones, 1998), resistance is triggered when the R protein detects modifications to its guardee, which is presumably targeted and modified by the Avr factor to create a favourable environment for the pathogen. Accordingly, R proteins that guard virulence targets are more likely to be maintained in the plant population than R proteins that rely on a direct physical interaction between R protein and Avr factor (Van der Hoorn *et al.*, 2002). In this respect, it is tempting to speculate on the age of the *Rpi-blb1* gene cluster and on the mode of

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action of the Rpi-blb1 protein. The extraordinary high levels of synonymous divergence among paralogues of the *Rpi-blb1* haplotype reflects longevity of the *Rpi-blb1* gene cluster and thereby the possible occurrence of balancing selection at this locus in natural *S. bulbocastanum* populations. This would implicate a high relative contribution of the matching *Avr* gene to the virulence of *P. infestans*. This is in line with the broad spectrum of the resistance conferred by *Rpi-blb1* and the observation that amino acid replacement changes have not accumulated faster than synonymous changes in those regions of the gene that, in well-studied *R* genes, have been pinpointed as the major determinants of recognition specificity for Avr factors (Ellis *et al.*, 2000).

Evolution of new pathogen specificity has been traced to shifts in solvent-exposed LRR residues that are caused by single-base changes, insertion or deletion events, and by unequal-exchange meiotic recombination events within *R* genes or between closely linked homologous *R* genes in a cluster (Ellis *et al.*, 2000). Comparison of the Rpi-blb1 protein sequence with those of seven other members of the Rpi-blb1 protein family identified, in addition to various indels, a total of 51 Rpi-blb1-specific amino acid residues, the majority of which (70%) are located in the LRR region. Targeted or random mutagenesis of *Rpi-blb1* is necessary to determine which of these residues are essential for recognitional and/or signalling capacity of the Rpi-blb1 protein. Recent structure–function analysis of the CC-NBS-LRR protein encoded by *Rx* implies that conserved motifs in the NBS and LRR regions of Rx regulate a signalling domain in the amino-terminal region possibly through intramolecular interactions (Bendahmane *et al.*, 2002). Interestingly, one of the Rpi-blb1-specific amino acid residues is situated at the same position as an Rx gain-of-function mutation close to the RNBS-D (CFLY) motif that is conserved in R proteins (Meyers *et al.*, 1999).

The majority of *R* genes are members of tightly linked multigene families. This is thought to facilitate the generation of novel *R* gene specificities through re-assortment of sequence polymorphism by (un)equal inter- and/or intragenic meiotic recombination between family members resulting in duplication, partial deletion or formation of new chimaeric genes. Comparative sequence analysis of several *R* gene haplotypes supports this idea (reviewed in Hulbert *et al.*, 2001). The two homologous chromosomes of *S. bulbocastanum* 8005-8 contain different numbers of RGAs, the *Rpi-blb1* gene being present as an extra copy in the *Rpi-blb1* haplotype. Shared polymorphisms between *Rpi-blb1* and the two flanking paralogues *RGA3-blb* and *RGA1-blb* indicate that *Rpi-blb1* is likely to have evolved through unequal intragenic meiotic recombination between ancestral genes of *RGA3-blb* and *RGA1-blb*. The presence of a truncated RGA between *RGA3-blb* and *Rpi-blb1* suggests that additional unequal recombinational events have occurred in the evolution of the *Rpi-blb1* gene cluster.

The cloning of *Rpi-blb1* has paved the way to develop late blight-resistant potato and tomato varieties through a transgene approach. A drawback to the use of *R* genes to obtain resistance in crop plants is that their effects often appear not to be durable because of shifts in *Avr* gene frequencies in pathogen populations. Indirect evidence that *Rpi-blb1*-mediated resistance might be stable is the observation that it confers full resistance to a range of *P. infestans* isolates carrying multiple virulence factors. Race specificity has also not been observed with breeding material containing the *RB* locus. Somatic hybrids of *S. bulbocastanum* and *S. tuberosum* and backcrossed germplasm were found to be highly resistant to late blight, even under extreme disease pressure in repeated trials (Helgeson *et al.*, 1998). One reason *Rpi-blb1* may remain effective is if the loss of the corresponding *Avr* gene from the pathogen has a significant cost in terms of fitness or pathogenicity (Leach *et al.*, 2001). An example of this is the isolated *Bs2* gene from pepper (Tai *et al.*, 1999), whose cognate *Avr* gene that codes for a virulence factor is widespread in *Xanthomonas campestris* pathovars (Kearney and Staskawicz, 1990). The possible occurrence of balancing selection at the *Rpi-blb1* locus may however imply the existence of resistance-breaking isolates. Cloning of *Avr-blb1* and subsequent analysis of its frequency and variation within *Phytophthora* species will help in predicting the stability of the *Rpi-blb1*-derived resistance in crop plants. However, to date, no *Avr* gene from *Phytophthora* has been cloned. Another way to increase the stability of late blight resistance is by combining *R* genes that actually require the pathogen to lose or mutate several *Avr* genes simultaneously. This would be especially important when introducing *R* genes that, in natural populations, are under balancing selection. Manipulation of *R* genes as transgenes facilitates the pyramiding of *R* genes at a single linkage block in the genome. This could promote durability of the complex by preventing the single deployment of the component genes in other cultivars by various breeding programmes.

Experimental procedures

Disease assay

The phenotypes of *S. bulbocastanum*, *S. tuberosum* and tomato genotypes for resistance to *P. infestans* were determined by detached leaf assays (El-Kharbotly *et al.*, 1994). Each leaf was inoculated with two droplets (25 μl each) of sporangiospore solution (30 000–50 000 sporangiospores per millilitre) on the abaxial side. After 6 days, the leaves were evaluated for the development of *P. infestans* disease symptoms. Plants with leaves that clearly showed sporulating lesions 6 days after inoculation were considered to have a susceptible phenotype, whereas plants with leaves showing no visible symptoms or necrosis at the site of inoculation

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in the absence of clear sporulation were considered to be resistant. Plants initially displaying unclear phenotypes could, in nearly all cases, be classified as resistant or susceptible after a second test. The assays were performed with *P. infestans* isolates mentioned in Table 1.

Plant DNA marker screening

Genomic DNA was extracted from young leaves according to Bendahmane *et al.* (1997). For PCR analysis, 15-μl reaction mixtures were prepared containing 0.5 μg DNA, 15 ng of each primer, 0.2 mM of each dNTP, 0.6 units Taq polymerase (15 U μl^{-1}, SphaeroQ, Leiden, the Netherlands), 10 mM Tris–HCl (pH 9), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100 and 0.01% (w/v) gelatin. The PCRs were performed using the following cycle profile: 25-sec DNA denaturation at 94°C, 30-sec annealing and 40-sec elongation at 72°C. As a first step in PCR amplification, DNA was denatured for 5 min at 94°C and finalised by an extra 5-min elongation step at 72°C. The amplification reactions were performed in a Biometra® T-Gradient or Biometra® Uno-II thermocycler (Westburg, Leusden, the Netherlands). Depending on the marker, the PCR product was digested with an appropriate restriction enzyme to generate polymorphisms. An overview of the markers including primer sequences, annealing temperature and restriction enzymes is given in Table 2. Subsequently, the (digested) PCR products were analysed by electrophoresis in agarose or acrylamide gels.

BAC library construction and screening

A resistant clone of *S. bulbocastanum* (blb) BGRC accession 8005, heterozygous for the *Rpi-blb1* locus, was used as source DNA for the construction of the 8005-8 BAC library. High-molecular weight DNA preparation and BAC library construction were carried out as described by Rouppe van der Voort *et al.* (1999). Approximately 130 000 clones with an average insert size of 100 kbp, which corresponds to 15 genome equivalents, were obtained. A total of approximately 83 000 clones were individually stored in 216 384-well microtitre plates at −80°C. Another 50 000 clones were stored as bacterial pools containing approximately 1000 white colonies. These were generated by scraping the colonies from the agar plates into LB medium containing 18% glycerol and 12.5 μg ml^{-1} chloramphenicol using a sterile glass spreader. These so-called super pools were also stored at −80°C. Marker screening of the BAC library harbouring the individually stored BAC clones was carried out as described by Rouppe van der Voort *et al.* (1999). For the screening of the BAC library stored as 50 pools of approximately 1000 clones, plasmid DNA was isolated from each pool of clones using the standard alkaline lysis protocol and PCR was carried out to identify positive pools. Bacteria corresponding to positive pools were diluted and plated on LB agar plates containing chloramphenicol (12.5 μg ml^{-1}). Individual white colonies were picked into 384-well microtitre plates and single positive BAC clones subsequently identified as described above. Names of BAC clones isolated from the super pools carry the prefix SP (e.g. SPB33).

DNA sequencing and computer analysis

The DNA sequence of BAC clone SPB4 was determined by shotgun sequence analysis as described by Van der Vossen *et al.* (2000). The automated assembly of the shotgun reads was carried out using the PHRED–PHRAP programs (Ewing and Green, 1998; Ewing *et al.*, 1998). A total of 835 reads provided an overall BAC sequence coverage equal to 5x. Gaps between contigs were closed by primer walking or through a combinatorial PCR approach. The sequence was finally edited at PHRED quality 40 (1 error every 10 000 nt) by manual inspection of the assembly using the Gap4 contig editor and re-sequencing of all low-quality regions. The complete sequence of the insert of BAC SPB4 consisted of 77 283 nt. Positions of putative genes were predicted using GENSCAN (Burge and Karlin, 1997) and GENEMARK (Lukashin and Borodovsky, 1998). Homology searches were carried out using the BLAST program (Altschul *et al.*, 1990). DNAstar (Lasergene, Madison, WI, USA) and Genetics Computer Group (Madison, WI, USA) software packages were used for sequence alignments and sequence comparisons. Nucleotide substitution rates were calculated using the DIVERGE program in the Genetics Computer Group software package.

Subcloning of candidate genes

Candidate RGAs were subcloned from BAC clone SPB4 as follows. Aliquots of approximately 1 μg BAC DNA were digested with 1, 0.1 or 0.01 U of *Sau3*AI restriction enzyme for 30 min. The partially digested BAC DNA was subjected to CHEF electrophoresis at 4°C in 0.5× TBE using a linear increasing pulse time of 1–10 sec and a field strength of 6 V cm^{-1} for 16 h. After electrophoresis, the agarose gel was stained with ethidium bromide to locate the region of the gel containing DNA fragments of approximately 10 kbp in size. This region was excised from the gel and treated with GELASE (Epicentre Technologies, USA) according to the manufacturer. The size-selected DNA was ligated to the *Bam*HI-digested and dephosphorylated binary vector pBINPLUS (Van Engelen *et al.*, 1995) followed by transformation to ElectroMAX *E. coli* DH10B competent cells (Life Technologies, UK). A total of 480 clones were PCR screened for the presence of RGA sequences using primers SPB42LF and SPB42LR or RGA4F and RGA4R (Table 2). Positive clones were selected for further characterisation. Identification of clones harbouring *RGA1-blb, RGA2-blb, RGA3-blb* or *RGA4-blb* was carried out by sequencing the SPB42L PCR fragments derived from positive clones. The relative position of the RGAs within a subclone was determined by sequencing the ends of the clone and subsequent comparison of the sequences to the complete BAC insert sequence.

Agrobacterium tumefaciens-mediated transformation of potato and tomato

Binary plasmids harbouring the candidate genes were transformed to *A. tumefaciens* strains LBA4404 (Hoekema *et al.*, 1983), AGL0 (Lazo *et al.*, 1991) or UIA143 (Farrand *et al.*, 1989), the latter containing the helper plasmid pCH32 (Hamilton *et al.*, 1996). Overnight cultures of the transformed *A. tumefaciens* strains were used to transform potato tuber discs (cv. Impala) or tomato leaf discs (cv. Moneymaker) according to standard protocols (Fillati *et al.*, 1987; Hoekema *et al.*, 1989). Three plants per transformed regenerant were transferred to the greenhouse.

Rapid amplification of amplification of cDNA ends (RACE)

The 5′- and 3′-ends of the *Rpi-blb1* cDNA were determined by RACE using the GeneRacer™ kit (Invitrogen™, the Netherlands). 3′

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RACE was carried out with the nested primers GSP1 (5′-GAGGAATCCATCTCCCAGAG) and GSP2 (5′-GTGCTTGAAGAGATGATAATTCACGAG) in combination with the GeneRacer™ 3′ primer and GeneRacer™ 3′ nested primer, respectively. 5′ RACE was carried out on cDNA synthesised with the primer GSP3 (5′-GTCCATCTCACCAAGTAGTGG) using primers GSP4 (5′-GAAATGCTCAGTAACTCTCTGG) and GSP5 (5′-GGAGGACTGAAAGGTGTTGG) in combination with the GeneRacer™ 5′ primer and GeneRacer™ 5′ nested primer, respectively.

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Understanding and Exploiting Late Blight Resistance in the Age of Effectors

Vivianne G.A.A. Vleeshouwers,[1] Sylvain Raffaele,[2] Jack H. Vossen,[1] Nicolas Champouret,[1] Ricardo Oliva,[2] Maria E. Segretin,[2] Hendrik Rietman,[1] Liliana M. Cano,[2] Anoma Lokossou,[1] Geert Kessel,[3] Mathieu A. Pel,[1] and Sophien Kamoun[2]

[1]Wageningen UR Plant Breeding, 6700 AJ, Wageningen, The Netherlands; email: vivianne.vleeshouwers@wur.nl, jack.vossen@wur.nl, nicolas.champouret@sainsbury-laboratory.ac.uk, hendrik.rietman@wur.nl, Anoma.Lokossou@syngenta.com, m.pel@enzazaden.nl

[2]The Sainsbury Laboratory, Norwich Research Park, Norwich, NR4 7UH, United Kingdom; email: sylvain.raffaele@sainsbury-laboratory.ac.uk, ricardo.oliva@sainsbury-laboratory.ac.uk, maria.segretin@sainsbury-laboratory.ac.uk, liliana.cano@sainsbury-laboratory.ac.uk, sophien.kamoun@sainsbury-laboratory.ac.uk

[3]Plant Research International, 6700 AA, Wageningen, The Netherlands; email: geert.kessel@wur.nl

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Abstract

Potato (*Solanum tuberosum*) is the world's third-largest food crop. It severely suffers from late blight, a devastating disease caused by *Phytophthora infestans*. This oomycete pathogen secretes host-translocated RXLR effectors that include avirulence (AVR) proteins, which are targeted by resistance (R) proteins from wild *Solanum* species. Most *Solanum R* genes appear to have coevolved with *P. infestans* at its center of origin in central Mexico. Various *R* and *Avr* genes were recently cloned, and here we catalog characterized R-AVR pairs. We describe the mechanisms that *P. infestans* employs for evading R protein recognition and discuss partial resistance and partial virulence phenotypes in the context of our knowledge of effector diversity and activity. Genome-wide catalogs of *P. infestans* effectors are available, enabling effectoromics approaches that accelerate *R* gene cloning and specificity profiling. Engineering *R* genes with expanded pathogen recognition has also become possible. Importantly, monitoring effector allelic diversity in pathogen populations can assist in *R* gene deployment in agriculture.

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INTRODUCTION

Effectors: pathogen molecules that alter host cell structure and function, thereby facilitating infection and/or triggering defense responses

The potato (*Solanum tuberosum* L.) is one of the three most consumed crops, along with wheat and rice (44). The regions of the globe with the largest areas of potato cultivation are in Asia and Europe, and production of potatoes approached 330 Megatons in 2009 (27). However, potato plants are susceptible to many pests and pathogens that seriously hamper crop production, and extensive use of chemicals is required to reach sufficient yield. The most devastating disease of potato is late blight, which results in global yield losses of 16% (44). Late blight is caused by the notorious oomycete pathogen *Phytophthora infestans* Mont. de Bary, which can infect the entire plant, including stems, leaves, and tubers (32).

P. infestans, renowned for triggering the Irish potato famine in the 1840s, is a destructive pathogen that when left unchecked can destroy a potato crop within a few days. This pathogen success is not only due to its elevated virulence but also to its remarkable capacity to rapidly adapt to resistant plants (32, 41, 84). Indeed, this feature has led authors to describe *P. infestans* as a pathogen with a "high evolutionary potential" (84) and an "*R* gene destroyer" (41). Remarkably, evolutionary and comparative analyses of the *P. infestans* genome revealed a peculiar architecture that underpins accelerated adaptation to host plants (41, 108). The *P. infestans* genome shows an unusual discontinuous distribution of gene density in which disease effector genes and other virulence factors are localized to repeat-rich and gene-sparse regions of the genome. In contrast, housekeeping genes occupy repeat-poor and gene-dense regions (41, 108). The repeat-rich, gene-sparse regions appear to promote evolutionary plasticity and enhance genetic variation of the subset of genes that determine pathogenicity and host-specificity (108).

P. infestans has the capacity to reproduce sexually, a feature that is associated with increased genetic diversity and survival in many parts of the world (32). However, late blight epidemics are often caused by asexual clones that rapidly spread and amplify through aerial dispersion of sporangia, the asexual spores that are abundantly produced on infected plants (33, 63). In agricultural settings, pathogen migration appears to define population dynamics of *P. infestans*. Population displacement by genotypes with increased fitness is a recurrent event. For instance, in the summer of 2007, the late blight season in Great Britain was the worst in 50 years due mainly to the emergence and rapid spread of an aggressive clone termed genotype_13 (Blue13) (33). In the United States and Canada, another *P. infestans* clone that is particularly aggressive on tomato first appeared in the summer of 2009 and spread further in 2010 (118). The impact of these epidemics is illustrated by the significant media and internet coverage. We used Google Trends to track the usage of the word "blight" as an internet search word. Web searches in the United Kingdom and the United States peaked in 2007 and 2009, respectively, during the late blight epidemics (**Figure 1**).

Resistance to *P. infestans* occurs in many tuber-bearing wild *Solanum* species that belong to the highly diverse section *Petota* Dumort. The center of origin of *Solanum* section *Petota*

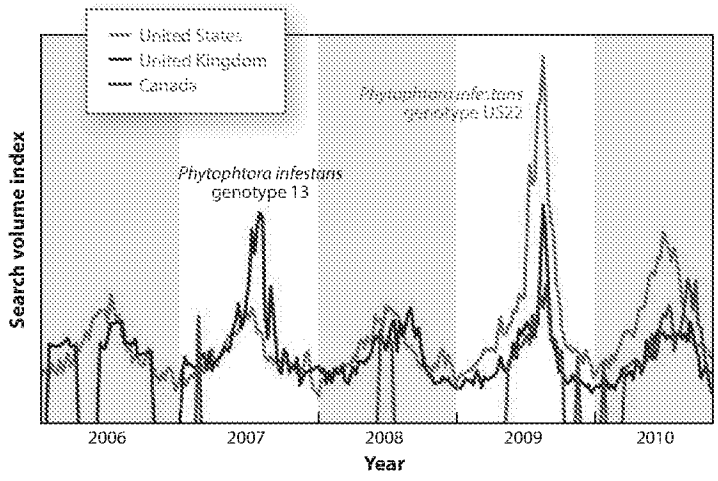


Figure 1

Public concern about late blight reflected in internet searches and news. The graphic displays the search volume index for the term "blight" by region (United Kingdom, United States, and Canada) from January 2006 to the end of 2010. Adapted from Google Trends. **http://www.google.co.uk/trends**.

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is thought to be in central Mexico, from which species migrated southward and evolved in a separate gene pool in South America. A return migration to Mexico was followed by hybridizations and allopolyploidizations within Central American taxa (45). Currently, a large number of diploid and polyploid *Solanum* species occur from the most southern parts of Argentina and Chile to the southern United States (57) (**Figure 2*a***). *Solanum demissum*, a common species in central Mexico, has been used in the earliest potato breeding efforts since the first half of the twentieth century. Eleven *S. demissum* resistance (*R*) genes designated *R1-R11* are distinguished in a potato differential set by Black and Mastenbroek (8, 80). *R1*, *R3*, and *R10*, and to a lesser extent *R2* and *R4*, have widely been used for introgression in European breeding programs (62). The exploitation of new cultivars containing these *R* genes was initially successful, but rapidly changing populations of *P. infestans* overcame them (32, 84, 105, 148). Durability in the field of a particular *R* gene is, however, variable (74), and a wealth of novel *R* genes are being discovered from other wild *Solanum* species (23, 51, 113, 115, 122, 124, 140). To date, 21 *R* genes that confer differential resistance specificities to *P. infestans* isolates have been cloned from various *Solanum* species (**Table 1**, **Figure 2**). In relatively short time frames, it should be possible to deploy these *R* genes using genetic engineering approaches that circumvent the problem of linkage drag, which can be a serious problem during classical introgression breeding (46, 59, 98). Because the cloned *R* genes originate in wild species related

R: resistance

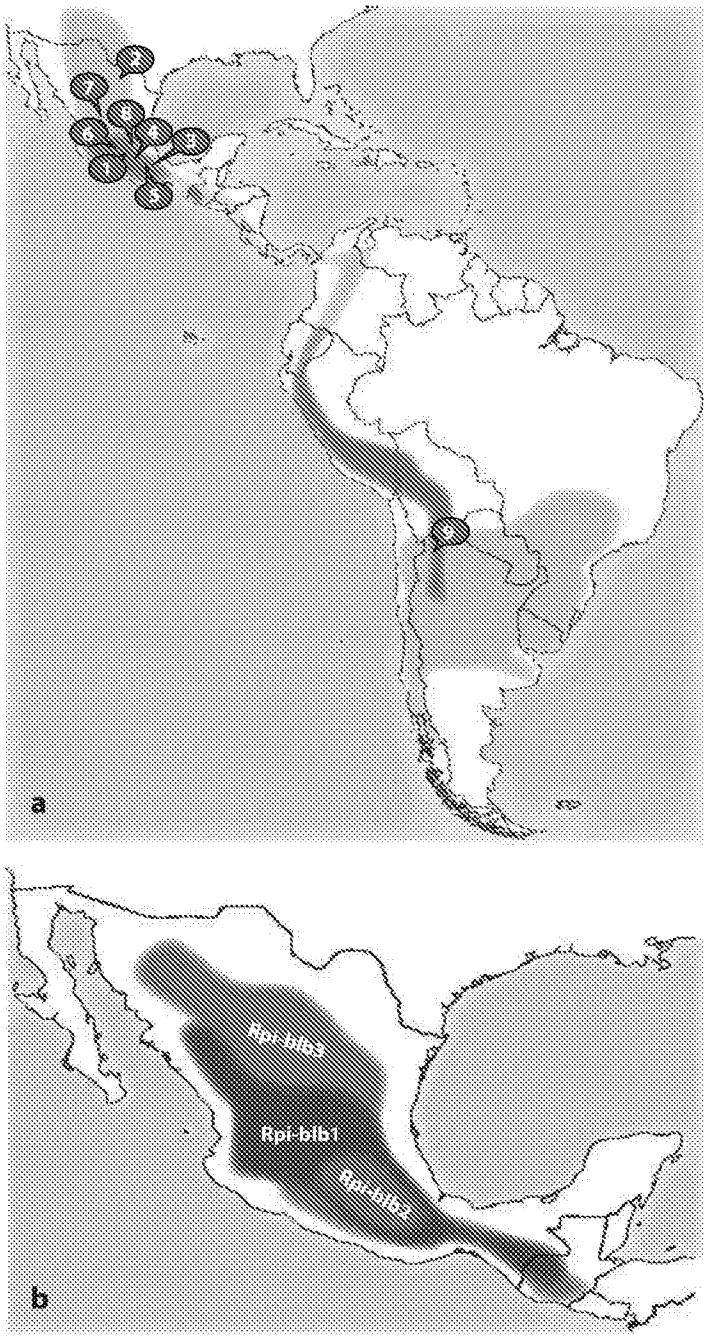


Figure 2

The origin of *R* genes and spread of *Solanum* species. (*a*) *Solanum* section *Petota* species and isolated *R* genes against *Phytophthora infestans* presented on a geographical map, based on 50, 52, 122. Regions that harbor more than five *Solanum* species are yellow, and the two *Solanum* centers of diversity are dark green. Accessions with known *R* genes are depicted on the map: ① *Solanum demissum* (*R1*, *R2*, *R3*, *R4*); ② *Solanum hjertingii* (*Rpi-hjt1*); ③ *Solanum schenckii* (*Rpi-snk1*); ④ *Solanum edinense* (*Rpi-edn1*); ⑤ *Solanum bulbocastanum* (*Rpi-blb1*); ⑥ *Solanum stoloniferum* (*Rpi-sto1*); ⑦ *S. stoloniferum* (*Rpi-pta1*); ⑧ *S. bulbocastanum* (*Rpi-blb2*); ⑨ *Solanum venturii* (*Rpi-vnt1*). (*b*) Geographical spread of *Rpi-blb1*, *Rpi-blb2*, *Rpi-blb3* of *S. bulbocastanum* in central Mexico, adapted from (79).

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Table 1 *Solanum R* genes that confer resistance to *Phytophtora infestans*

R gene	RGH[a]	*Solanum* species[b]	Origin[c]	Chr[d]	Reference
***R1* family**	None				
R1		*demissum*	Mexico	V	(3, 86)
***R2* family**	None				
R2		*demissum*	Mexico	IV	(8, 78, 82)
Rpi-blb3		*bulbocastanum*	Mexico	IV	(78, 95)
Rpi-abpt		Unknown[e]	Mexico	IV	(49, 78, 97)
R2-like		*edinense*	Mexico	IV	(17, 78, 96)
Rpi-edn1.1		*edinense*	Mexico	IV	(17)
Rpi-snk1.1		*schenckii*	Mexico	IV	(17)
Rpi-snk1.2		*schenckii*	Mexico	IV	(17)
Rpi-hjt1.1		*hjertingii*	Mexico	IV	(17)
Rpi-hjt1.2		*hjertingii*	Mexico	IV	(17)
Rpi-hjt1.3		*hjertingii*	Mexico	IV	(17)
Rpi-mcd1		*microdontum*	Argentina	IV	(77)
***R3a* family**	*I2* (94)				
R3a		*demissum*	Mexico	XI	(55, 56)
Rpi-sto2		*stoloniferum*	Mexico	XI	(17)
***R4* family**	Unknown[f]				
R4		*demissum*	Mexico	XI	(136, 142)
***Rpi-blb1* family**	None				
Rpi-blb1, RB		*bulbocastanum*	Mexico	VIII	(123, 134)
Rpi-sto1		*stoloniferum*	Mexico	VIII	(145)
Rpi-pta1		*stoloniferum*[g]	Mexico	VIII	(145)
***Rpi-blb2* family**	*Mi* (114)				
Rpi-blb2		*bulbocastanum*	Mexico	VI	(135)
***Rpi-vnt1* family**	*Tm2*^{2} (72)				
Rpi-vnt1.1		*venturii*	Argentina	IX	(100)
Rpi-vnt1.2		*venturii*	Argentina	IX	(30)
Rpi-vnt1.3		*venturii*	Argentina	IX	(100)

[a]RGH (resistance gene homolog) indicates the closest *R* (resistance) gene homolog from tomato.
[b]Species indicates the donor *Solanum* species from which the *R* gene originated.
[c]Origin indicates the geographic origin of the accession.
[d]Chr indicates the *Solanum* chromosome to which the gene was genetically localized.
[e]*Rpi-abpt* was cloned from breeding material, which was derived from quadruple hybrids ABPT of *Solanum acaule, S. bulbocastanum, Solanum phureja*, and *Solanum tuberosum*.
[f]Not yet cloned but genetically mapped in vicinity of *N*-like and *Rx*-like sequences.
[g]*Rpi-pta* was originally described to be isolated from *Solanum papita*, which was later renamed to *S. stoloniferum*.

to potato, it will be possible to introduce them using cisgenesis, therefore bypassing some of the issues associated with classical transgenic crops.

Durability: the period of time before the emergence of a pathogen strain that overcomes resistance

Resistance to *P. infestans* is characterized by a cell death–associated defense reaction known as the hypersensitive response (HR) (65, 146). At the first stage of infection, *P. infestans* penetrates the plant and translocates effectors inside host cells (6, 149). Specific effectors can act as avirulence (Avr) factors and activate corresponding *R* genes according to the gene-for-gene model (29). Upon recognition of the effector by an R protein, effector-triggered immunity is activated, often resulting in the HR (61). The *R* genes against *P. infestans* (known as *Rpi* genes)

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Table 2 *Phytophthora infestans* effectors with avirulence activity

Effector	Alternative ID	Number of homologs in genome of T30—4 strain	Types of allelic variants in virulent races	Reference
Avr1		1	Unknown	(131; Francine Govers, personal communication)
Avr2	PexRD11	18	Mutation	(17, 78; Paul Birch, personal communication)
Avr3a		3	Mutation	(2)
Avr4		1	Pseudogenization, mutation	(137)
Avrblb1	IpiO	1	Mutation, suppression by another effector	(42, 145)
Avrblb2	PexRD39/40	8	Not known	(90)
Avrvnt1		3	Gene silencing	(99)

typically encode immune receptor proteins of the coiled coil, nucleotide binding, leucine rich repeat (CC-NB-LRR) class of intracellular plant proteins (3, 30, 55, 78, 100, 123, 134, 135) (**Table 1**). These *R* genes have been proposed to follow either of two distinct evolutionary patterns, i.e., fast- or slow-evolving, designated type I or type II *R* genes, respectively.

All known *P. infestans Avr* genes that have been identified thus far belong to the RXLR effector class. These genes encode modular, secreted proteins with a RXLR motif for translocation into the host cell, followed by diverse, rapidly evolving C-terminal effector domains (2, 41, 60, 78, 90, 99, 137, 145, 151) (**Table 2**). Typically, *P. infestans Avr* genes (*a*) function inside the host cell, (*b*) reside in gene-sparse, repeat-rich regions that are thought to contribute to genome plasticity (109), and (*c*) are highly upregulated during the early biotrophic phase of infection in potato (**Figure 3**). These effectors promote pathogen virulence, for example, by suppressing plant immunity (53, 64, 117).

Effectoromics, a high throughput, functional genomics approach that uses effectors to probe plant germplasm for specific recognition by R proteins, has recently emerged as a powerful tool for identification of *Avr* and *R* genes (26, 90, 145). The availability of genome sequence reso urces for *P. infestans* enabled the generation of effector libraries cloned in vectors designed for in planta expression (41, 90). The effectors are transiently expressed in *Solanum* germplasm by agroinfiltration with *Agrobacterium tumefaciens* and/or a virus vector such as *Potato virus X* (PVX) (**Figure 4*a***), and plants are monitored for the occurrence of macroscopic cell death responses to the individual effectors (17, 145) (**Figure 4*b***). Effectors triggering cell death represent candidate *Avr* genes. These are subsequently validated for Avr activity by genetic analyses (**Figure 4*c***). Co-segregation of the cell death response to the effectors correlates with HR-based resistance to *P. infestans* isolates in genetic populations (**Figure 4*c***, *top*). If the matching *R* gene has been cloned, additional verification of R-AVR pairs can be obtained by coexpression of *R*-gene and *Avr*-gene candidates in leaves of tester plants such as *Nicotiana benthamiana* (**Figure 4*c***, *bottom*).

A catalog of *R* and *Avr* gene pairs has recently become available for the potato-*P. infestans* pathosystem (**Table 1, 2**). In this review, we describe these *R-Avr* pairs in more detail. In addition, we provide an update on different aspects of late blight resistance and discuss how we can exploit disease resistance in the age of effectors (92).

R1-AVR1

R1

The first late blight *R* gene to be cloned was *R1*, which originates from *S. demissum* (3)

Cisgenesis: a marker free recombinant DNA-based gene transfer of natural genes from the crop plant itself or from crossable plant species

Avr: avirulence

Rpi: resistance gene against *Phytophthora infestans*

NB-LRR: nucleotide binding site, leucine-rich repeat

Type I *R* genes: rapidly evolving *R* genes that are characterized by frequent sequence exchanges between paralogs, resulting in obscured allelic relationships

Type II *R* genes: slowly evolving *R* genes that are characterized by infrequent sequence exchange between paralogs, resulting in tractable allelic relationships

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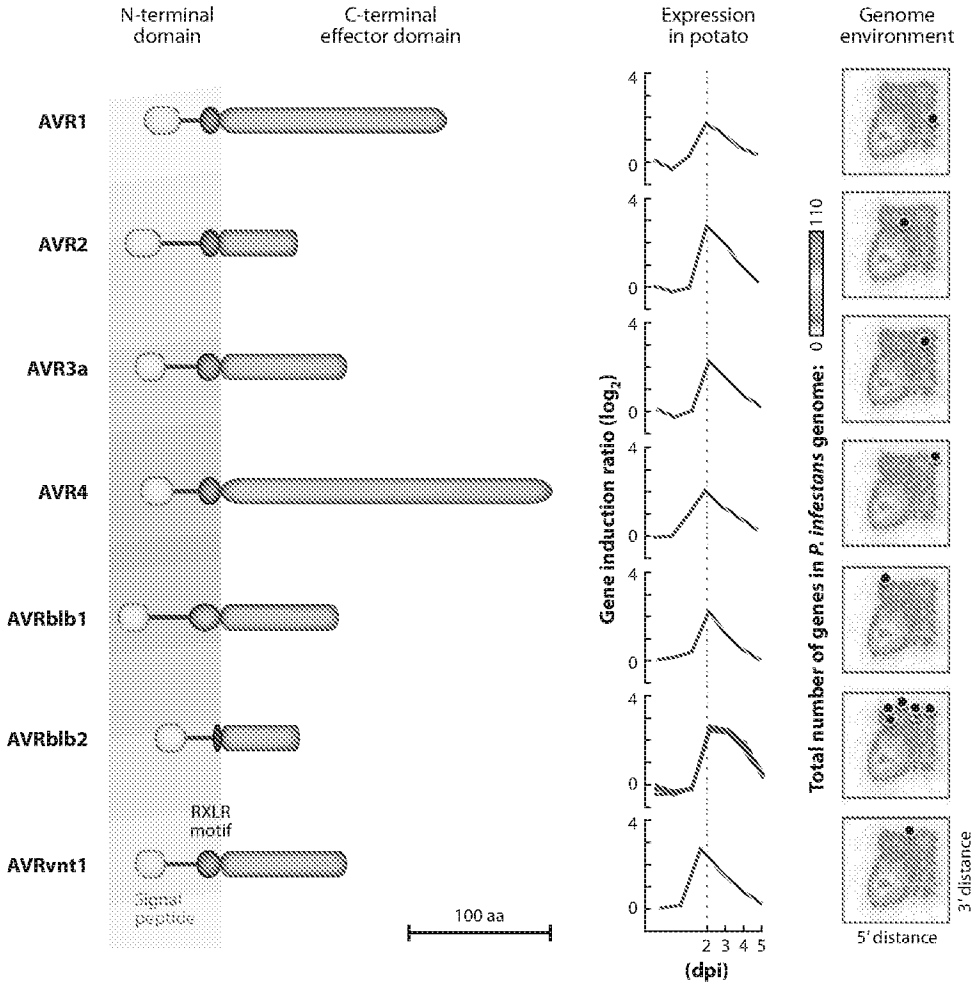


Figure 3

Features of characterized *Phytophthora Avr* gene products. The figure depicts AVR1, AVR2, AVR3a, AVR4, AVRblb1, AVRblb2, and AVRvnt1. The domain structure of *P. infestans* AVR proteins shows a typical RXLR effector modular structure with N-terminal (signal peptide) domain, RXLR motif, and C-terminal effector domain. The N-terminal domain functions in secretion and host translocation whereas the variable C-terminal domain carries the effector biochemical activity. Expression in potato panels illustrate a time course expression pattern of the *Avr* genes during infection of potato [2–5 days post infection (dpi)], with the y-axis showing gene induction (for details see 41). Each of the *Avr* genes is maximally induced at 2 dpi in potato during the early phase of the disease. Genome environment heat maps are two-dimensional plots of 5′ and 3′ intergenic distance for all *P. infestans* genes (for details see 41). The *Avr* genes reside in the gene-sparse regions (upper right corner of the map) with longer distance to their neighboring genes.

RXLR: Single-letter amino acid code for arginine, any amino acid, leucine, and arginine; constitutes a motif found in oomycete effector proteins that are translocated into the host.

(**Table 1**, **Figure 2*a***). The *R1* gene was genetically localized on the short arm of chromosome V of potato and isolated by map-based cloning. The gene contains three introns and encodes a CC-NB-LRR protein of 1,293 amino acids (aa). The C-terminal leucine-rich region is unusually short and comprises only approximately 400 aa. Also, the spacing of the leucine residues does not fit the consensus for leucine rich repeats. To our knowledge, there are no known functional homologs of *R1* identified thus far.

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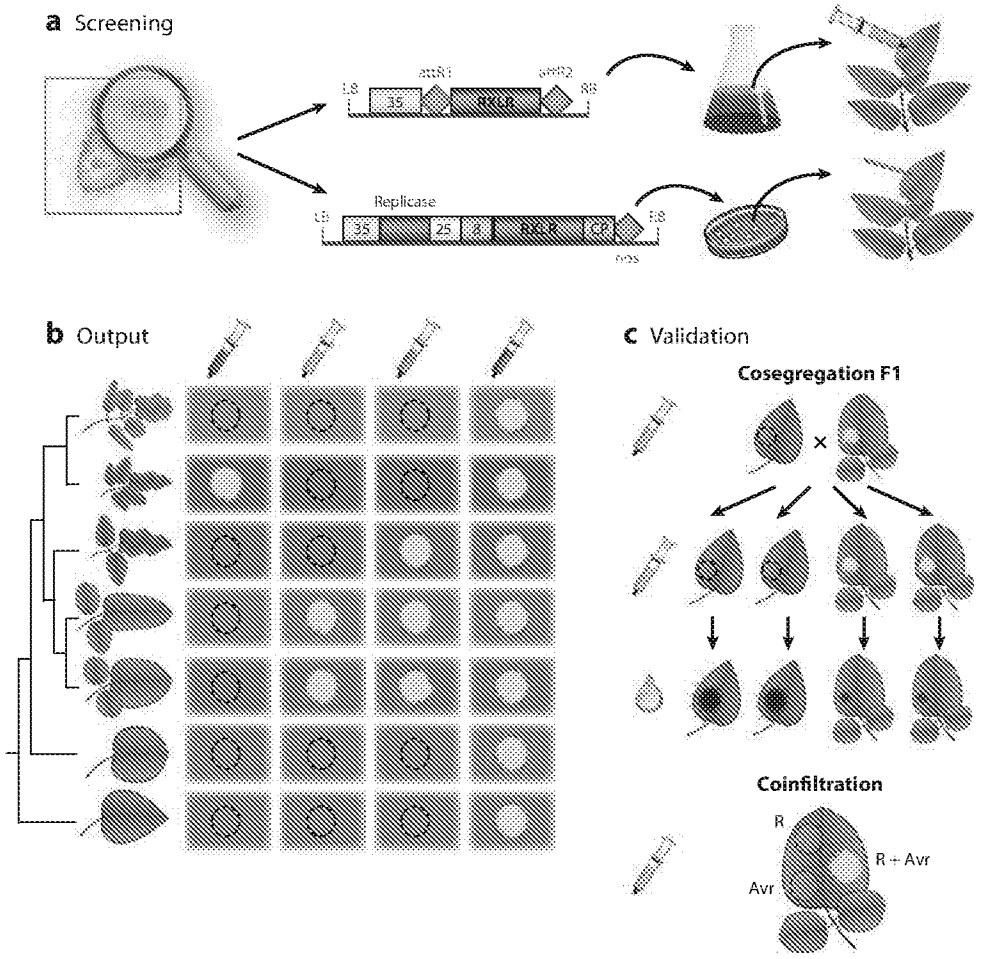


Figure 4

Diagram of effectoromics strategy for identification of corresponding *R* genes and *Avr* genes. (*a*) RXLR effectors are retrieved from *Phytophthora infestans* genome sequence and cloned into expression vectors. Constructs are introduced in *Agrobacterium tumefaciens* for functional screening in plants by agroinfiltration or *Potato virus X* agroinfection (90, 144, 145). (*b*) Output of screening of *Solanum* germplasm with a range of RXLR effectors depicted in syringes containing different *A. tumefaciens* suspensions (different effectors are illustrated by different liquid colors in the syringes). The different *Solanum* species are shown with different leaf shapes and colors. Columns in the matrix represent patterns of cell death responses that are specific to genotypes, species, or groups, up to nonspecific responses for the *Solanum* section *Petota* (57), as depicted from left to right. Specific responses represent candidate *R-Avr* pairs. (*c*) Validation of *R-Avr* interactions by cosegregation of responses to the effector (syringe) with resistance to *P. infestans* isolates (inoculum droplet) in F1 populations (*top*), and coinfiltration of cultures of *A. tumefaciens* that express *R* and *Avr* gene resulting in specific cell death response (*bottom*).

The *R1* gene is located in an area of the genome where multiple other disease resistance traits have been mapped and resides in a large cluster of paralogous sequences. Substantial structural variation exists among the three *R1* haplotypes from the allohexaploid *S. demissum*. Three groups of independently fast-evolving (type I) *R* genes are represented

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Effectoromics: high-throughput approaches to assign activities to computationally predicted effector genes

PVX: *Potato virus X*

aa: amino acids

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and characterized by chimeric structures that result from frequent sequence exchange between the paralogs (68).

R1 has been intogressed into many potato cultivars and has been widely deployed in agriculture (62). However, its agricultural value is limited, and it is currently classified as a narrow spectrum resistance gene because the majority of *P. infestans* isolates are virulent on *R1* plants (130).

Avr1

The *Avr1* gene, which encodes the cognate effector of R1, has been isolated using a positional cloning approach (39, 131, 133) (**Table 2**). However, a full description of AVR1 has not yet been published. AVR1 has a RXLR motif in its N-terminal effector domain (131). Similar to other *P. infestans Avr* genes, *Avr1* resides in a repeat-rich and expanded region of the *P. infestans* genome (**Figure 3**). Also, the expression of *Avr1* is highly induced during the biotrophic phase of potato infection (**Figure 3**).

R2-AVR2

R2

R2 is a representative of a highly diverse gene family located at a major late blight (MLB) resistance locus on chromosome IV of potato (**Table 1**). Eleven *R2* orthologs conferring resistance against *P. infestans* have been identified: *R2*, *R2-like*, *Rpi-blb3*, *Rpi-abpt*, *Rpi-mcd1.1*, *Rpi-snk1.1*, *Rpi-snk1.2*, *Rpi-edn1.1*, *Rpi-hjt1.1*, *Rpi-hjt1.2*, and *Rpi-hjt1.3* (8, 17, 58, 76–78, 82, 95–97, 127). *Rpi-blb3* and *Rpi-abpt* were isolated by map-based cloning, and *R2* and *R2-like* by allele mining (78). The others were identified using effectoromics screens of candidate *R2* gene homologs (R2GH) (17). The R2 family proteins have a leucine-zipper (LZ)-NB-LRR structure and homologs that confer resistance to *P. infestans* range in size from 844 to 847 aa. Outside of *Solanums*, R2 homologs share the highest similarity (35% aa) with RPP13 from *Arabidopsis thaliana* (7), which confers resistance to the *Arabidopsis* downy mildew *Hyaloperonospora arabidopsidis*.

The *R2GH* conferring resistance to *P. infestans* originate from diverse *Solanum* species, including *S. demissum*, *Solanum bulbocastanum*, *Solanum hjertingii*, *Solanum edinense*, *Solanum schenckii*, and *Solanum microdontum* (**Figure 2**). Interestingly, recognition of members of the *Avr2* family is geographically restricted to *Solanum* species originating from Mexico (17). Also, multiple *R2/Rpi-blb3* haplotypes have been detected in Mexican *Solanum* species, suggesting that the gene is polymorphic in these plant populations (79) (**Figure 2*b***). In contrast, the *R2* homolog *Rpi-mcd1*, originating from the Argentinean species *S. microdontum*, does not show HR when coagroinfiltrated with *Avr2* family members, indicating that it may have evolved to recognize a different *P. infestans* effector (79). This difference could be explained by adaptive evolution of *R* loci driven by local *P. infestans* populations in South versus Central America, which may have led to distinct recognition spectra between late blight *R* genes from the two centers of diversity (77, 111).

R2GH can differ in the degree of resistance that they confer. For example, in contrast to the high levels of resistance conferred by the Mexican *R2* homologs, the Argentinean *Rpi-mcd1* exhibits partial resistance to late blight (14, 15, 75, 116). A potential explanation for the observed difference is discussed in a paragraph below.

In addition to *R2GH* with resistance activity against *P. infestans* isolates, up to 27 other *R2GH* were identified in the potato *R2* breeding lines and wild *Solanum* species (8, 17, 78, 82). Structural analyses of these *R2GHs* revealed clear blocks of sequence exchange between paralogs (78), suggesting that *R2* is a class I fast-evolving *R* gene (17, 79). In wild *Solanum* genotypes, up to four active *R2* homologs are conserved (17), which suggests that *R2* is still useful to sustain resistance to late blight in the natural ecosystem of the host, conceivably in combination with other endemic *R* genes.

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R2 has been exploited in agriculture (62) but has been overcome by late blight in the field. However, *R2* still confers resistance to at least part of the local *P. infestans* populations in various geographic regions, including some provinces in China (S. Zhu, personal communication), Russia (43), and in the Netherlands (G. Kessel, unpublished results). Also, *R2* has been shown to delay infection in France (104). It remains possible that a subset of the *R2GH* may confer broader spectrum resistance than the canonical *R2* gene.

Avr2

Avr2 of *P. infestans* (also called *PiAvr2* to distinguish it from homonyms in fungal plant pathogens) is a member of a highly diverse family of 18 RXLR effectors, represented by the *PITG_22870* gene in the genome of *P. infestans* strain T30-4 (17, 78) **(Table 2, Figure 3)**. *PITG_22870* was identified as *Avr2* by map-based cloning (E. Gilroy, P. Birch, personal communication); family member *PexRD11* was detected by effectoromics screens, and the other family members by sequence similarity searches of the *P. infestans* genome (41). *PexRD11* and *PITG_22870* share a low level of aa sequence identity sensu stricto, but 63% similarity based on identical, conserved, and semiconserved aa substitutions. From the *PexRD11/PITG_22870* effector family, 13 candidate effectors are nonredundant, and four of these (including PEXRD11 and *PITG_22870*) induce cell death when coexpressed with *R2*, *R2-like*, *Rpi-abpt*, or *Rpi-blb3*. A fifth member of the family is recognized by 6 additional *R2* homologs. The other *PexRD11/PITG_22870* family members are not recognized and correspond to truncated or mutated proteins, or are not expressed during infection. Detailed population and functional studies will reveal whether diversity seen in *R2* family members in central Mexico reflects a coevolutionary arms race between this family and the diverse family of related RXLR effector genes in *P. infestans*.

R3A-AVR3A

R3a

R3a is a well-characterized *R* gene originating from Mexico that occurs at an MLB resistance locus on the short arm of chromosome XI from *S. demissum* (55) **(Table 1, Figure 2*a*)**. Originally, *R3*-specific resistance derived from the potato R3 differential (83) was mapped in this locus (24). Fine-mapping studies revealed that the phenotype was conferred by two tightly linked *R* genes, *R3a* and *R3b*, a gene that has a distinct recognition specificity (56). The *R3a* locus is highly expanded in *S. demissum* and harbors 30 to 45 *R3a* homologs per haplotype (31). This MLB resistance locus also contains *R5* through *R11*, making it a hot spot of *Rpi* genes of *S. demissum* origin (13, 25, 54)

Comparative genomic studies with the tomato *I2* locus, which harbors major genes encoding resistance to the fungus *Fusarium oxysporum* (94, 120), enabled the isolation of *R3a*. The *R3a* gene contains a single exon, which belongs to the CC-NB-LRR class, encoding a protein of 1,283 aa with nine imperfect LRR. *R3a* is characterized as a typical type I *R* gene (69) and seems to represent an ancient *R* gene of Mexican origin (17, 54). *R3a* gene homologs (R3aGH) with high sequence conservation were also detected in *Solanum stoloniferum* using functional allele mining with Avr3a. The R3aGH *Rpi-sto2* shares 99.9% aa identity with *R3a* and confers resistance to *P. infestans* with the same specificity as *R3a* (17).

R3a has been widely used since early potato breeding (62), but many *P. infestans* isolates overcome *R3a* in potato growing areas as well as central Mexico (43, 112, 139).

Avr3a

Avr3a encodes an RXLR cytoplasmic effector (5, 88) that was cloned based on the interaction between candidate *P. infestans* extracellular proteins (*Pex*) (128) and potato genotypes carrying *S. demissum R* genes (2) **(Table 2, Figure 3)**. Homologs of *Avr3a* have

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been found in *Phytophthora sojae* and *Phytophthora capsici* (10), and the genomic region containing *Avr3a* shows collinearity with the *ATR1* locus in *Hyaloperonospora parasitica* (2, 110). In *P. infestans* populations, two alleles of *Avr3a* have been identified that encode secreted proteins $AVR3a^{K80/I103}$ ($AVR3a^{KI}$) and $AVR3a^{E80/M103}$ ($AVR3a^{EM}$), which differ in two aa in their effector domains (2) **(Table 2)**. This difference profoundly affects host response: $AVR3a^{KI}$ but not $AVR3a^{EM}$ triggers effector-triggered immunity by activating the potato resistance protein R3a (2, 47, 55). Both forms of AVR3a can suppress the cell death response induced in potato by the *P. infestans*-secreted protein INF1, although $AVR3a^{KI}$ is stronger (10, 11). Recently, AVR3a was found to suppress immunity by binding and stabilizing the host E3 ubiquitin ligase CMPG1 (9). Interestingly, these different activities of AVR3a can be uncoupled as mutants that are deficient in either R3a activation or suppression of cell death have been recovered (reviewed in 91). Armstrong et al. (2) proposed that $Avr3a^{EM}$ arose from an *Avr* allele after gene duplication and positive selection. It was recently shown that *Avr3a* is essential for full virulence of *P. infestans* (9), which suggests that this effector can be an important target for durable resistance breeding if *R* genes that target all the allelic forms can be identified (see below).

When *R3a* was bred into potato cultivars in the 1950s, virulent races of *P. infestans* rapidly emerged to overcome the resistance in multiple regions of the world (32). It was assumed that the *Avr3a* gene must be highly mutable and that mutated *Avr3a* must arise very rapidly in *P. infestans* populations. The cloning of *Avr3a* revealed that, surprisingly, this gene has little allelic diversity and that only two major haplotypes occurr ($Avr3a^{KI}$ and $Avr3a^{EM}$) (2). It appears that the great majority of avirulent races of *P. infestans*, including the US1 clonal lineage that dominated pathogen populations in the 1950s, are heterozygous for the *Avr3a* gene (2). Therefore, virulence on *R3a* potatoes did not evolve by repeated independent mutation of *Avr3a* but by allele reassortment in sexual populations. In asexual populations, strains homozygous for $Avr3a^{EM}$ probably evolved from heterozygous strains through mitotic gene conversion, which is known to occur at relatively high frequency in *Phytophthora* (16).

R4-AVR4

R4

The fourth resistance gene from *S. demissum*, *R4* **(Figure 2*a*)**, has not yet been cloned; however, it has been placed on the genetic map. The *R4* gene is linked to a molecular genetic marker with sequence homology to the *Rx* gene, which confers resistance to PVX (4, 136). In another study, response to AVR4 was localized on the long arm of chromosome XI (142). Indeed, this chromosomal region contains *Rx*-like sequences in the *Solanum phureja* genome sequence (101, 143). This suggests that *R4* resides in an *R* gene cluster containing *Rx*-like sequences on chromosome XI.

Similar to *R1*, *R2*, and *R3*, *R4* is classified as a narrow spectrum *R* gene, which was introgressed into potato cultivars in the mid-twentieth century (62). It remains of limited agricultural value because of the high frequency of virulent pathogen races (43, 139).

Avr4

Avr4 of *P. infestans* (also called *PiAvr4*) was cloned using a combination of map-based cloning (133, 150) and cDNA-AFLP (amplified fragment length polymorphism)-based transcriptional profiling (40, 137). *Avr4* encodes a typical RXLR effector of 287 aa. It is a single-copy gene residing in an approximately 100-kb expanded repeat-rich region of the *P. infestans* genome **(Table 2, Figure 3)** (41, 138). Homologs that are related to *Avr4* exist in *P. sojae* and *Phytophthora ramorum* (137, 138). Unlike *Avr3a*, *Avr4* is highly polymorphic in *P. infestans*. Allelic variants in virulent races show deletions causing a premature stop codon, resulting in truncated, probably nonfunctional, proteins. Strains that carry a full-length copy of

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Avr4 are always avirulent on plants carrying *R4*. Interestingly, nonsense or premature termination mutations of *Avr4* do not seem to penalize fitness of *P. infestans*, which explains why virulent races evolved repeatedly and at a somewhat high frequency (137). Therefore, *Avr4* is an example of a dispensable effector that evades resistance through pseudogenization of the full-length gene. R proteins recognizing such dispensable effectors cannot provide durable resistance and are not considered as useful targets in resistance breeding programs.

RPI-BLB1-AVRBLB1

Rpi-blb1

Rpi-blb1, also known as *RB*, was isolated from the Mexican wild potato species *S. bulbocastanum* using a map-based cloning approach in combination with a long-range polymerase chain reaction strategy (**Table 1**, **Figure 2*a***). *Rpi-blb1* encodes a CC-NB-LRR protein of 970 aa. Functional homologs of *Rpi-blb1* were also detected in *S. stoloniferum* following effectoromics screens and resulted in the rapid isolation of *Rpi-sto1* and *Rpi-pta1* (145). *Rpi-blb1* was originally described as a broad spectrum *R* gene conferring race-nonspecific resistance to all tested *P. infestans* isolates. However, virulent races on *Rpi-blb1* potatoes have recently been identified in Mexico (18). Nonetheless, virulent races appear to be rare, particularly in Europe and North America, and thus *Rpi-blb1* appears to resist a broader spectrum of *P. infestans* isolates than most of the other exploited *R* genes.

Rpi-blb1 locates to a cluster of four CC-NB-LRR paralogs on chromosome VIII. *Rpi-bt1*, another *S. bulbocastanum R* gene that confers resistance to late blight, also localizes on chromosome VIII and is 78% similar at the aa level to *Rpi-blb1* (93). *Rpi-blb1* is described as an ancient *R* gene and has an evolutionary pattern similar to Type II *R* genes, which are predicted to evolve slowly and show significant orthologous relationships in diverse species (69, 123, 134). Allele mining and gene-specific marker studies in diverse *Solanum* germplasm demonstrated that *Rpi-blb1* is geographically restricted to Mexico (**Figure 2*b***) and is limited to only a few *Solanum* species (79, 125, 147).

S. bulbocastanum cannot directly be crossed with cultivated potato *S. tuberosum*, and complicated techniques that require several years to complete, such as bridge crosses or somatic hybridizations, are required to introgress *Rpi-blb1* into cultivated potato (48, 49). For these reasons, *Rpi-blb1* is not yet prevalent in potato cultivars. A more rapid introgression became recently feasible by the identification of the functional homolog *Rpi-sto1* from *S. stoloniferum*, which can be directly crossed with *S. tuberosum* cultivars (145, 147).

Besides classical breeding, the cloning of *Rpi-blb1/RB* has opened the possibility of producing resistant potatoes with increased resistance to late blight by direct transfer of the gene using recombinant DNA techniques (70). However, the exploitation of *Rpi-blb1* may be complicated by the observation that when introduced into cultivated potato, this gene confers only partial resistance to aggressive isolates. In a collection of independent *Rpi-blb1* transgenic lines, the level of resistance was positively correlated to the amount of *Rpi-blb1* transcript and negatively correlated with the aggressiveness of the applied *P. infestans* isolate (12, 67). In detached leaf assays, highly aggressive isolates can infect most transgenic potatoes, but not the wild *S. bulbocastanum*, which showed the highest endogenous *Rpi-blb1* transcript levels as well as a dramatic transcriptional increase after inoculation (12, 18, 67). These studies suggest that expression of *Rpi-blb1* alone may not be sufficient to provide satisfactory resistance for potatoes in the field and that stacking with other *R* genes may be necessary (see below).

Avrblb1

Avrblb1 was identified following effectoromics screens of a collection of late blight resistant *Solanum* with candidate RXLR effectors (145) (**Figure 4**). *Avr-blb1* turned out to be identical to the in planta induced gene *ipiO*, which was initially postulated to be involved

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in pathogenicity based on its expression profile (102, 103, 141) (**Table 2**, **Figure 3**). In the genome of particular *P. infestans* strains, *ipiO* is present in two copies in inverted orientation (41, 103). The *ipiO* gene family is highly diverse among a series of *P. infestans* isolates that were collected worldwide (18, 42). The *ipiO* variants are grouped into three classes, I, II, and III, from which class I contains most variants, including the well-characterized *ipiO1* and *ipiO2* (18). Absence of class I *ipiO* was correlated with virulence on *Rpi-blb1* plants. In line with this, class I and II, but not class III *ipiO*, induce cell death when coexpressed with *Rpi-blb1*. Interestingly, class III *ipiO* can suppress *Rpi-blb1*-mediated cell death, which could explain virulence phenotypes on *Rpi-blb1* plants (42). The observation that class I *ipiO* occurs in most *P. infestans* isolates collected worldwide explains why *Rpi-blb1* is functional against the majority of *P. infestans* isolates (18). Therefore, *Rpi-blb1* is a useful *R* gene to deploy in agriculture.

RPI-BLB2-AVRBLB2

Rpi-blb2

Rpi-blb2 was isolated by positional cloning and originates from *S. bulbocastanum* (135) (**Table 1**). The deduced open reading frame encodes a predicted polypeptide of 1,267 aa and belongs to the CC-NB-LRR proteins. *Rpi-blb2* is located on the short arm of chromosome VI, in a hot spot of NB-LRR genes. In tomato, this same region harbors the *Mi-1* gene, which confers resistance to nematodes, aphids, and whiteflies (87, 89, 114), and Mi-1 shares 82% of the aa sequence identity with Rpi-blb2.

The origin of *Rpi-blb2* lies in central Mexico, and its occurrence is confined to accessions from that region (**Figure 2**). Sequences similar to *Rpi-blb2* occur at low frequency in *Solanum*, and the lack of allelic diversity among *S. bulbocastanum* accessions suggests that this *R* gene evolved recently (79).

Rpi-blb2 confers effective resistance against the *P. infestans* strains tested so far, making it a desired gene for breeding (135). Only on rare occasions has infection been found on *Rpi-blb2*-containing *Solanum* species in the Netherlands (G. Kessel, unpublished results). Introgression of *Rpi-blb2* has resulted in the resistant potato varieties Bionica and Toluca after 46 years of strenuous breeding efforts that utilized bridge-crosses and successive backcrosses to diminish linkage drag (44, 132). The broad spectrum activity associated with *Rpi-blb2* has also motivated the use of genetic engineering to produce plants carrying a combination of *Rpi-blb1* and *Rpi-blb2* by companies and governmental programs (44). A genetically engineered potato variety with the trade name Fortuna that carries both *Rpi-blb1* and *Rpi-blb2* genes has entered the commercialization pipeline in Europe but will require a few more years before final approval for cultivation (145).

Avrblb2

Avrblb2 was identified after allele mining and functional screening of candidate RXLR effector genes (90). *Avrblb2* belongs to a multi-gene family with at least seven duplicated copies in the genome of *P. infestans* strain T30–4 (41) (**Table 2**, **Figure 3**). The gene encodes a secreted protein of 100 aa that is highly polymorphic and exhibits high rates of nonsynonymous substitutions. Among 13 variable residues in the mature protein, Oh and colleagues (90) found a key residue at position 69 that compromises activation of Rpi-blb2. When this residue was mutated from Ala, Ile, or Val to Phe_{69} in the *Avrblb2* background, activation was lost. Interestingly, natural $Avrblb2^{Phe69}$ variants coexist with avirulence copies of the gene in the genome of several isolates of *P. infestans* (90; R. Oliva & S. Kamoun, unpublished results). This suggests a potential benefit for *P. infestans* in maintaining duplicated copies of *Avrblb2*. Under these conditions, it is less likely that consecutive point mutations or deletions would enable gain of virulence in the short term. Whether other mechanisms such as epigenetic gene silencing or suppression may play a more predominant role in the attenuation of avirulence is unpredictable.

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RPI-VNT1-AVRVNT1

Rpi-vnt1

Rpi-vnt1 was the first late blight *R* gene to be cloned from a plant of South American origin **(Figure 2*a*)**. Three highly similar allelic variants, *Rpi-vnt1.1*, *Rpi-vnt1.2*, and *Rpi-vnt1.3*, were isolated from three different accessions of *Solanum venturii* (30, 100) **(Table 1)**. *Rpi-vnt1* contains a single exon encoding a 891 aa CC-NB-LRR protein with a C-terminal leucine rich region that is relatively short (377 aa) and loosely fits the consensus for LRR. *Rpi-vnt1* shares 75% aa identity with the mosaic virus resistance protein $Tm2^2$ from tomato (72).

Rpi-vnt1.1, *Rpi-vnt1.2*, and *Rpi-vnt1.3* are located on the long arm of chromosome IX. From *Solanum mochiquense*, *Rpi-mcq1* (formerly *Rpi-moc1)* was mapped to a similar genetic location (121) and was found to be homologous to $Tm2^2$ and *Rpi-vnt1* (30). Spectrum analyses with *P. infestans* isolates and functional assays using effectors suggest that *Rpi-mcq1* and *Rpi-vnt1* have distinct recognition specificities (H. Rietman, unpublished results).

Genomic analysis revealed the presence of many related sequences in the genome, of which only one copy is located at the *Rpi-vnt1* locus. Pel (99) hypothesized that allelic variants of *Rpi-vnt1* evolved through illegitimate recombination, and the conservation of functionally equivalent allelic variants suggests that *Rpi-vnt1* is a type II *R* gene (69). The low distribution of *Rpi-vnt1* allelic variants through the section *Petota* and the confinement of this gene to a geographic area in the Andes suggest that *Rpi-vnt1* emerged recently.

Rpi-vnt1 has not widely been used in agriculture, even though it confers resistance to a broad spectrum of isolates from potato-growing areas in Europe (100). A three year field trial with a genetically engineered potato based on the popular variety Desirée expressing *Rpi-vnt1* started in 2010 in Norfolk, United Kingdom (66) and will determine whether *Rpi-vnt1*-based resistance to late blight is realized under field conditions in Europe.

Avrvnt1

Avrvnt1 was recently identified using effectoromics (99) **(Figure 4)**. A genome-wide set of expressed RXLR effectors of *P. infestans* was functionally screened in a resistant S. *venturii* containing *Rpi-vnt1*, and few candidate *Avr* genes were found to induce defense responses. One effector, designated *Avrvnt1*, specifically induced cell death in the resistant offspring of a population that segregated for *Rpi-vnt1*. In the reference genome of *P. infestans* T30-4, *Avrvnt1* locates in a gene-sparse region at a single locus (41) **(Figure 3)**. Only three homologous RXLR effectors were detected in the T30-4 genome, pointing to a small gene family **(Table 2)**. The genetic diversity of *Avrvnt1* among a broader set of *P. infestans* isolates was limited to four variants. In *P. infestans* strains that are avirulent on *Rpi-vnt1* plants, *Avrvnt1* is upregulated during the early biotrophic phases of the infection **(Figure 3)**. In virulent strains of the South American EC1 lineage however, *Avrvnt1* coding sequence was intact but its transcript was not detected. This suggests that *Avrvnt1* evades *R* gene recognition by reduced expression, and conserved sequence between the avirulent and virulent alleles points to epiallelic variation (99).

Broad spectrum: effective against a wide range of pathogen isolates

DIVERSITY OF LATE BLIGHT *R* GENES IS HIGHEST IN CENTRAL MEXICO

The antagonistic interplay between the pathogen and host is driven by *R* and *Avr* genes that are expected to be the direct targets of coevolutionary forces (20, 88). Therefore, one would expect diversity of late blight *R* genes to be high in regions where coevolution between *P. infestans* and *Solanum* has occurred over a long period of time. In contrast, host plants from geographical regions that have been only relatively recently invaded by the pathogen are expected to display reduced levels of late blight *R* gene diversity. Many of the Mexican *R* genes that confer high levels of

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QTL: quantitative trait loci

race-specific resistance, including *R1* through *R4* of *S. demissum*, appear to represent products of ancient and extensive coevolution (122) (**Table 1**, **Figure 2*a***). In addition, virulent alleles of the corresponding Avr effectors have been detected in Mexican populations of *P. infestans*. These observations are consistent with the hypothesis that central Mexico, not South America, is the primary center of origin of *P. infestans* (34, 37, 38). This said, several distinct lineages of *P. infestans* occur in South America and matching *R* genes have been detected in South American *Solanum* species (1, 36).

R GENES THAT CONFER PARTIAL RESISTANCE

Some *R* genes appear to confer partial resistance to *P. infestans*. These *R* genes often segregate as quantitative trait loci (QTL) in genetic mapping populations and breeders tend to wrongly assume that their mode of action differs from classical gene-for-gene interactions (reviewed in 35, 46, 106, 111). In these cases, coevolution between pathogen and host may be less advanced than for genes conferring full resistance. An example is *Rpi-mcd1* (77), which was originally described as a QTL from an Argentinean *S. microdontum* (127). Initially, this gene was assumed to be similar to other QTL identified in South America genotypes, but it was later shown to be a classical *R* gene of the *R2* family (78). An example from Mexico is *Rpi-blb1*, which confers partial resistance in transgenic or introgressed potatoes (12, 18, 22, 67). Perhaps this slowly evolving (Type II) *R* gene has not fully unleashed its evolutionary potential and the partial level of resistance may reflect a weaker recognition of the *Avrblb1* effector. Consistent with this proposal, the *Rpi-blb1* donor species, *S. bulbocastanum* and *S. stoloniferum*, typically occur in more arid habitats that are less conducive to *P. infestans* infections, compared with *S. demissum* (18, 54).

PARTIAL EVASION OF R PROTEIN RECOGNITION BY *P. INFESTANS*

As described above, there are many examples of *P. infestans* races that have evolved to overcome *R* gene–mediated resistance. In all cases reported so far, the virulent races are equally aggressive on genotypes that differentially carry/lack the cognate *R* gene (**Figure 5*a***). This is explained by the *Avr* effector gene being usually mutated or pseudogenized to an inactive form. The impact of such mutations on pathogen virulence is negligible, possibly due to functional redundancy in the effector repertoire (5).

Recently, we have noted that some isolates of *P. infestans* are partially virulent on *R* gene–containing plants (G. Kessel, R. Oliva, J. Vossen, V. Vleeshouwers & S. Kamoun, unpublished results). In these cases, although the virulent race of the pathogen causes expanding lesions on the *R* gene–containing genotype, these lesions are smaller and expand less rapidly than on the *R* gene–lacking genotype (**Figure 5*b***). This indicates that the *R* gene confers partial resistance against the virulent race of *P. infestans*. Therefore, when assaying virulent races of *P. infestans*, it is critical to perform side-by-side assays with *R* gene differential plant genotypes to discriminate between fully and partially virulent strains as described in **Figure 5**.

What is the molecular basis of partial evasion of *R* gene mediated resistance by *P. infestans*? The observation that the *R* gene retains a residual effect against the partially virulent *P. infestans* indicates that the virulent strain carries a functional *Avr* effector gene. One explanation is that the *Avr* gene has not pseudogenized but may have mutated to an allele that partially evades activation of the *R* gene. An alternative is that the timing and level of expression of the *Avr* effector may be altered enough to delay or reduce the intensity of recognition by the *R* gene. A third possibility is that the partially virulent race evolved a suppressor gene

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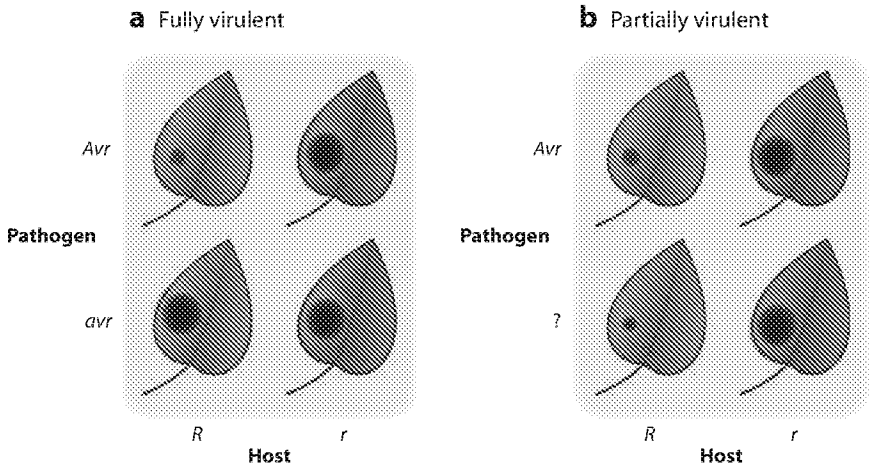


Figure 5

Phenotypic distinctions between fully versus partially virulent strains of *Phytophthora infestans*. (*a*) Fully virulent *P. infestans* strains grow at the same rate irrespective of *R* gene presence. (*b*) For partially virulent strains, the presence of the *R* gene has an impact on the overall performance of the strain. In the scheme, small circles represent typical nonexpanding hypersensitive response lesions, big circles represent expanding lesions with sporulation of *P. infestans*, and the intermediate-size circle represents a slowly expanding lesion. A difference in the degree of disease caused by the virulent race between *R* versus *r* plants indicates that the *R* gene still confers partial resistance.

of the *R* gene response. This situation is more likely to arise in cases where the effector is essential for full virulence of *P. infestans* (73).

Importantly, *R* genes that have been only partially defeated remain useful for deployment in agriculture, especially when stacked with other *R* genes. Targeting a critical pathogen effector (Achilles heel hypothesis), as for instance in the tomato–*Fusarium oxysporum* system (*I* and *Avr1* interaction) (126), might be beneficial for maximizing resistance durability in the field. But even under such Achilles heel conditions, *P. infestans* appears able to evolve into a somewhat virulent race in line with its reputation as a pathogen with a high evolutionary potential (84). Nevertheless, this theoretical extension of the gene-for-gene model provides a first insight to explain the partially resistant phenotypes often observed in the potato–*P. infestans* pathosystem.

EXPLOITING EFFECTORS IN RESISTANCE BREEDING

The emerging field of pathogen effector biology has valuable implications for breeding and deployment of disease resistance (26, 90, 145). Here, we present four ways by which the knowledge on pathogen effector diversity and mode of action can be utilized to improve the use and deployment of late blight disease resistance.

First, the cloning of *R* genes is accelerated when matching *Avr* genes are available. *R* gene activity can efficiently be assessed by rapid functional assays that consist of coexpressing the *Avr* gene in planta with candidate *R* genes, e.g., by agroinfection or agroinfiltration (**Figure 4**) (90, 145). This means that the slow and tedious process of generating stable transformants for complementation studies in potato is no longer a limiting step.

Second, functional allele mining with *Avr* genes in large collections of germplasm can quickly lead to identification of functional resistance gene homolog (*RGH*) in a variety of species. This can accelerate resistance breeding as sexually more compatible species with a particular resistance specificity can be identified and used for introgression (145). In addition, redundant breeding or cloning efforts can be avoided by classifying germplasm or *R* genes

RGH: resistance gene homolog

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based on their responses to effectors. This is particularly important for *R* genes with broad spectrum effects for which diagnostic pathogen races are not available.

Third, expanding the effector recognition specificity of a given *R* gene to new virulent alleles is another strategy to increase the pool of genes available for breeding. Artificial evolution by random mutagenesis is a potential tool to accomplish this goal, as previously demonstrated for the PVX resistance gene *Rx* (4, 28). Expanded recognition specificity by a new *R* gene variant could be due to a single aa change in the R protein, as we recently discovered for *R3a* (M.E. Segretin & S. Kamoun, unpublished results). In this scenario and whenever the original *R* gene is present in the crop species, targeted mutagenesis (genome editing) by new technologies, such as zinc finger nuclease–based approaches (119, 129), can be implemented. This would potentially provide a nontransgenic resistant variety that does not carry extraneous pieces of DNA (81). A key requirement for the success of this strategy is to have basic knowledge of the pathogen effectors. The right choice of the *R-Avr* pair will influence the probabilities of success in terms of durability once the new *R* gene is deployed in the field. Also, knowledge related to the effector protein is needed in order to design an efficient screening system to identify the mutated *R* gene candidates. Moreover, if homologs of the targeted *Avr* gene are present in other species, the R protein recognition specificity could be potentially expanded to confer resistance to related pathogens.

EXPLOITING EFFECTORS IN DEPLOYMENT OF RESISTANCE

A fourth aspect of exploiting pathogen effectors is to improve the spatio-temporal deployment of *R* gene–based disease resistance. The geographical structure and dynamics of *P. infestans* populations impact the effectiveness of *R* gene–based late blight management. For instance, sub-Saharan Africa is dominated by few locally adapted *P. infestans* asexual lineages (85, 107), whereas more dynamic or complex populations tend to occur in Europe or North America (33). Effector assessment can assist with evaluating the potential of a given *R* gene by informing about the distribution of virulence alleles in local *P. infestans* populations. Such analysis would detect emerging virulent races of *P. infestans* before they reach epidemic proportions. Along with genetic adaptation and selection, emergence of highly aggressive clones is accelerated because primary inoculum will be increased in the following season. In Europe, for instance, genotype_13 appeared in 2004 and increased in frequency from 12% to 71% of populations in the United Kingdom over three seasons (19, 33). One alternative to manage these types of epidemics is to buffer the occurrence of the clone by choosing appropriate cultivars. With the availability of the *P. infestans* genome sequence (41) and next-generation sequencing technology, rapid profiling of the effector repertoires of emerging genotypes of *P. infestans* is possible. Within a couple of months it is possible to generate a list of *Avr* gene targets for a given isolate (or genotype) of *P. infestans*. This can assist decision making in the next potato growing season. In-season monitoring for virulence frequencies of *Avr* genes is also possible, and in an experiment in 2010, a strong reduction of fungicide input on field plots with *R* gene–carrying potatoes was achieved through effector monitoring (G. Kessel, unpublished results). Given that important efforts to understand and characterize Avr effector function and variability are underway (18, 42, 90, 145), we will soon be in a position to monitor potential changes in pathogen populations.

CONCLUSION

The most sustainable strategy to manage late blight is to breed broad-spectrum disease resistance into potato. However, traditional disease resistance breeding approaches are slow, inefficient, and have taken little advantage of emerging knowledge of pathogen mechanisms. Late blight resistance genes have been

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identified, bred, and deployed in agriculture in a 'blind' fashion without detailed knowledge of the effectors they are recognizing. It remains problematic to predict the extent to which an *R* gene will be durable until it is actually deployed in agriculture. Nonetheless, it is relatively easy to point to non-durable *R* genes, i.e. *R* genes such as *R4* that target dispensable effectors. Therefore, the first step in a breeding program should be to avoid wasting efforts on such ineffective *R* genes to maximize the potential for resistance durability. This is already a step beyond traditional "pathogen-blind" resistance breeding approach.

SUMMARY POINTS

1. Map-based cloning, allele mining, effectoromics, and sequencing of the potato genome led to isolation of 21 *R* genes conferring resistance to late blight.
2. Late blight *R* gene families occur in several different genomic locations. *R* genes differ in resistance spectrum, geographic origin, evolution rate, and in the degree of resistance they confer.
3. Most *R* genes identified thus far confer high levels of race-specific resistance and were retrieved from *Solanum* species originating from Central America, the center of origin of *P. infestans*.
4. *Avr* effector genes reside in gene-sparse regions of the *P. infestans* genome, belong to diverse gene families that vary in size between isolates and are typically upregulated at early stages of the biotrophic interaction. They encode modular proteins with a signal peptide, an RXLR motif, and a C-terminal effector domain.
5. *Avr* genes avoid recognition by diverse mechanisms, e.g., mutation, gene loss, suppression, and gene silencing.
6. Exploiting effectors in breeding has led to accelerated cloning of *R* genes and enables accurate profiling of *R* gene specificities.
7. Effectors can be exploited in deployment of resistance by monitoring pathogen populations for effector allelic diversity.
8. Eliminating ineffective *R* genes that target dispensable *Avr* effectors maximizes the potential for resistance durability in the field and is a step beyond traditional "pathogen-blind" approaches.

FUTURE ISSUES

1. Next-generation sequencing and new approaches are expected to further accelerate *R* gene cloning.
2. Next-generation resistance breeding will enhance resistance spectrum and durability by incorporating engineered, synthetic *R* genes with expanded pathogen recognition specificities.
3. Investigating the molecular mechanisms of partial resistance and partial virulence will contribute to understanding observed phenotypes and lead to educated deployment of available resistance genes and QTL.

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4. Exploiting another layer of defense, based on pathogen molecular pattern recognition by recognition receptors could contribute to producing a defense response that is more durable and broad spectrum (71).

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

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

Commissioner:

In compliance with the duty of disclosure under 37 C.F.R. § 1.56, it is respectfully requested that this Supplemental Information Disclosure Statement be entered and the documents listed on attached 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 4, 2021

PTO/SB/08a (02-18)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

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

U.S.PATENTS

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	10524436		2020-01-07	Lindhout et al.	

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U.S.PATENT APPLICATION PUBLICATIONS

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	Application Number	16541071
	Filing Date	2019-08-14
	First Named Inventor	Willem Hendrik Lindhout
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EXAMINER SIGNATURE

Examiner Signature		Date Considered	

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

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

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

CERTIFICATION STATEMENT

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.**

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

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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 4, 2021

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING
TRANSMITTAL OF COPY OF INTERNATIONAL
PRELIMINARY REPORT ON PATENTABILITY
(CHAPTER I OF THE PATENT COOPERATION
TREATY)
(PCT Rule 44bis.1(c))

To:

JANSEN, C.M.
V.O.
Johan de Wittlaan 7
NL-2517 JR Den Haag
PAYS-BAS

Date of mailing *(day/month/year)* 31 October 2013 (31.10.2013)	
Applicant's or agent's file reference P94557PC00	**IMPORTANT NOTICE**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NL2012/050269	20 April 2012 (20.04.2012)	21 April 2011 (21.04.2011)

Applicant
AGVENTURE B.V. et al

The International Bureau transmits herewith a copy of the international preliminary report on patentability (Chapter I of the Patent Cooperation Treaty)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. +41 22 338 82 70

Authorized officer
Philippe Bécamel

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Page 1644

PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY
(Chapter I of the Patent Cooperation Treaty)

(PCT Rule 44*bis*)

Applicant's or agent's file reference P94557PC00	**FOR FURTHER ACTION**	See item 4 below
International application No. PCT/NL2012/050269	International filing date *(day/month/year)* 20 April 2012 (20.04.2012)	Priority date *(day/month/year)* 21 April 2011 (21.04.2011)
International Patent Classification (8th edition unless older edition indicated) See relevant information in Form PCT/ISA/237		
Applicant AGVENTURE B.V.		

1. This international preliminary report on patentability (Chapter I) is issued by the International Bureau on behalf of the International Searching Authority under Rule 44 *bis*.1(a).

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   In the attached sheets, any reference to the written opinion of the International Searching Authority should be read as a reference to the international preliminary report on patentability (Chapter I) instead.

3. This report contains indications relating to the following items:

☒	Box No. I	Basis of the report
☐	Box No. II	Priority
☐	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☐	Box No. VII	Certain defects in the international application
☒	Box No. VIII	Certain observations on the international application

4. The International Bureau will communicate this report to designated Offices in accordance with Rules 44*bis*.3(c) and 93*bis*.1 but not, except where the applicant makes an express request under Article 23(2), before the expiration of 30 months from the priority date (Rule 44*bis* .2).

	Date of issuance of this report 22 October 2013 (22.10.2013)
The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. +41 22 338 82 70	Authorized officer Philippe Bécamel e-mail: pt03.pct@wipo.int

Form PCT/IB/373 (January 2004)

PATENT COOPERATION TREATY

From the
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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NL2012/050269	20.04.2012	21.04.2011

International Patent Classification (IPC) or both national classification and IPC
INV. C12N15/82 A01H1/04 A01H4/00 A01H5/04

Applicant
AGVENTURE B.V

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notifed the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA:

European Patent Office

D-80298 Munich
Tel. +49 89 2399 - 0
Fax: +49 89 2399 - 4465

Date of completion of this opinion

see form PCT/ISA/210

Authorized Officer

Mundel, Christophe

Telephone No. +49 89 2399-7314

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Page 1646

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed

   ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43bis.1(a))

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing filed or furnished:

   a. (means)

      ☐ on paper

      ☒ in electronic form

   b. (time)

      ☐ in the international application as filed

      ☐ together with the international application in electronic form

      ☒ subsequently to this Authority for the purposes of search

4. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-18
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-18
Industrial applicability (IA)	Yes:	Claims	1-18
	No:	Claims	

2. Citations and explanations

see separate sheet

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1 The present application refers to a diploid, fertile, self-compatible and essentially homozygous potato line comprising plants having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant and at least one agronomically desirable trait or gene. The application also refers to hybrid potato plants and seeds resulting from a cross from said potato line with another line, to a method of producing a diploid, fertile, self-compatible and essentially homozygous potato line comprising plants having an average tuber yield expressed in grams of fresh weight of at least 200 grams.

2 Reference is made to the following documents:

D1 CHALERMPOL PHUMICHAI ET AL: "Cryptic improvement for fertility by continuous selfing of diploid potatoes using Sli gene", EUPHYTICA, KLUWER ACADEMIC PUBLISHERS, DO, vol. 149, no. 1-2, 2 June 2006 (2006-06-02), pages 251-258, ISSN: 1573-5060, DOI: 10.1007/S10681-005-9072-5

D2 CHALERMPOL PHUMICHAI ET AL: "Expression of S-locus inhibitor gene (Sli) in various diploid potatoes", EUPHYTICA, KLUWER ACADEMIC PUBLISHERS, DO, vol. 148, no. 3, 28 April 2006 (2006-04-28), pages 227-234, ISSN: 1573-5060, DOI: 10.1007/S10681-005-9004-4

D3 PHUMICHAI CHALERMPOL ET AL: "Toward the development of highly homozygous diploid potato lines using the self-compatibility controlling Sli gene", GENOME, OTTAWA, CA, vol. 48, no. 6, 1 December 2005 (2005-12-01), pages 977-984, ISSN: 0831-2796, DOI: 10.1139/G05-066

D4 MARIS B: "COMPARISON OF DIPLOID AND TETRAPLOID POTATO FAMILIES DERIVED FROM SOLANUM-PHUREJA X DIHAPLOID SOLANUM-TUBEROSUM HYBRIDS AND THEIR VEGETATIVELY DOUBLED COUNTERPARTS", EUPHYTICA, KLUWER ACADEMIC PUBLISHERS, NL, vol. 46, no. 1, 1 March 1990 (1990-03-01), pages 15-33, ISSN: 0014-2336, DOI: 10.1007/BF00057615

D5 SHELLEY HERMUNDSTAD ET AL: "Tuber yield and tuber traits of haploid-wild species F1 hybrids", POTATO RESEARCH, ASSOCIATION FOR POTATO RESEARCH, WAGENINGEN, NL, vol. 29, 1 September 1986 (1986-09-01), pages 289-297, ISSN: 0014-3065, DOI: 10.1007/BF02359958

3 **Novelty; Article 33(2) PCT.**

None of the prior art documents unambiguously discloses a diploid, fertile, self-compatible and essentially homozygous potato line comprising plants having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant and comprising at least one of the cited traits or genes. Therefore, the claims have to be considered as novel in the sense of Article 33(2) PCT.

4 **Inventive step; Article 33(3) PCT.**

The documents D1 to D3 disclose the generation of diploid, fertile, self-compatible and essentially homozygous potato line by using the Sli gene present in diploid potato lines. Said line have been obtainedby introduction of the Sli gene originally derived from a self-compatible variant of Solanum chacoense Bitter by crossing (**D1** : p. 252 : Plant material; **D2** : p. 228 : Plant material; **D3** : Figure 1 and p. 978 : Plant material)

In the light of any of these documents, the problem to be solved by the present application can be seen as the provision of improved diploid, fertile, self-compatible, essentially homozygous potato lines.

It is clear from the teaching of any of D1 to D3 that the introduction of the Sli gene in diploid potato lines will result in the generation of a fertile, self-compatible potato line. Such diploid, fertile, self compatible lines are useful for gene analysis, genetic fixation of traits, production of TPS (True Potato Seeds), varieties with genetic uniformity and heterosis breeding of potato (**D1** : p. 251, right-hand column, line 8 to p. 252, left-hand column, line 2; **D2** : p. 227, left-hand column, line 4 to p. 228, left-hand column, line 3; **D3** : p. 977, right-hand column, lines 13-17).

At the priority date of the present application, diploid potato lines having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant were already well-known (see for example documents D4 : p. 16-17 and Table 8; D5 : p. 290 : Material and Methods, Results and Figure 1).

The skilled person willing to improve the potato line disclosed in any of D1 to D3 would have needed no inventive activity to consider introducing the Sli gene in a diploid genotype having high tuber yield. D3 already discloses the introduction of the Sli gene in different diploid genotypes (Abstract) thus demonstrating the feasibility of this approach.

The introduction of further well-known traits/genes in such a line, using well-known breeding and/or transformation techniques, cannot be considered as inventive. In this context, it has to be noted that documents D1 to D3 already state that diploid, fertile, self-compatible potato lines would be useful in breeding.

Therefore, claims 1-18 are considered to lack an inventive step (Article 33(3) PCT).

Re Item VIII

Certain observations on the international application

Lack of clarity; Article 6 PCT.

1 In claim 1, the plant is only characterised by the result to be achieved by said plant, i.e. being self-compatible, essentially homozygous and having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant and at least one additional agronomically desirable trait or gene.

According to the PCT International Search and Preliminary Examination Guidelines as in force from March 25, 2004, chapter 5.35 : "The are defined by the claims must be as precise as the invention allows. As a general rule, claims which attempt to define the invention, or a feature thereof, by a result to be achieved should be objected to as lacking clarity. [...]".

2 The plants of claim 1 are characterized, inter alia, by the presence of one or more of a list of traits/genes. For some of the claimed traits, it is not clear which gene or genes are implicated in the trait and how the trait should be transmitted. Therefore, claim 1 further lacks clarity (Article 6 PCT).

3 No plant according to claim 1 has been exemplified in the present application. For the moment, it is considered that the skilled person would not be able to generate the plants of the present application over the whole scope of claim 1 without undue burden of experimentation. Therefore, claim 1 cannot be considered as fully supported by the description of the present application (Article 6 PCT in combination with Article 5 PCT).

The attention of the Applicant is drawn to the fact that the only plants which fulfill the definition of claim 1 are the deposited lines under breeder reference AGVD1, AGVD2, AGVD3 and AGVD17 which all derive from the Sli gene donor IVP07-1004-1. However the claims are by no way restricted to such plants and encompass completely uncharacterized plants.

4 Claim 2 refers to a hybrid potato seed resulting from the cross between a plant according to claim 1 and an undefined essentially homozygous potato breeding line. Claim 3 refers to hybrid potato plants grown from said seed. It is not clear what should be the characteristics of such a hybrid potato seed or plant what renders the scope of the claim unclear. It has to be noted that such a hybrid plant will not necessarily comprise the characteristics of the plant of claim 1.

These remarks applies mutatis mutandis to claims 9-15 and 17.

Electronic Acknowledgement Receipt

EFS ID:	42080311
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
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Application Type:	Utility under 35 USC 111(a)

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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
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National Stage of an International Application under 35 U.S.C. 371

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

New International Application Filed with the USPTO as a Receiving Office

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

Theor Appl Genet (2003) 106:1524–1531
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E. Bakker · P. Butterbach · J. Rouppe van der Voort ·
E. van der Vossen · J. van Vliet · J. Bakker · A. Goverse

Genetic and physical mapping of homologues of the virus resistance gene *Rx1* and the cyst nematode resistance gene *Gpa2* in potato

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Abstract Nine resistance gene homologues (RGHs) were identified in two diploid potato clones (SH and RH), with a specific primer pair based on conserved motifs in the LRR domain of the potato cyst nematode resistance gene *Gpa2* and the potato virus X resistance gene *Rx1*. A modified AFLP method was used to facilitate the genetic mapping of the RGHs in the four haplotypes under investigation. All nine RGHs appeared to be located in the *Gpa2/Rx1* cluster on chromosome XII. Construction of a physical map using bacterial artificial chromosome (BAC) clones for both the *Solanum tuberosum* ssp. *tuberosum* and the *S. tuberosum* ssp. *andigena* haplotype of SH showed that the RGHs are located within a stretch of less than 200 kb. Sequence analysis of the RGHs revealed that they are highly similar (93 to 95%) to *Gpa2* and *Rx1*. The sequence identities among all RGHs range from 85 to 100%. Two pairs of RGHs are identical, or nearly so (100 and 99.9%), with each member located in a different genotype. Southern-blot analysis on genomic DNA revealed no evidence for additional homologues outside the *Gpa2/Rx1* cluster on chromosome XII.

Keywords *Globodera pallida* · Potato cyst nematodes · Potato virus X · Resistance gene homologues

Introduction

Plants are constantly under attack by a wide range of pathogens and pests. To defend themselves, plants have evolved an innate surveillance system consisting of a large set of resistance genes. Most resistance genes are single-dominant and confer resistance in a gene-for-gene specific manner (Flor 1971). More than 30 resistance genes (*R* genes) have been cloned from different plant species and can be divided into four classes based on common structural motives (Takken and Joosten 2000). The majority of the *R* genes are characterised by a leucine-rich repeat (LRR) and a nucleotide binding site (NBS) domain. *R* genes that belong to this superfamily confer resistance to completely unrelated taxonomic groups like bacteria, fungi, viruses and nematodes (Mindrinos et al. 1994; Whitham et al. 1994; Lawrence et al. 1995; Milligan et al. 1998; Vos et al. 1998; Bendahmane et al. 1999; Van der Vossen et al. 2000).

In potato, 19 *R* genes have been mapped to 11 chromosomal regions (Gebhardt and Valkonen 2001). Four of them, *Rx1*, *Gpa2*, *Rx2* and *R1*, have been isolated and they all belong to the NBS-LRR class. *Rx1* and *Gpa2* originate from *Solanum tuberosum* ssp. *andigena* and have been identified by map-based cloning (Bendahmane et al. 1999; Van der Vossen et al. 2000). *Rx2* has been isolated from *Solanum aucaule* using a PCR-based approach (Bendahmane et al. 2000) and *R1* has been isolated from *Solanum demissum* by using a combination of map-based cloning and the candidate gene approach (Ballvora et al. 2002). *Rx1* and *Gpa2* are highly homologous, yet they confer resistance to two completely unrelated pathogens, namely potato virus X and the potato cyst nematode *Globodera pallida*. The genes are tightly linked on chromosome XII of potato (Bendahmane et al. 1997; Rouppe van der Voort et al. 1999). Sequencing a 187-kb region revealed that *Gpa2* and *Rx1* are part of a complex locus containing at least two other closely related resistance gene homologues (RGHs): *RGC1* and the pseudogene *RGC3* (Bendahmane et al. 1997; Bendahmane et al. 1999; Rouppe van der Voort et al. 1999; Van der Vossen et al. 2000). In the LRR domain, more variation is observed between the RGHs than in the NBS domain. The mean K_a/K_s ratio for the LRR region is larger than 1, whereas in the NBS regions the ratio is smaller than 1, indicating that the LRR domain is subject

Communicated by G. Wenzel

E. Bakker · P. Butterbach · J. Rouppe van der Voort ·
E. van der Vossen · J. van Vliet · J. Bakker · A. Goverse (✉)
The Graduate School of Experimental Plant Sciences,
Wageningen University, Department of Nematology,
P.O. Box 8123, 6700 ES Wageningen, The Netherlands
e-mail: aska.goverse@wur.nl
Tel.: +31-317-485086
Fax: +31-317-484-254

to diversifying selection and that specificity is determined by this domain (Van der Vossen et al. 2000).

In this paper we describe the use of LRR-specific primer combinations for both the identification and mapping of nine *Gpa2/Rx1* homologues in two diploid potato clones (SH and RH). The value of using LRR-specific primers for the dissection of *R* gene clusters in different haplotypes will be discussed.

Materials and methods

Plant material and DNA extraction

A mapping population for *S. tuberosum* ssp. *tuberosum* was available from the diploid potato clones SH83-92-488 × RH89-039-16 (Rouppe van der Voort et al. 1997). The female parent SH83-92-488 contains an introgression segment originating from the wild accession *S. tuberosum* ssp. *andigena* CPC1673 on which the *G. pallida* resistance gene *Gpa2* and the PVX resistance gene *Rx1* are located. The male parent RH89-039-16 has been selected for its fertility and the production of vigorous offspring. SH83-92-488 will be referred to as SH, and RH89-039-16 will be referred to as RH. The mapping population F_1 SH × RH consists of 136 vigorous F1 genotypes. Genomic DNA was extracted from frozen leaf tissue of in vitro plants as described (Van der Beek et al. 1992).

BAC library and DNA extraction

The construction of the BAC library from the diploid potato clone SH83-92-488 has been described by Rouppe van der Voort et al. (1999). The library has been extended with 30,000 clones and now comprises 90,000 clones. Pooling and preparation for screening was done as described (Kanyuka et al. 1999), and resulted in 255 plate pools. DNA extracted from these BAC clones was used as a template for PCR and sequence analysis. For this, clones were cultured overnight at 37 °C in 500 ml of LB medium, supplemented with 170 μg/ml of chloramphenicol for selection. Plasmid DNA was isolated using the "very low-copy plasmid DNA purification protocol" of the plasmid midi-kit according to the manufacturer's instructions (Quiagen, Hilden, Germany). For each BAC, 1 μl of DNA was digested with *Hin*dIII to check the concentration and the purity.

PCR analysis

A cluster-specific primer pair was designed based on conserved DNA sequences in the LRR domains of *Gpa2*, *Rx1* and *SH-RGH1* [previously designated as *RGC1* (Van der Vossen et al. 2000)]. The forward primer (LRR-F: ttg gtg tcg taa cag tga gg) starts at position +1,533 of *Gpa2* and the reverse primer (LRR-R: ctg gct agt cct cag aac ac) at position +3,192 of *Gpa2* (UTR). A PCR was performed with this primer pair using the Expand High Fidelity PCR System according to the manufacturer's instructions (Roche, Mannheim, Germany). The following PCR cycle file was applied: 3 min at 94 °C followed by 30 s at 94 °C, 30 s at 55 °C, 90 s at 72 °C for 10 cycles, 30 s at 94 °C, 30 s at 55 °C, 90 s at 72 °C with an extension of 5 s/cycle for 25 cycles and finally a 5-min elongation at 72 °C.

Cloning and digestion analysis of the PCR products

PCR products were cloned into the PCR2.1TOPO-vector for transformation of *Escherichia coli* TOP10 cells according to the manufacturer's instructions (Invitrogen, San Diego, Calif., USA). For each PCR product, 12 positive clones were cultivated overnight at 37 °C in 2 ml of liquid LB medium with 100 μg/ml of ampicillin for selection. Each clone was re-amplified with the same LRR primer pair and the resulting PCR products were digested with *Taq*I. PCR was performed using an adjusted PCR buffer containing 100 mM of Tris HCl pH 8.3, 500 mM of KCl, 25 mM of MgCl and 10% Triton X-100 to avoid additional cleaning steps of the PCR products prior to digestion. The DNA fragments were separated on a 4% agarose gel consisting of 1% ultra-pure agarose (Life Technologies, Breda, The Netherlands) and 3% NuSieve GTG agarose (FMC, Philadelphia, Pa., USA) in 1 × TAE buffer at 120 V. Clones were selected for further analysis based on differences in the digestion patterns.

Sequence analysis

Sequence analysis of the PCR products was carried out by Greenomics, PRI, Wageningen, The Netherlands. BAC-end sequencing was performed using approximately 1 μg of template DNA in a cycle-sequencing reaction using either 100 ng of sp6 or t7 primer and 8 μl of Big Dye terminator mix (PerkinElmer, Wellesley, Mass., USA) in a total volume of 20 μl. The PCR protocol consisted of 25 cycles of 30 s at 96 °C, 15 s at 50 °C and 4 min at 60 °C. After ethanol precipitation at room temperature for 10 min and recovery, the labelled DNA was dissolved in 3 μl of formamide. DNA was then heated for 2 min at 96 °C and directly cooled on ice. Approximately 1.5 μl was then loaded on a 6% TBE (pH 8.3) polyacrylamide gel. Sequence electrophoresis was carried out on either an ABI 373XL or ABI 377 sequencer. DNA sequence analysis and comparisons were employed using DNAstar software.

CHEF-gel electrophoresis

BAC insert sizes were determined with CHEF electrophoresis on a 1% agarose gel (Seakem Gold, FMC, Philadelphia, Pa., USA) in 0.5 × TBE buffer at 4 °C using a BIO-RAD CHEF DR II system (Bio-Rad Laboratories, Hercules, Calif., USA) at 200 V, with a pulse time of 5 to 15 s for 18 h.

Southern-blot analysis

Approximately 20 ng of BAC DNA and 3 μg of genomic DNA was digested with *Hin*dIII. The digested DNA was separated on a 1% agarose (Agarose NA, Pharmacia, Peapack, N.J., USA) gel in 1 × TAE buffer at 50 V. The gels were blotted overnight on hybridisation filters using capillary forces (Sambrook et al. 1989). Southern analysis was performed based on the DIG Application Manual for filter hybridisation (Roche, Basel, Switzerland). An LRR-based probe [position +1,533 to position +1,936 (403 bp)] and an NBS- and LRR-based probe [position +1,367 to position +1,552 (185 bp)], were amplified from *Gpa2* BAC DNA. The primer pairs used are PLRR-F (ttg gtg tcg taa cag tga gg) and PLRR-R (gtt ctc tgt agg ctc atg ac) at an annealing temperature of 60 °C, and PNBSLRR-F (gtg gaa tgc atg atg tga cc) and PNBSLRR-R (ctc act gtt acg aca cca ag) at an annealing temperature of 55 °C. The results were visualised on an autoradiogram after 5 min up to 2-h exposure.

Genetic mapping

Mapping was performed using a modified method (Fig. 1) based on the AFLP technique (Vos et al. 1995). The template used was the PCR product generated from gDNA using the iPM4 primers as described (Kanyuka et al. 1999). The PCR product was digested with *Taq*I, and a *Taq*I adapter as described (Vos et al. 1995) was ligated to the digestion products. This was followed by fragment amplification on a 1:25 diluted template using a labelled *Taq*I primer [gat gag tcc tga ccg a (Vos et al. 1995)] and the original iPM4R primer. The AFLP thermal cycle conditions were applied as

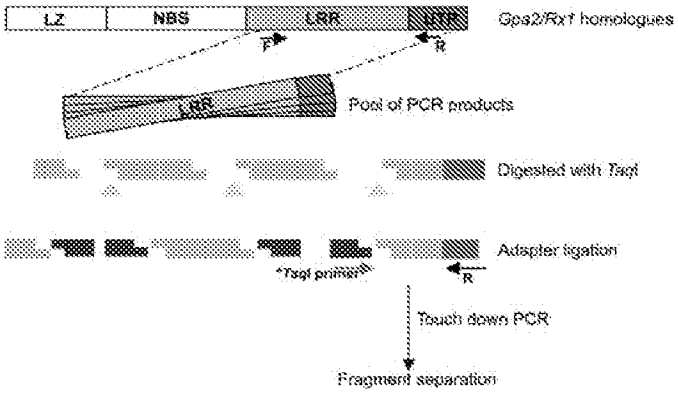


Fig. 1 Schematic depiction of the method used to genetically map the *Gpa2/Rx1* homologues. The LRR-specific primers iPM4F and iPM4R are used to amplify a pool of RGH sequences from either genomic DNA or BAC DNA. Subsequently, the PCR products are used as a template in a modified AFLP reaction. After digestion with the restriction enzyme *Taq*I the products are ligated to a *Taq*I adapter. A second touch-down PCR reaction is performed using the *Taq*I primer and the original iPM4R primer

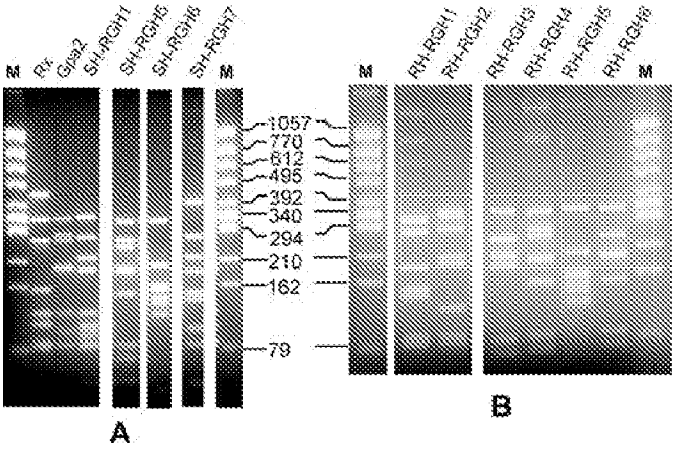


Fig. 2 Identification of *Gpa2/Rx1* resistance gene homologues (RGHs) in the diploid potato clone SH (**A**) and RH (**B**). RGHs were selected based on differences in their *Taq*I digestion pattern after PCR-amplification with specific LRR primers. The *Taq*I digestion patterns are related to a DNA base-pair ladder (*M*). Bands larger then 612 bp are the result of partially digested DNA

Table 1 Primer sequences based on BAC end sequences to detect overlap between BAC clones

BAC end	Primers
7E16sp6	5'- cgg ggt gta atg tga tga gc-3' 5'- ggc ctg caa gtc tgt gca c-3'
7E16t7	5'- gtt cgt atg agc gag tat gg-3' 5'- tcc acg atg gtc tcc tcg-3'
25G18sp6	5'-cca att tca agc ttc ttc ata g-3' 5'-cag tca agg tgc ttt gga gg-3'
25G18t7	5'- gtt acc tgc tat gtg agc tc-3' 5'- cat cag ctg cct tgc agt tg-3'
36G3sp6	5'- gcc caa cat gat agg tcg c-3' 5'- ctt ggt atc aga gca cag ag -3'
36G3t7	5'-tgt atg aat tgg gtc att ccg-3' 5'- gcc caa tat tcc tcc atc tg-3'

described (Vos et al. 1995). Separation of labelled fragments and autoradiography was done as described by Van Eck et al. (1995).

Linkage analysis of pair-wise recombination frequencies between segregating RGHs and markers were performed using JOINMAP 1.4 (Stam 1993).

Physical mapping

BAC-end sequences were employed to design PCR primers to study overlap between BAC clones. The primers designed for each BAC-end are listed in Table 1. PCR conditions used in the amplifications are equal for all primer pairs and are as follows: 3 min at 94 °C, followed by 30 s at 94 °C, 30 s at 60 °C, 90 s at 72 °C for 35 cycles and 5 min at 72 °C.

For the alignment of the contigs in SH, we used primers as described (Kanyuka et al. 1999) and 187-kb sequence information derived from four overlapping BAC clones harbouring *Gpa2*, *Rx1*, *SH-RGH1* and *SH-RGH3* (previously designated *RGC3*) (Van der Vossen et al. 2000).

Results

Identification of *Gpa2/Rx1* homologues in SH and RH

To identify homologues of *Gpa2* and *Rx1*, a specific primer set was designed based on conserved regions in the LRR domain. This primer set was used to screen a BAC library of the diploid potato clone SH, which harbours the *R* genes *Gpa2* and *Rx1* on a *S. tuberosum* ssp. *andigena* introgression segment. For each positive BAC clone, amplification products were cloned and 12 transformants were used for re-amplification. After *Taq*I digestion, resistance gene homologues (RGHs) were selected based on their unique digestion pattern (Fig. 2a). This resulted in the identification of three RGHs: *SH-RGH5* (BAC clone 7E16), *SH-RGH6* and *SH-RGH 7* (both located on BAC clones 25G18 and 36G3). Also *Gpa2* (BAC 85N1), *Rx1* (BAC 43) and *SH-RGH1* (BAC 60K18) were detected based on their expected *Taq*I digestion pattern (Bendahmane et al. 1999; Van der Vossen et al. 2000). No additional RGHs were found after screening the genomic DNA of SH.

The diploid potato clone RH was studied using genomic DNA. PCR analysis resulted in three bands at 800 bp, 1,300 bp and 1,600 bp. The complete PCR product was cloned and restriction analysis of the *Taq*I patterns of the re-amplified PCR products of 25 clones resulted in the identification of six *Gpa2/Rx1* homologues: *RH-RGH1* to *RH-RGH6* (Fig. 2b). Interestingly, *RH-RGH4* and *RH-RGH5* resembled the digestion patterns of *SH-RGH5* and *SH-RGH6*, respectively. In total, 12 RGHs were identified in potato including *Gpa2*, *Rx1* and *SH-RGH1* using a *Gpa2/Rx1* cluster-specific primer set (Table 2). Another *Gpa2/Rx1* homologue present in the cluster, the pseudogene *SH-RGH3*, was not amplified with the cluster primer set. The annealing site of the

Table 2 Resistance gene homologues (RGHs) identified in BAC and genomic DNA of the diploid potato clones SH and RH using a primer pair based on the LRR domain of *Gpa2* and *Rx1*

RGH	PCR product (kb)	BAC clones	gDNA
SH-RGH1	1.6	85N1	+
Gpa2	1.6	85N1, 60K18	–[a]
Rx1	1.6	BAC 43	–
SH-RGH5	1.5	7E16	+
SH-RGH6	1.3	25G18, 36G3	+
SH-RGH7	1.6	25G18, 36G3	–
RH-RGH1	1.6	–	+
RH-RGH2	1.6	–	+
RH-RGH3	1.6	–	+
RH-RGH4	1.5	–	+
RH-RGH5	1.3	–	+
RH-RGH6	0.8	–	+

[a] Not detected in genomic DNA

reverse primer is not present in *SH-RGH3*, because the 3' end of its sequence is truncated.

The *Gpa2/Rx1* homologues of SH and RH were sequenced to determine their sequence resemblance with *Rx1* and *Gpa2*. Comparison of the nucleotide sequence of the LRR domains of the RGHs showed that they are highly similar to each other, with sequence identities ranging from 85.5% to 100% (Table 3). The sequences of *RH-RGH4* and *RH-RGH5* were indeed virtually identical to those of *SH-RGH5* and *SH-RGH6*, respectively (99.9% and 100% sequence identity).

Genetic mapping of the *Gpa2/Rx1* homologues in SH × RH

Gpa2/Rx1-specific primers were used to perform PCR on the parents SH and RH, and a progeny of 100 individuals, to determine the genetic position of the *Gpa2/Rx1* homologues in the potato genome. The selected BAC clones harbouring *Gpa2/Rx1* homologues were included as a control. On the resulting pools of PCR products, a modified AFLP analysis was performed that makes use of sequence polymorphisms in the last *Taq*I restriction sites of the PCR products. Using the sequence information of the RGHs we calculated the length of the expected fragments from the *Taq*I restriction site closest to the 3' end of the PCR products. In this way we were able to determine the positions in the gels of the RGHs. Figure 3 shows the autoradiogram with segregating bands for *Gpa2*, *Rx1*, *SH-RGH5*, *SH-RGH6* and *RH-RGH1-RH-RGH6* at the expected positions in the gel (summarised in Table 4). *SH-RGH1* and *SH-RGH7* cannot be linked to a segregating band, because they co-migrate with a thick band in the gel. However, *SH-RGH1* is present on the same BAC as *Gpa2*, and *SH-RGH7* on the same BAC as *SH-RGH6*. All the segregating RGHs were linked to the *Gpa2/Rx1* resistance-gene cluster with a logarithm of odds (LOD) score between 8.2 and 12.6. Other DNA fragments visible on the autoradiogram either co-segregate with RGHs, or do not segregate at all. They may be

Table 3 Sequence identities (%) of the LRR domains of the RGHs in SH and RH

RGH	Gpa2	Rx1	SH-RGH1	SH-RGH3*	SH-RGH5	SH-RGH6	SH-RGH7	RH-RGH1	RH-RGH2	RH-RGH3	RH-RGH4	RH-RGH5
Rx1	93.5											
SH-RGH1	93.6	93.2										
SH-RGH3*	86.5	86.8	87.3									
SH-RGH5	92.9	92.8	93.5	87.4								
SH-RGH6	94.3	93.2	93.1	92.6	92.4							
SH-RGH7	92.8	93.3	91.9	86.5	91.9	93.1						
RH-RGH1	92.9	93.8	91.8	87.0	91.6	92.8	98.5					
RH-RGH2	93.7	94.8	93.1	88.3	93.5	94.8	93.4	92.2				
RH-RGH3	94.1	93.7	93.9	87.7	93.1	94.5	91.2	93.3	93.9			
RH-RGH4	93.1	92.9	93.5	87.5	99.9	92.6	92.1	91.7	93.4	93.1		
RH-RGH5	94.3	93.2	93.1	92.6	92.4	100.0	93.0	92.8	94.7	94.5	92.5	
RH-RGH6	93.6	94.0	85.5	85.5	93.3	90.8	92.3	92.2	94.1	94.3	93.3	90.8

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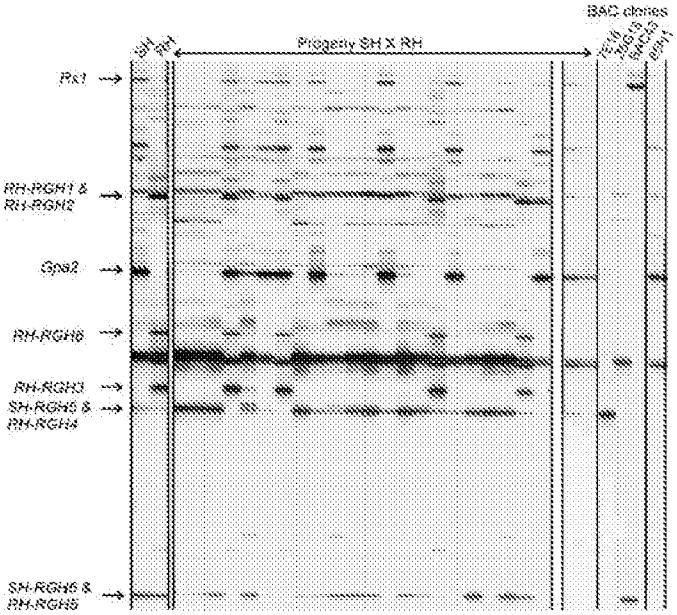


Fig. 3 Mapping of RGHs in a SH × RH cross and in a number of BAC clones using a modified AFLP method on a pool of RGH sequences. All the indicated RGHs map on chromosome XII and are linked to *Gpa2* with a LOD score between 88.2 and 12.6. *Rx1* was also identified by the co-migrating band on BAC43 from which *Rx1* was originally cloned (Bendahmane et al. 1999). Likewise, we were able to verify the expected bands for *Gpa2* and *SH-RGH1* by comparing the pattern of the mapping population with the bands in BAC 85N1. Furthermore, we could relate the bands present on BAC clones 7E16 and 25G18 with bands in the progeny. The majority of the remaining unassigned bands are artefacts of the PCR procedure

Table 4 Segregation of the RGHs in a progeny of 136 individuals. RGHs with the same mark (++ or – –) are in coupling

SH		RH	
RGH	Segregation	RGH	Segregation
Gpa2	++	*RH-RGH1*	++
Rx1	++	*RH-RGH2*	++
SH-RGH5	– –	*RH-RGH3*	++
SH-RGH6	– –	*RH-RGH6*	++
		RH-RGH4	– –
		RH-RGH5	– –

artefacts caused by the double PCR and the limited complexity of the template used.

SH-RGH5 and *SH-RGH6* are in repulsion with *Gpa2*. This implies that *SH-RGH5*, *SH-RGH6* and *SH-RGH7* are all derived from the *S. tuberosum* ssp. *tuberosum* haplotype and that no additional RGHs have been found on the *Gpa2/Rx1* cluster of the *S. tuberosum* ssp. *andigena* introgression segment. Furthermore, *RH-RGH1*, *2*, *3* and *6* are in coupling with each other and in repulsion with *RH-RGH4* and *5* (Table 4). From these data we can conclude that all the identified homologues are genetically linked to the *Gpa2/Rx1* cluster on chromosome XII in SH and in RH. Based on these and

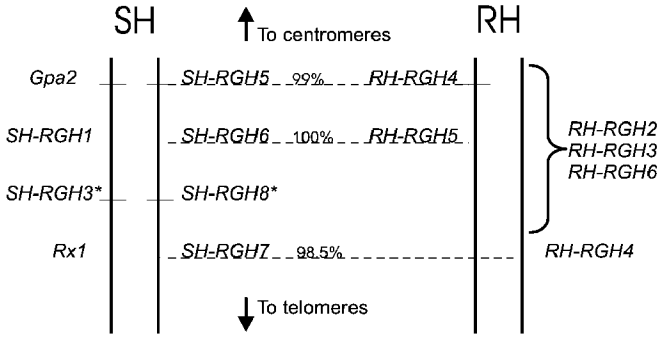


Fig. 4 Schematic drawing of the *Gpa2/Rx1* homologues on the four chromosomes of SH and RH. The relative order and orientation of a number of RGHs has been postulated as follows (see also Results). *SH-RGH5* is the only RGH present on a BAC clone positioned at the top of the cluster. *RH-RGH4* has a 99.9% identity to *SH-RGH5*, and therefore we assume that this RGH is also positioned at the top of the cluster in RH. Because the intron positions of *SH-RGH7* are the same as in *Rx1* we assume that this RGH, like *Rx1*, is at the bottom of the cluster. *RH-RGH1* and *SH-RGH7* have a sequence identity of 98.5 and therefore we assume that *RH-RGH1* is also at the bottom of the cluster in RH. We also assume that the two pseudogenes *SH-RGH3* and *SH-RGH8* that could not be amplified in the PCR (marked with an *asterisk*) occupy the same position in the cluster. Finally, we assume, based on their 100% sequence identity, that *RH-RGH5* has the same position in the cluster as *SH-RGH6*. No relative order for *RH-RGH2*, *RH-RGH3* and *RH-RGH6* could be postulated

other data (see also below), we postulated the position of the RGHs as shown in Fig. 4.

Physical mapping of the *Gpa2/Rx1* cluster in SH

The BAC clones containing RGHs were used to construct a physical map of the *Gpa2/Rx1* cluster for both the *S. tuberosum* spp. *tuberosum* and the *S. tuberosum* spp. *andigena* haplotypes of SH. Genetic mapping revealed that all these RGHs are located on chromosome XII and, hence, the BAC inserts correspond with genomic fragments of this chromosomal region of the potato genome. *SH-RGH5* and *SH-RGH6* co-migrate with markers that are in repulsion with *Gpa2*, implicating that they are located on the homologous chromosome. Based on this information we could asign the origin of the BAC clones to any of the two haplotypes: BAC 43, BAC 85N51 and BAC 34F16 were derived from the homologous chromosome in *S. tuberosum* ssp. *andigena* introgression segment, and BAC clones 25G18, 7E16 and 36G3 were derived from *S. tuberosum* ssp. *tuberosum*. The BAC clones derived from *S. tuberosum* ssp. *andigena* formed a closed contig and could be easily aligned with the original physical map based on the presence and absence of *Gpa2*, *SH-RGH1* and *Rx1*, and the markers 73L, 111R, 111L, 221R, 45L, 77R and 77L (Kanyuka et al. 1999). In order to make a contig of the *S. tuberosum* ssp. *tuberosum* haplotype, the BAC ends were sequenced and primers were designed to perform PCR on the other BAC clones of this haplotype. Additionally, BACs 25G18 and 36G3

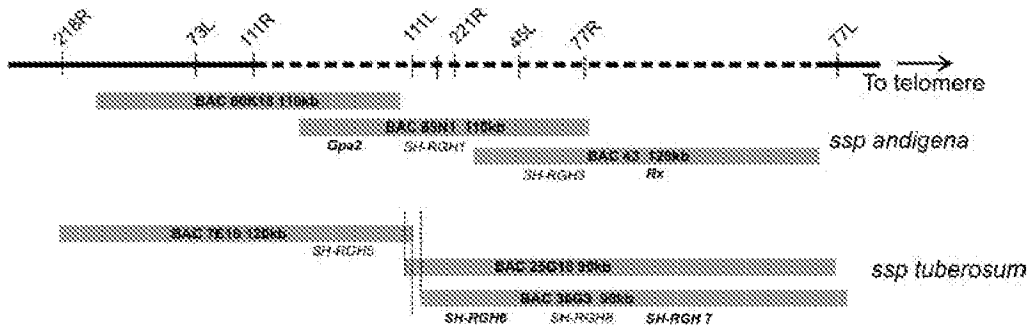


Fig. 5 Physical map of the *Gpa2/Rx1* cluster in the diploid potato clone SH. The region (187 kb) around the resistance genes *Gpa2*, *Rx1* and *SH-RGH1* and the pseudogene *SH-RGH3* on the haplotype derived from *S. tuberosum* ssp *andigena*, has been sequenced. On the *S. tuberosum* ssp. *tuberosum* haplotype four *Gpa2/Rx1* homologues are also present: *SH-RGH5*, *6*, *7* and *8*. Like *SH-RGH3*, the homologue *SH-RGH8* could not be identified by PCR, but was detected by Southern-blot analysis. The BAC clones are represented in *grey rectangles* and the RGHs are indicated alongside the BACs on which they were detected. RGHs are indicated in *bold* when intron positions are known. *Vertical lines* indicate the marker positions used to align the BAC contig from the *S. tuberosum* ssp. *tuberosum* haplotype with the *S. tuberosum* ssp. *andigena* introgression segment. *Vertical dotted lines* indicate an overlap between BAC ends. The *dotted horizontal line* in the *S. tuberosum* ssp. *andigena* chromosome indicates the position of the sequenced region of 187 kb

harbour the same RGHs. These data enabled us to construct a closed contig of these BAC clones (Fig. 5). CHEF electrophoresis of the BAC inserts (data not shown) showed that BAC 7E16 is approximately 120 kb. Both 36G3 and 25G18 are approximately 90 kb in size resulting in a physical map of *S. tuberosum* ssp. *tuberosum* of about 200 kb containing *SH-RGH5*, *6* and *7*.

Moreover, we were also interested in comparing the genomic organisation of the *Gpa2/Rx1* cluster derived from the *S. tuberosum* spp. *tuberosum* haplotype and the one derived from the *S. tuberosum* ssp. *andigena* introgression segment. A segment of 187 kb of the *S. tuberosum* ssp. *andigena* haplotype has previously been sequenced for cloning *Rx1* and *Gpa2* (Van der Vossen et al. 2000). This sequence information was used to align the BAC end-sequences of the BAC clones derived from *S. tuberosum* ssp. *tuberosum* with the contig of the *S. tuberosum* ssp. *andigena* haplotype. The approximate 80% sequence identity for the BAC ends of the left arms of BAC clones 7E16, 25G18 and 36G3 with regions between *Gpa2* and *SH-RGH1* led to the orientation of the BAC contig as proposed in Fig. 5. The order of the homologues present on BAC clones 25G18 and 36G3 is not clear. However, a comparison of intron positions between the RGHs, showed that *SH-RGH7* has similar intron positions as *Rx1* and *SH-RGH6* has not (Bakker et al., unpublished data). Therefore we presented the order of the homologues as depicted in Fig. 5.

Southern-blot analysis

To confirm the specificity of the *Gpa2/Rx1*-specific primer set, Southern-blot analysis was carried out at high stringency conditions on *Hin*dIII-digested DNA of the BAC clones from SH harbouring the RGHs (data not shown). Hybridisation with a 400-bp fragment derived from the 5'-end of the LRR domain of *Gpa2* resulted in a single band for 7E16 and four bands for 25G18 and 36G3. *SH-RGH7* has a *Hin*dIII site in the probe region which accounts for one of the two additional bands detected in 25G18 and 36G3. The other additional band indicates the presence of an extra RGH in the *Gpa2/Rx1* cluster in the haplotype derived from *S. tuberosum* ssp. *tuberosum*. Similar to the pseudogene *SH-RGH3*, this RGH is not amplified with the cluster-specific primer pair. Based on this information together with the information on the positions of BAC 25G18 and 36G3, we assume that this additional RGH (designated *SH-RGH8*) is also a pseudogene and that it is most-likely positioned between the homologues *SH-RGH6* and *SH-RGH7* (Figs. 4 and 5). It is noted that the sequence-homologous region in the haplotype of *S. tuberosum* ssp. *andigena* contains exactly the same number of homologues. In addition, a 185-bp fragment was used to determine the number of RGHs with an NBS domain. This probe reached 73 bp into the NBS domain and 70 bp into the LRR domain, and gave identical results as the LRR probe. These data show that all *Gpa2/Rx1* homologues identified in the PCR-based method also possess at least part of an NBS domain similar to *Gpa2* and *Rx1*.

The total number of *Gpa2/Rx1* homologues in the potato genome was also determined by Southern blotting on genomic DNA of SH and RH. Hybridisation with the LRR and NBS probe confirmed our previous data and resulted in the detection of eight bands for SH and six for RH. All the bands for SH were also present in the BAC clones harbouring the *Gpa2/Rx1* homologues. The observation that Southern blotting resulted in the same number of RGHs as the PCR approach indicates that no additional, slightly modified, RGHs are present in RH.

Discussion

In this paper, we describe the identification and mapping of nine resistance gene homologues (RGHs) of a single complex locus in four homologous chromosomes using a cluster-specific primer combination based on the LRR domain of *Gpa2* and *Rx1*. Eight of the nine RGHs could be mapped on chromosome XII in a single-step procedure using a modified AFLP method. Physical mapping revealed that the remaining homologue (*SH-RGH7*) was also located in the *Gpa2/Rx1* cluster on chromosome XII. The segregation of all six RH-RGHs in the mapping population shows that the two haplotypes of RH are heterozygous at the *Gpa2/Rx1* locus. This is confirmed by the fact that all six RH-RGHs have different sequences. Only two pairs of RGHs are (virtually) identical: SH-RGH5 and RH-RGH4 (99.9%), and SH-RGH6 and RH-RGH5 (100%). In both cases these identical RGHs are derived from different genotypes.

Gpa2 and *Rx1* are highly homologous. At the amino-acid level, they have a homology of 88% and at the nucleotide level their identity is even 93% (Van der Vossen et al. 2000). *SH-RGH1*, an RGH present on the same haplotype as *Gpa2* and *Rx1*, has also a similar sequence identity to the LRR domains of *Gpa2* and *Rx1*, respectively 93.6 and 93.5%. A complete open reading frame was detected for this putative *R* gene based on the 187-kb sequence of this region (Van der Vossen et al. 2000). This indicates that *SH-RGH1* could be a functional homologue of *Gpa2* and *Rx1* with unknown specificity. The nine RGHs identified in this study are all closely related to *Gpa2* and *Rx1* with sequence identities ranging from 93 to 95%. Southern analysis showed that all these RGHs have at least part of an NBS domain.

Our results indicate that all homologues are located in the *Gpa2/Rx1* cluster on chromosome XII. Surprisingly, in *S. aucaule* a functional *Gpa2/Rx1* homologue with the same specificity as *Rx1* is found (Bendahmane et al. 2000). This gene (*Rx2*), however, is genetically linked to RFLP marker Gp21 on chromosome 5 (Ritter et al. 1991; Bendahmane et al. 2000). Sequence identity between *Rx1* and *Rx2* is so high (97.9% for the complete genes and 99.4% for the LRR domains) that another *Rx1/Gpa2*-like cluster on chromosome V, if present in SH or RH, would certainly have been identified in this study. An explanation for this remarkable phenomenon, that highly homologous genes are located on different chromosomes in two closely related species, could be a recent translocation event after the speciation of *S. tuberosum* and *S. aucaule*. However, the synteny between the more distantly related species *S. tuberosum* and *Lycopersicon esculentum* is very high (Grube et al. 2000), indicating that such translocation events are rare within the genus *Solanum*.

In this paper, a PCR-based method has been used to identify *Gpa2/Rx1* homologues. Remarkably, some of the amplified RGHs gave conflicting results, among others, with regard to physical mapping. For example, PCR analyses of nearly completely overlapping BAC clones (25G18 and 36G3) resulted in totally different RGHs. Sequence alignments of these RGHs with other RGHs (two by two) revealed that they were the result of a chimaeric PCR product derived from two distinct RGHs. These artificial RGHs consisted of two extraordinary stretches of several-hundred nucleotides each. One stretch was 100% identical to one RGH and the other stretch was 100% identical to another, completely different, RGH. These results were obtained with BAC DNA and genomic DNA as well. Fortunately, chimaeric PCR products were typically less-frequent than genuine RGHs and occurred in most cases only once in a series of re-amplified colonies. Although PCR techniques are commonly used to identify and map RGHs, this phenomenon has to our knowledge not been described before in the literature.

Despite the importance of potato as a food crop and its vulnerability to various pests and diseases, only few *R* genes have been cloned. To facilitate cloning genes of interest, an Ultra High Dense genetic map comprising 10,000 AFLP markers has been constructed [http://www.dpw.wageningen-ur.nl/uhd/ (Van Os et al., unpublished)]. This has been accomplished by using the mapping population of SH and RH. However, not for all potato species that harbour interesting *R* genes, a dense genetic map and BAC library will become available. The production of these tools is still laborious and costly, and the possibility to dissect *R* gene clusters with specific primer combinations in different species is a promising alternative. Comparative analysis has shown that the genomes of members of the *Solanaceae* family have a large synteny (Grube et al. 2000). The results described in this paper indicate that characterising *R* genes from other potato (sub)species with PCR-based approaches may be feasible.

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The *R1* gene for potato resistance to late blight (*Phytophthora infestans*) belongs to the leucine zipper/ NBS/LRR class of plant resistance genes

Agim Ballvora[1], Maria Raffaella Ercolano[1], Julia Weiß[1], Khalid Meksem[2], Christina Angelika Bormann[1], Petra Oberhagemann[1], Francesco Salamini[1] and Christiane Gebhardt[1,*]

[1]*Max-Planck Institut für Züchtungsforschung, Carl von Linné Weg 10, 50829 Köln, Germany, and*

[2]*Southern Illinois University at Carbondale, Department of Plant, Soil and General Agriculture, Carbondale, IL62901-4415, USA*

Received 22 November 2001; revised 7 February 2002; accepted 15 February 2002

*For correspondence (fax: +49 221 5062 430; email: gebhardt@mpiz-koeln.mpg.de)

Summary

Late blight caused by the oomycete *Phytophthora infestans* is the most destructive disease in potato cultivation worldwide. New, more virulent *P. infestans* strains have evolved which overcome the genetic resistance that has been introgressed by conventional breeding from wild potato species into commercial varieties. *R* genes (for single-gene resistance) and genes for quantitative resistance to late blight are present in the germplasm of wild and cultivated potato. The molecular basis of single-gene and quantitative resistance to late blight is unknown. We have cloned *R1*, the first gene for resistance to late blight, by combining positional cloning with a candidate gene approach. The *R1* gene is member of a gene family. It encodes a protein of 1293 amino acids with a molecular mass of 149.4 kDa. The *R1* gene belongs to the class of plant genes for pathogen resistance that have a leucine zipper motif, a putative nucleotide binding domain and a leucine-rich repeat domain. The most closely related plant resistance gene (36% identity) is the *Prf* gene for resistance to *Pseudomonas syringae* of tomato. *R1* is located within a hot spot for pathogen resistance on potato chromosome V. In comparison to the susceptibility allele, the resistance allele at the *R1* locus represents a large insertion of a functional *R* gene.

Keywords: late blight (*Phytophthora infestans*), potato (*Solanum tuberosum*), resistance gene, positional cloning, candidate gene approach

Introduction

Late blight is the most destructive disease in potato cultivation worldwide, causing billion-dollar losses every year (Kamoun *et al.*, 2001). The causal pathogen is *Phytophthora infestans*, an oomycete which also infects tomatoes (Judelson, 1997). Complete destruction of the potato crop by late blight caused the 'Irish potato famine' in the middle of the 19th century (Salaman, 1985) and initiated the search for resistant plants. Single genes for resistance to late blight (*R* genes) were discovered nearly 100 years ago in *S. demissum*, a wild potato species indigenous to Mexico. However, introgression of *R* genes conferring race-specific resistance into potato cultivars provided only transient resistance to late blight, as new races rapidly overcame the *R* gene-mediated resistance (Fry and Goodwin, 1997; Wastie, 1991). Quantitative or field resistance to late blight has also been identified in wild potato species (Ross, 1986). This resistance is more durable than that mediated by *R* genes, but is difficult to move into cultivated varieties by crossing and phenotypic selection. Late blight is mostly controlled by the frequent application of fungicides which lose their efficiency by selection of fungicide-resistant isolates. Improving the genetic resistance to late blight is therefore a major issue in breeding new varieties of potato.

Several *R* genes originating from introgressions of *S. demissum*, *S. bulbocastanum* and *S. berthaultii* have been mapped to potato chromosomes using DNA markers (El-Kharbotly *et al.*, 1994; El-Kharbotly *et al.*, 1996; Ewing *et al.*, 2000; Leonards-Schippers *et al.*, 1992; Li *et al.*, 1998; Naess *et al.*, 2000). *R1* is located on potato chromosome V

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(Leonards-Schippers *et al.*, 1992) in a hot spot for resistance to various pathogens. Single genes for resistance to potato virus X have been mapped to the same region (De Jong *et al.*, 1997; Ritter *et al.*, 1991). This region also contains major quantitative trait loci (QTL) for resistance to late blight (Collins *et al.*, 1999; Leonards-Schippers *et al.*, 1994; Oberhagemann *et al.*, 1999) and the parasitic root cyst nematode *Globodera pallida* (Kreike *et al.*, 1994; Rouppe van der Voort *et al.*, 1997, 2000). The clustering of functional genes for qualitative and quantitative resistance to various pathogens suggests their evolution from common ancestors by local gene duplication followed by functional diversification (Gebhardt and Valkonen, 2001; Leister *et al.*, 1996; Leonards-Schippers *et al.*, 1994; Oberhagemann *et al.*, 1999). Molecular cloning of the *R1* gene should therefore facilitate study at the molecular level of several factors participating in the control of qualitative and quantitative resistance to various potato pathogens.

Here we report the molecular cloning of *R1*, a first gene for resistance to late blight that is located in the resistance hot spot on potato chromosome V. The gene was identified by a combined positional cloning and candidate gene approach. The molecular structure of the gene allows classification of *R1* among plant resistance genes containing a conserved nucleotide binding domain (NBS), a leucine-rich repeat domain (LRR) and a leucine zipper motif (Dangl and Jones, 2001; Ellis *et al.*, 2000).

Results

High-resolution genetic mapping of the R1 *locus*

A high-resolution genetic map of the 3 cM interval between RFLP markers *GP21* and *GP179* containing the *R1* locus (Leonards-Schippers *et al.*, 1992) has been constructed based on 15 plants with recombination events in the *GP21–GP179* interval (Meksem *et al.*, 1995). To further facilitate physical mapping of the *R1* locus, 16 additional recombinants in the same interval were selected from 588 new plants and tested for resistance to a *P. infestans* isolate with the corresponding avirulence factor *Avr1*. Together with the previous experiment, 31 recombinants were available from 1049 plants, corresponding to 3.0% recombination frequency between *GP21* and *GP179*. Recombination frequencies between *GP21* and *R1* and between *R1* and *GP179* were 2.2% and 0.8%, respectively (Table 1). Two further markers *SPUD237* and *AFLP1*, both mapping in the interval *GP21–GP179* (De Jong *et al.*, 1997; Meksem *et al.*, 1995) were positioned relative to the *R1* locus using the recombinants. These markers flanked *R1* at a genetic distance of one recombination event in 1049 plants (0.1 cM, Figure 1).

Table 1. Number of recombinant individuals in the intervals *GP21–R1, GP179–R1* and *GP21–GP179,* selected from 1049 plants of a segregating F1 population

	GP21–R1	*GP179–R1*	*GP21–GP179*
Number of recombinants	23	8	31
Recombinants with genotype *R1r1*	12	4	16
Recombinants with genotype *r1r1*	11	4	15
Recombination frequency (%)	2.2	0.8	3.0

Chromosome walking towards the R1 *locus and identification of candidate genes*

Marker *SPUD237* was used to probe the cosmid library. One positive clone CosS was identified. End-sequencing of the CosS insert generated a new marker that was, like *SPUD237,* separated by one recombination event (0.1 cM) from the *R1* locus. Screening the BAC library with this marker identified BAC clone BA100e13 (Figure 1). End-sequencing of the BAC insertion was used to develop a new PCR marker that mapped three recombination events distal to *R1*, thereby orienting BA100e13 relative to *R1* on the genetic map. Using as a probe the BA100e13 end proximal to *R1* for screening the BAC library, clone BA47f2 was identified. Both ends of BA47f2 were mapped relative to *R1*. The BA47f2 end overlapping BA100e13 was separated from *R1* by one recombination event. The proximal end co-segregated with *R1*, as did all genetically mapped ends of the BACs that extended the contig further (right part of Figure 1). The BA47f2 end that co-segregated with *R1* identified BAC clone BA27c1. The BA27c1 end not overlapping with BA47f2 identified clones BA122p13 and BA121o1. The end of BA121o1 that did not overlap with BA27c1 showed highly significant sequence similarity (37% identity, 56% similarity of translated amino acid sequence) to the tomato *Prf* gene for resistance to *Pseudomonas syringae* (Salmeron *et al.*, 1996). RFLP mapping using this resistance-gene-like (RGL) fragment as a marker probe on a mapping population that did not segregate for *R1* (Leister *et al.*, 1996) identified a small gene family with several members located on chromosome V in the *R1* region and at least one member located on chromosome XI (not shown). The RGL fragment was used as probe to re-screen the BAC library. In addition to BA122p13, several new positive clones were identified, two of which, BA87d17 and BA76o11, extended the contig on chromosome V (Figure 1). Both BACs contained full-length copies of the candidate gene family as judged from the end sequences which were unrelated to the RGL fragment. The end of BA87d17 that did not overlap with BAC BA121o1 still co-segregated with the *R1* locus (Figure 1).

The partially overlapping BACs in the contig shown in Figure 1 had insertions of between 70 and 100 kb, as

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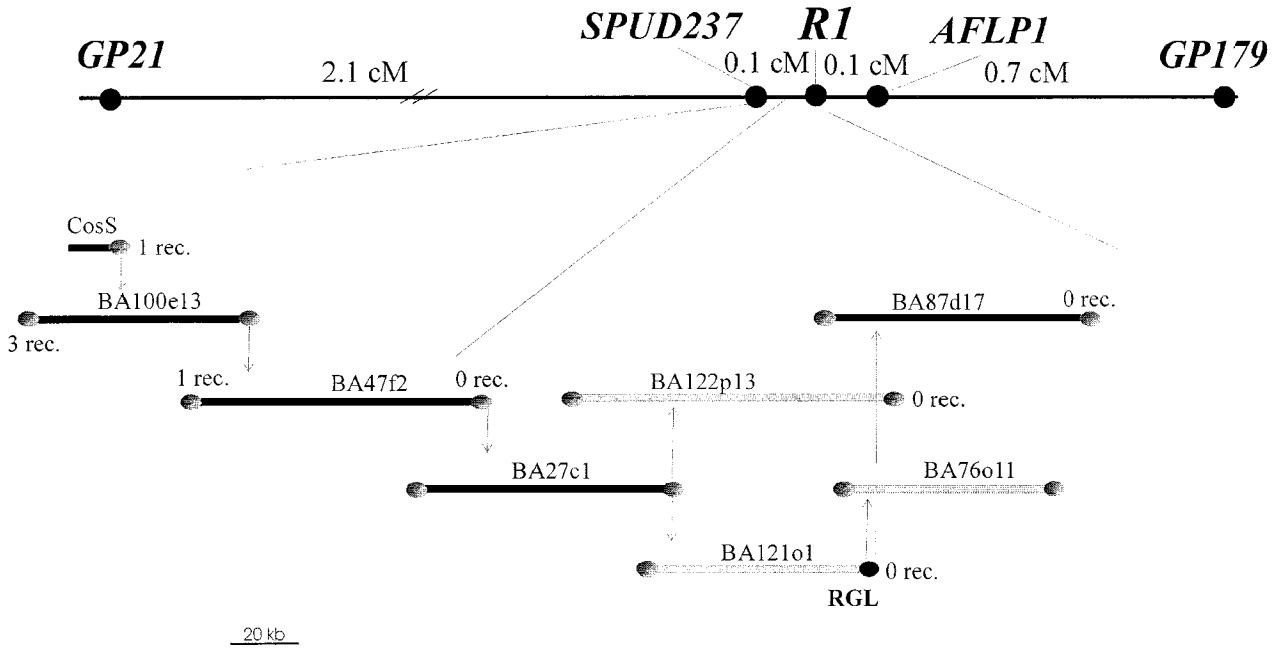


Figure 1. Genetic and physical map of the *R1* region.
GP21 and *GP179* are the markers used to construct the high-resolution map of the *R1* region. *SPUD237* and *AFLP1* are converted AFLP markers (De Jong *et al.*, 1997; Meksem *et al.*, 1995) flanking *R1*. Genetic distances are given in cM. CosS is a cosmid clone selected with *SPUD237*. Remaining clones in the physical map are BACs with lengths between 70 and 100 kb. Solid black bars: BACs from the chromosome carrying *R1*. Grey bars: BACs from the chromosome carrying *r1*. Mapped BAC ends are indicated by the number of recombinants separating the BAC end from *R1*. Cosmid and BAC ends used for chromosome walking are indicated by the vertical arrows. RGL, resistance-gene-like fragment.

estimated from BAC DNA digested with *Not*I and size-separated by pulsed-field gel electrophoresis. Based on the length of the individual BACs, the length of the physical map was estimated at 250–300 kb. The region co-segregating with the *R1* locus covered around 200 kb of the contig.

Line P6/210 from which the BAC library originated is heterozygous for the *R1* resistance allele (*R1r1*). BACs in the contig therefore corresponded to either the chromosome carrying the *R1* or the chromosome carrying the *r1* allele. The polymorphisms of BAC end markers that were instrumental in mapping and contig construction (data not shown) were used to assign BAC clones to either the *R1* or the *r1* allele. BAC clones BA100e13, BA47f2, BA27c1 and BA87d17 (Figure 1) were *in cis* with the *R1* allele, whereas clones BA121o1, BA122p13 and BA76o11 were derived from the homologue with the *r1* allele (Figure 1).

Of all BACs in the contig, clone BA87d17 was the best candidate for hosting *R1* because (i) it co-segregates with the *R1* locus, (ii) it contains at least one RGL, and (iii) it is derived from the *R1*-containing chromosome.

Complementation analysis

Complementation analysis of susceptible cultivar Desirée with whole BAC DNA confirmed that clone BA87d17 contained the *R1* gene (unpublished data). To identify the *R1* gene within the BAC insertion, a random genomic sub-library with, on average, 10 kb insertions was constructed from BA87d17. The library was screened by colony hybridization for clones containing the candidate gene. Positive clones were evaluated for the presence of a complete copy of the candidate gene (including the putative promoter region)by the size of the amplification products obtained by PCR with forward primers from the vector borders and reverse primers from the candidate gene (data not shown). Subclone g10 was selected, which had an insertion of about 10 kb and contained at least 2 kb sequence upstream and 4 kb downstream of the candidate gene. Subsequent sequence analysis confirmed the presence of a single open reading frame in g10. Clone g10 was transformed into *Agrobacterium tumefaciens* and three different bacterial colonies were used to transform the susceptible cultivar Desirée. From three transformation experiments, 15 independent transgenic lines were regenerated and tested in four independent experiments for expression of hypersensitive resistance to *P. infestans* race 4 (*Avr1*) (Table 2). Nine transgenic lines consistently showed a typical HR response, similar to the resistant line P41 hosting *R1* (Figure 2); three lines gave inconsistent results and the remaining three were susceptible, like the untransformed Desirée control. When transgenic lines that expressed HR upon inoculation with *P. infestans* race 4 (*Avr1*) were infected with race 1,4 (*avr1*)

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Table 2. Test for resistance to *P. infestans* race 4 of transgenic potato lines transformed with clone g10

Transgenic	Line number	Resistance[c]
10-2[a]	1[b]	S
10-2	2	R
10-2	3	R
10-2	4	R
10-5	1	R
10-5	2	S
10-5	3	nd
10-5	4	nd
10-5	5	R
10-23	1	nd
10-23	2	R
10-23	3	R
10-23	4	R
10-23	5	R
10-23	6	S

Transgenic lines were tested in four independent experiments with three leaflets from each line for expression of hypersensitive resistance to *P. infestans* race 4
[a]Numbers represent three different transformation experiments; [b]Numbers refer to each plant regenerated from independent calli; [c]Plants were scored as resistant if HR was visible 8–10 days after inoculation with *P. infestans* race 4; R, resistant; S, sensitive; nd, resistance status not determined as lines showed inconsistent results.

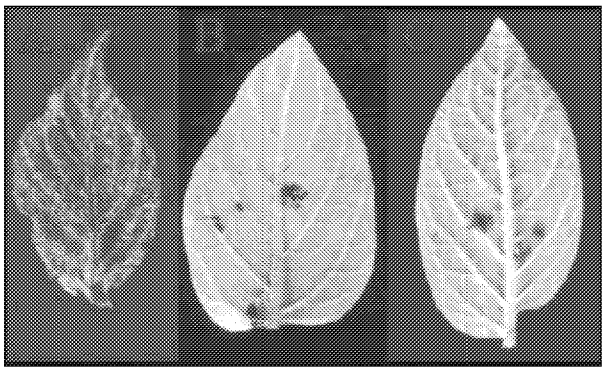


Figure 2. *R1* complementation test.
Disease symptoms are shown 9 days post-inoculation on leaflets from susceptible cultivar Desirée (A), transgenic Desirée line no. 10-5-5 transformed with clone y10 (B), and the resistant parent P41 (*R1r1*) (C).

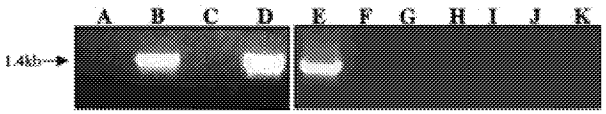


Figure 3. *R1*-specific PCR.
PCR amplification of a 1.4 kb fragment of the *R1* gene using allele-specific primers 76-2sf2 and 76-2SR and template DNA of (A) Desirée; (B) resistant parent P41; (C) susceptible parent P40; (D) transgenic Desirée plant 10-5-5; (E) BAC clone BA87d17 carrying the *R1* allele; (F–J) BAC clones BA122p13, BA12101, BA76011, BA47F2 and BA27c1, respectively; (K) negative control.

they were susceptible (not shown). The candidate gene present in clone g10 was therefore necessary and sufficient to transfer the HR response upon infection with *P. infestans* to the susceptible cultivar Desirée. The observed response was as expected for the *R1* gene according to the nomenclature of Black *et al.* (1953). As line P41 from which the transgene originated carried only the *R1* gene (Leonards-Schippers *et al.*, 1992), we concluded that the candidate gene present in g10 corresponded to *R1*.

All transgenic lines that showed the HR response contained the gene corresponding to cDNA 76-2 (see below), as demonstrated by the presence of a 1.4 kb PCR product amplified by the sequence-specific primers 76-2sf2 and 76-2SR. This product was absent in untransformed Desirée (Figure 3).

Candidate cDNA clones

The whole insertions of BACs BA121o1 and BA76o11 both containing copies of the candidate RGL family were used to screen a cDNA library prepared from infected leaves of genotype P41 (*R1r1*). Fourteen cDNA clones were isolated, eight of which were, based on DNA sequence analysis, similar to known plant resistance genes. The highest similarity was obtained with the tomato *Prf* gene for resistance to *Pseudomonas syringae* (Salmeron *et al.*, 1996). The sequences of the eight candidate cDNAs shared approximately 80–90% identity among each other. The sequence of one cDNA clone, c76-2, was 2292 nucleotides long and was identical, with the exception of the introns, to the sequence of the genomic clone g10 used for complementation analysis, thus indicating that the gene present in clone g10 was functional and expressed in leaves infected with an incompatible race of *P. infestans*. Sequence comparison to known resistance genes in the database indicated that c76-2 was not full-length. Using RACE analysis, the cDNA was extended at the 5′ end by 1943 nucleotides, resulting in a full-length cDNA sequence of 4235 nucleotides including a 5′ untranslated region of 59 nucleotides and 297 nucleotides of 3′ untranslated sequence between the stop codon and the poly(A) tail. The cDNA included a start codon at position 2223 of the genomic sequence corresponding to the first methionine in the amino acid sequence deduced from the genomic clone g10 (Figure 4b). Two adenines were identified at positions –3 and +4 (with the A of the ATG initiation codon at position +1), referred to as the ribosome recognition sequence in plants, insects, yeast and mammals (Kozak, 1991).

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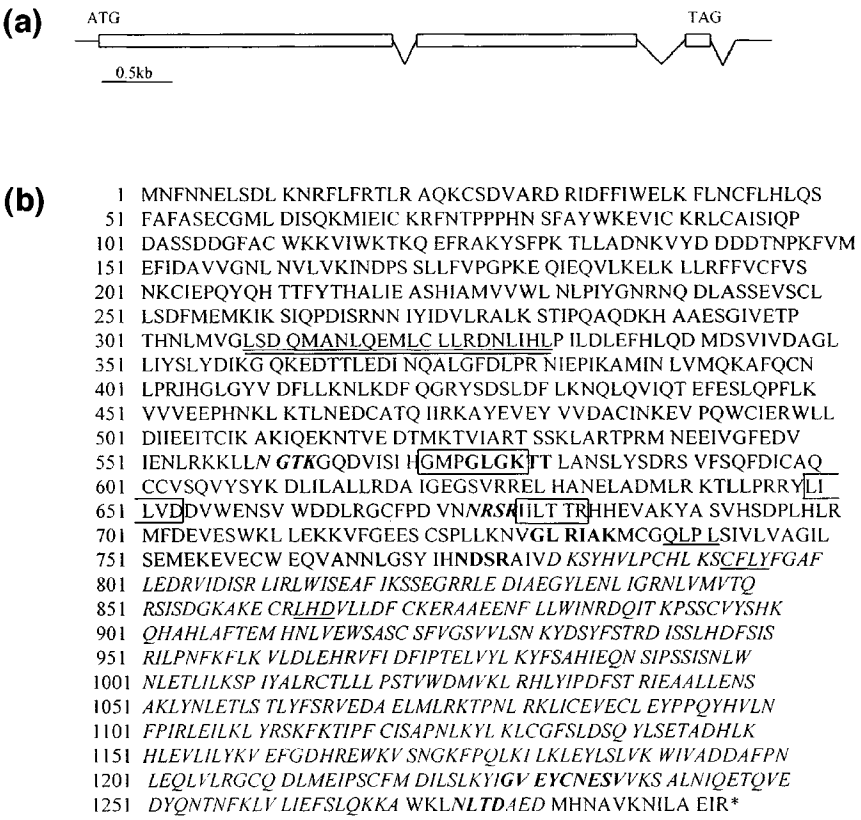


Figure 4. The *R1* gene.
(a) Structural organization. Exons are shown as boxes and introns as angled lines. (b) The deduced amino acid sequence. The leucine zipper motif is underlined twice. The LRR region is indicated in italics. The predicted kinase motifs are indicated inside the boxed regions and N-glycosylation sites are indicated in bold. The conserved motifs QLPL, CFLY and LHD specific for plant resistance proteins are underlined.

PCR primers specific for cDNA c76-2 were designed based on sequence alignment with the other seven candidate cDNAs (not shown). Primers 76-2sf2 and 76-2SR (see Experimental procedures) generated a 1.4 kb PCR product that was present in the diploid line P41 (*R1r1*), the source of the *R1* gene (Leonards-Schippers *et al.*, 1992), in BAC clone BA87d17 hosting *R1*, and in all g10 transgenic plants showing hypersensitive resistance. The 1.4 kb fragment was not amplified in the susceptible genotypes P40 (*r1r1*) and cultivar Desirée (*r1r1r1r1*). The product was also absent in the other BACs in the contig (Figure 3).

Structure of the R1 *gene*

The sequence of the complementing genomic clone g10 (GenBank accession no. AF447489) was 10 388 nucleotides long. It contained one open reading frame (ORF) with sequence similarity to other plant resistance genes, and included 2222 base pairs of the putative promoter region (Figures 4 and 5). No other ORFs or sequence homology were identified in the public databases. Sequence alignment with cDNA c76-2 and the 5′ RACE product revealed the presence of three exons and three introns. Two introns of 92 bp (positions 4878–4970) and 126 bp (positions 6103–6229) interrupt the coding region. The third intron of 81 bp (positions 6323–6404) is located in the 3′ untranslated region immediately downstream of the stop codon (Figure 4a). The deduced amino acid sequence suggests a polypeptide of 1293 amino acids with a molecular mass of 149.4 kDa (Figure 4b). The predicted R1 protein has a putative nucleotide binding site (NBS) domain consisting of P-loop (amino acids 572–578), kinase 2 (amino acids 649–653) and kinase 3a (amino acids 677–682) motifs (Figure 4b). Downstream of the kinase motifs were other sequences with similarity to domains of unknown function conserved among resistance genes: GLPL (QLPL in *R1*), CKLY (CFLY in *R1*) and MHD (LHD in *R1*). Searching for conserved motifs by using the ExPASY algorithm, four myristylation, nine glycosylation, 43 phosphorylation and one amidation putative sites were found in the deduced R1 amino acid sequence. The putative leucine-rich repeat (LRR) domain of R1 has 15/16 imperfect repeats located in the C-terminal part of the gene. Like some other plant R proteins with cytoplasmic LRRs, the deduced R1 protein contains a leucine zipper motif at amino acid positions 308–329, which may fold into a coiled-coil structure (Hammond-Kosack and Jones, 1997; Lupas, 1996). The deduced amino acid sequence of the *R1* gene is most similar (36% identity) to the *Prf* gene for resistance to *P. syringae* of tomato (Salmeron *et al.*, 1996). The sequence similarity extends throughout the deduced polypeptide sequences with the exception of the first 240 and 785

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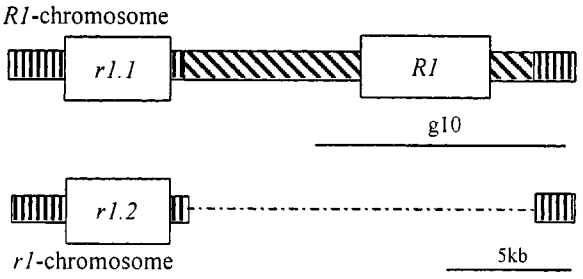


Figure 5. Schematic representation of the potato chromosome V region around the *R1* locus.
Boxes filled with vertical lines represent regions homologous between the chromosomes bearing the *R1* and *r1* alleles. The functional *R1* allele and the neighbouring locus with the *r1.1* and *r1.2* alleles are shown as open boxes. The boxes with diagonal lines including the *R1* gene show the 15 kb insertion present on the RI chromosome when compared to the *r1* chromosome (deletion marked with dashed line). The genomic clone g10 (10 kb) used for functional complementation is shown relative to the *R1* gene as straight line.

N-terminal amino acids of *R1* and *Prf*, respectively. Like *Prf*, *R1* has all sequence characteristics of coiled coil (CC)-NBS-LRR genes, including a highly conserved tryptophan at position 656 and a second putative coiled-coil domain between amino acids 536 and 559, corresponding to amino acids 1087–1110 of *Prf* (Pan *et al.*, 2000).

Genomic organization of the R1 *locus*

DNA sequence analysis of BACs BA87d17 (*R1*) and BA122p13 (*r1*) (unpublished data) revealed that BA87d17 contained two highly similar members of the *R1* gene family, one corresponding to the expressed functional *R1* gene and the second one being allelic with a single member present in BA122p13. The functional *R1* gene was part of a 15 kb insertion present in the *R1*-bearing chromosome in the region covered by BA87d17, but absent in the chromosome bearing *r1* (Figure 5).

Discussion

R1 was cloned by using a positional cloning strategy in combination with searching for candidate genes with DNA sequence similarity to known plant resistance genes (Ellis *et al.*, 2000; Hammond-Kosack and Jones, 1997). A similar approach was successful in cloning potato genes for resistance to potato virus X (*Rx1*, Bendahmane *et al.*, 1999) and the root cyst nematode *Globodera pallida* (*Gpa2*, Van der Vossen *et al.*, 2000).

Complementation analysis showed that one of two candidate genes present in BAC clone BA87d17 was able to transfer a typical HR response to the susceptible cultivar Desirée in 60% of all transgenic plants tested. Failure of the transgene to express the HR response may have been due to position effects, co-suppression or rearrangements after transformation, and has also been observed in other complementation experiments with resistance genes (Grant *et al.*, 1995; Whitham *et al.*, 1994).

The HR response after transformation of the susceptible cultivar Desirée was observed upon infection with a *P. infestans* strain incompatible with *R1*. Genetic analysis using compatible and incompatible races of *P. infestans* had demonstrated earlier that, of the 11 known *R* genes introgressed from *S. demissum* (Black *et al.*, 1953), only *R1* is present in the interval *GP21–GP179* on chromosome V of the parental clone P41 from which the cloned *R1* gene originated (Leonards-Schippers *et al.*, 1992). At the molecular level, however, there is at least one additional member of the *R1* gene family that also co-segregates with the *R1* locus. Further paralogous members may be located in the region between *R1* and marker *AFLP1* that is not covered by the physical map. There is the possibility that the *R1* locus hosts more than one *R* gene with the same race specificity, or, in addition to *R1*, other *R* genes with so far unknown specificities. Further complementation studies are required to elucidate the function of the other members of the *R1* family in resistance to *P. infestans* and other pathogens.

Based on the deduced protein sequence, *R1* is a member of the leucine zipper/NBS/LRR class of plant resistance genes (Hammond-Kosack and Jones, 1997). The leucine zipper motif in the N-terminal region is thought to feature in dimerization or interaction with other proteins. Leucine zippers may participate in a coiled-coiled secondary protein structure (Lupas, 1996). Plant R proteins containing these structural elements are referred to as the CC-NBS-LRR class to distinguish them from the class of NBS-LRR resistance genes with a Toll/interleukin receptor domain at the N-terminal part (TIR-NBS-LRR; Pan *et al.*, 2000; Young, 2000). The downstream putative NBS domain may be involved in the signal transduction pathway leading to the onset of the resistance response. The C-terminal LRR domain matches the consensus sequence for a cytoplasmic LRR domain as described by Jones and Jones (1997) and may function in protein–protein interactions and ligand binding. It has been shown that the LRR domains of alleles of the flax rust resistance gene *L* determine recognition of specific races of the pathogen (Ellis *et al.*, 1999). Prediction *in silico* of four myristylation and 43 phosphorylation sites in the *R1* sequence suggests a possible anchoring of the R1 protein in the plasma membrane and phosphorylation/dephosphorylation steps, respectively, participating in signal transduction (Dangl and Jones, 2001).

Upon infection of potato with the hemibiotrophic oomycete *Phytophthora infestans,* hypersensitive cell death occurs only when growth of the pathogen proceeds to the formation of a clearly identifiable haustorium that is separated from the plant cell cytoplasm by the haustorial

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and plasma membranes (Freytag *et al.*, 1994). The putative cytoplasmic localization of the R1 protein implies that the avirulence factor that interacts with R1 is translocated from the haustorium into the cytoplasm. In the comparable pathosystem of rice and the hemibiotrophic rice blast fungus *Magnaporthe grisea*, it has been shown that only the processed form of the extracellular avirulence protein AVR-Pita (Orbach *et al.*, 2000) interacts inside the host plant cell with the corresponding cytoplasmic resistance protein Pi-ta (Jia *et al.*, 2000). Alternatively, the R1 protein may be anchored intracellularly to the plasma membrane via a myristylation site and interacts directly or indirectly with the so far unknown product of the *Avr1* gene of *Phytophthora infestans*.

R1 is located on the short arm of chromosome V (Dong *et al.*, 2000; Leonards-Schippers *et al.*, 1992) and shows greatest sequence similarity to the tomato *Prf* gene for resistance to *P. syringae* that is located on tomato chromosome 5 within the *Pto/Fen* resistance gene cluster (Salmeron *et al.*, 1996). Chromosomes five of potato and tomato are co-linear with each other except for a paracentric inversion of the short arm (Tanksley *et al.*, 1992). The potato locus *StPto* corresponding to the tomato *Pto/Fen/Prf* gene cluster maps more than 10 cM proximal to *R1* (Leister *et al.*, 1996), excluding the possibility that *R1* and *Prf* occupy orthologous positions in the two genomes. This may be the case, however, when considering the tomato *Bs4* gene conferring resistance to the bacterial pathogen *Xanthomonas campestris* pv. *vesicatoria*. The position of the potato locus corresponding to tomato *Bs4* may be inferred from comparing the molecular maps of chromosomes five of potato and tomato based on common RFLP anchor markers (Ballvora *et al.*, 2001; Tanksley *et al.*, 1992).

Two potato genes for resistance to potato virus X, *Rx2* and *Nb*, map to similar positions as *R1* (De Jong *et al.*, 1997; Leonards-Schippers *et al.*, 1992; Ritter *et al.*, 1991). The *Rx2* gene has been cloned and is, like *R1*, a member of the leucine zipper/NBS/LRR class of resistance genes (Bendahmane *et al.*, 2000). The two resistance genes share 32% sequence identity and are therefore different members of the same super-family of genes. *Nb* is located in the interval *GP21–SPUD237* (De Jong *et al.*, 1997) not containing *R1*, and is therefore genetically separated from *R1*.

The resistance hot spot on potato chromosome V that includes *R1* also contains major QTL (quantitative trait loci) for resistance to *Phytophthora infestans* (Collins *et al.*, 1999; Leonards-Schippers *et al.*, 1994; Oberhagemann *et al.*, 1999) and the root cyst nematode *Globodera pallida* (Kreike *et al.*, 1994; Rouppe van der Voort *et al.*, 1997; Rouppe van der Voort *et al.*, 2000). Linkage disequilibrium mapping revealed strong association of markers in the 0.8 cM interval *SPUD237–GP179* containing *R1*, with the resistance of foliage and tubers to late blight (unpublished data), supporting the tight linkage between *R1* and factors controlling quantitative resistance to late blight. It has been suggested, based on the observed genetic linkage, that *R1* and the factors controlling quantitative resistance to late blight may be alleles of the same gene or members of a clustered gene family (Leonards-Schippers *et al.*, 1994; Oberhagemann *et al.*, 1999). The molecular analysis of the *R1* locus supports the latter option as *R1* is a member of a gene family with at least one additional member physically closely linked to *R1*. Further members might be present in those parts of the *SPUD237–GP179* interval not yet covered by the physical map and/or in other parts of the potato genome. Now that the *R1* sequence is available, those members can now be identified and characterized for structure and function. Allelic variants of *R1* in *S. tuberosum* and homologues in other Solanaceae species, that are involved in quantitative resistance to *P. infestans*, may be isolated.

R1 is present as an extra copy in a DNA insertion in the *R1*-bearing chromosome when compared to the *r1* chromosome. A similar finding has been reported for the *Rpm1* locus in *Arabidopsis* (Stahl *et al.*, 1999). The *R1* gene should have been introgressed into the *S. tuberosum* genome from the wild species *S. demissum* through heterogenetic chromosomal crossing-over. In crosses between wild and cultivated *Solanum* species, heterogenetic chromosome pairing is frequently found (Singh *et al.*, 1989).

The interaction between *R1* and the late blight pathogen is in accordance with the gene-for-gene concept (Flor, 1971; Person *et al.*, 1962). Transfer of a single gene was sufficient to elicit in a susceptible host plant the hypersensitive resistance response upon infection with a *P. infestans* race carrying the avirulence gene *Avr1*. *Avr1* segregates as single dominant factor in offspring of *P. infestans* strains heterozygous for *Avr1*, and was mapped to linkage group IV of the *P. infestans* molecular map (Van der Lee *et al.*, 2001). Further characterization of *R1* at the molecular level and cloning of the *Avr1* gene should allow clarification of how the resistance protein recognizes the avirulence effector molecule. Cloning of late blight resistance genes that recognize avirulence factors different from *Avr1* may allow identification of the molecular motifs that determine the specificity of effector recognition, and may help identification or engineering of R proteins with broader and more durable resistance to late blight.

Experimental procedures

Plant material

F1 offspring of a cross between the heterozygous diploid clones H79.1506/1 (*R1r1*) and H80.696/4 (*r1r1*), referred to as P41 and P40,

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respectively (Gebhardt *et al.*, 1989; Leonards-Schippers *et al.*, 1992), was used for high-resolution genetic mapping of *R1*. Recombinants in the marker interval *GP21–GP179* originating from the P41 (*R1r1*) parent were selected as described previously (Meksem *et al.*, 1995). Hybrid P6/210 derived from the cross P41 × P40 (Leister *et al.*, 1996) which carries *R1* in the heterozygous state was used for constructing genomic cosmid and BAC libraries. Parent P41 (*R1r1*) was used for cDNA library construction.

Test for resistance to Phytophthora infestans

Resistance to *P. infestans* race 4 (*Avr1*) and race 1,4 (*avr1*) (kindly provided by Dr Francine Govers, Wageningen University, The Netherlands) was determined as described previously (Leonards-Schippers *et al.*, 1992), except that whole leaflets instead of leaf disks were used for inoculation. The presence or absence of a hypersensitive response (HR) was scored 8–10 days post-inoculation.

Potato genomic libraries

The BAC library was supplied by LION Bioscience AG (Heidelberg, Germany). The library was constructed from *Hin*dIII partially digested high-molecular-weight genomic DNA of the potato genotype P6/210 in the binary vector pCLD04541 (Jones *et al.*, 1992) as described previously (Meksem *et al.*, 2000). The BAC library consists of approximately 100 000 clones with an average insert size of 70 kb. With the size of a haploid potato genome being approximately 10^9 base pairs, the genome coverage was 6–7 times per haploid genome. The colonies were stored in 264 384-microtitre plates (Genetix, Oxford, UK) in 2YT medium (Sambrook *et al.*, 1989) with freezing buffer (5.5% w/v glycine, 7 mM $(NH_4)SO_4$, 1.5 mM sodium citrate, 0.3 mM $MgSO_4$, 13 mM KH_2PO_4, 27 mM K_2HPO_4).

A cosmid library of approximately 150 000 clones was constructed using standard procedures (Sambrook *et al.*, 1989) from *Sau*3AI partially digested genomic DNA (17–23 kb fragments) of P6/210 in the same vector (*Bam*HI cloning site) as the BAC library. Cosmids were packaged using Gigapack II Gold Packaging extract (Stratagene, California, USA) and transfected into *E. coli* strain SURE (Stratagene). Plasmid DNA was extracted from pools of about 1500 bacterial colonies (Sambrook *et al.*, 1989). One hundred and three cosmid pools were generated and screened by PCR using *SPUD237*-specific primers (De Jong *et al.*, 1997). Positive pools were plated and screened by colony hybridization using standard protocols (Sambrook *et al.*, 1989).

BAC library screening and contig construction

High-density colony filters for hybridization-based screening of the BAC library were prepared using a BioGRID robot (Oxford, UK). Clones were gridded in double spots using a 5 × 5 array with 6 × 384 arrays per 22.5 × 22.5 cm nylon membrane (PALL, Biodyne, Portsmouth, UK). Each 5 × 5 array contained 2 × 12 colonies with the control position of the array occupied by the clone pSW1 (PE Biosystems, Foster City, California, USA). This gridding pattern allowed 27 648 colonies to be represented twice on each filter. Library screening was performed using a set of four filters carrying 101 376 clones. Colony filters were incubated on LB medium for 15 h at 37°C and processed for colony hybridization using standard techniques (Sambrook *et al.*, 1989). Filter hybridization was performed as described previously (Gebhardt *et al.*, 1989), except that 300 pg of pSW1 control insert were labelled and hybridized together with the probe to facilitate the determination of addresses of positive clones. Plasmid DNA was purified from positive clones and insertions were sequenced from both ends using T3 and T7 oligonucleotides as sequencing primers. DNA sequence information for BAC insertion ends was used to design specific PCR primer pairs. PCR products amplified with these primers and the respective BACs as template were used as probes for a new filter hybridization to identify overlapping BAC clones, for orientation of overlapping BAC clones relative to each other, and for mapping in the recombinant plants. Overlaps were confirmed by sequencing the PCR products. Direction of contig extension was verified by genetic mapping using the recombinant plants and RFLP- or PCR-based marker analysis. To determine the size of BAC insertions, the BAC DNA was digested with *Not*I and the fragments were separated by pulsed-field gel electrophoresis on a CHEF DRIII (Bio-Rad, Hercules, California, USA) for 12 h at 11°C with an initial pulse time of 5 sec and a final pulse time of 10 sec, at a 120° angle and 6 V cm^{-1}.

BAC DNA isolation

BAC DNA was extracted using QIAfilter Plasmid Purification Kit 100 (Qiagen, Hilden, Germany) according to manufacturer's instructions with minor modifications. A single colony was pre-cultured in liquid LB medium for 8 h at 37°C. A 75 µl aliquot of pre-culture was added to 75 ml LB medium and further incubated for 15 h at 37°C. A centrifugation step was introduced before passing the supernatant through the QIAfilter to remove bacterial cellular debris.

Preparation of probes from BAC insertions

A 1.5 µg aliquot of BAC DNA was digested to completion with *Hin*dIII plus *Ea*R1 and separated from the vector on 0.8% low-melting-temperature agarose (Sea Plaque GTG Agarose, Bioproducts, Rockland, Maine, USA). Inserted DNA was dissolved from the gel using the GELase system (Epicentre Technologies or Biozym) following the manufacturer's instructions. The DNA was ethanol-precipitated, dissolved in water and labelled with ^{32}P-dCTP by random primed labelling (Feinberg and Vogelstein, 1984).

Subcloning of BAC BA87d17

A 10 µg aliquot of BAC DNA was partially digested with 1 U *Tsp*509I for 15 min at 65°C and size-separated on a 0.8% low-melting-temperature agarose gel (Sea Plaque GTG Agarose). Fragments of about 10 kb in size were eluted using the GELase system (Epicentre Technologies, Madison, Wisconsin, USA), following the manufacturer's instructions. The purified fragments were cloned into the pCLD04541 binary vector linearized with *Eco*RI, dephosphorylated using SHRIMP phosphatase (Roche, Mannheim, Germany) and transformed into *E. coli* strain DH10B (Life Technologies, Rockville, MD, USA). Two hundred recombinant colonies were picked into microtitre plates.

CDNA library construction and screening

Cut shoots of approximately 8-week-old plants of parent P41 (*R1r1*) and of the susceptible cultivar Desirée were infected with *P.*

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infestans race 4 and maintained under a glass cylinder (to increase humidity) in water in a growth chamber at 17°C with 16 h light. Under these conditions, leaves of the susceptible control were overgrown by *P. infestans* mycelium after 8 days. Equal amounts of uninfected leaves of parent P41 and infected leaves 2 and 19 h and 3, 7 and 9 days after inoculation were collected. Poly(A)$^+$ RNA was isolated using the RNeasy Plant Mini Kit and the Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A λZAP II cDNA library (Stratagene) was constructed from the poly(A)$^+$ RNA according to the manufacturer's instructions. The different cDNA preparations were pooled prior to ligation into the λZAP vector. Approximately 5×10^5 plaque-forming units were plated and screened by plaque hybridization (Sambrook *et al.*, 1989) using as probe the insertions of BACs BA121o1 and BA76o11.

RACE analysis

Total RNA was isolated from uninfected leaf tissue of P41 (*R1r1*) using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. RACE analysis was performed with 1 μg total RNA using the SMART™ Race cDNA Amplification Kit (Clontech, California, USA) following the manufacturer's instructions. The nested gene-specific primers used for the PCR amplification were first RT1-1 (5′-AAACCCGGTGTTCCAAATCTAAC-ACT-3′) and second RT2-1 (5′- CATGTAGTGAGGATATG-TCACGAGTG-3′). The final PCR products of the RACE reaction were cloned into pGEM-T vector (Promega, California, USA). Two independent clones were sequenced.

DNA sequence analysis

Custom DNA sequencing was performed by the ADIS unit at the Max Planck Institute for Breeding Research. The dideoxy chain-termination sequencing method was employed using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit and an ABI377 automated DNA Sequencer (PE Biosystems, Foster City, California, USA).

DNA sequence analysis was performed using the Wisconsin Package, version 10.0 (Genetics Computer Group (GCG), Madison, Wisconsin, USA). Sequence databases were searched with BlastX and other algorithms available through the National Center for Biotechnology Information (Bethesda, Maryland, USA) and the ExPASY www server (Appel *et al.*, 1994).

Transformation of Agrobacterium tumefaciens

Subclone g10 of BAC BA87d17 was electroporated into *A. tumefaciens* strain LBA4404 according to the method described by Wen-jun and Forde (1989). Three *Agrobacterium* strains, LBAg10-2, LBAg10-5 and LBAg10-23, were used for potato transformation.

Agrobacterium tumefaciens*-mediated potato transformation and analysis of transgenic plants*

The susceptible potato cultivar Desirée was used in all transformation experiments. *Agrobacterium tumefaciens*-mediated transformation was performed as described by Rocha-Sosa *et al.* (1989), except that the MS medium contained 250 mg l^{-1} claforan. Kanamycin-resistant transgenic plants were tested by PCR for the presence of the g10 insert using the insert-specific primers 87e (5′-ATTACAATGGGTTGAACTCAG-3′) and 87s (5′-ACCTCTTTC-AATTGTTCTGGTG–3′). PCR conditions were: annealing at 55°C for 45 sec and polymerization at 72°C for 60 sec. Transgenic plants were screened with the *R1*-specific primers 76-2sf2 (5′-CACTCGTGACATATCCTCACTA-3′) and 76-2SR (5′-CAACCC-TGGCATGCCACG-3′) derived from cDNA c76-2. PCR conditions were: annealing at 55°C for 45 sec and polymerization at 72°C for 90 sec. Tests for resistance to *P. infestans* race 4 were performed using three leaflets per plant in each test.

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The *Rx* Gene from Potato Controls Separate Virus Resistance and Cell Death Responses

Abdelhafid Bendahmane,[1] Konstantin Kanyuka,[1] and David C. Baulcombe[2]

Sainsbury Laboratory, John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, United Kingdom

***Rx*-mediated extreme resistance against potato virus X in potato does not involve a necrotic hypersensitive response at the site of initial infection and thereby differs from the more usual type of disease resistance in plants. However, the Rx protein is structurally similar to products of disease resistance genes conferring the hypersensitive response. We show in both *Nicotiana* spp and potato that *Rx* has the potential to initiate a cell death response but that extreme resistance is separate and epistatic to necrosis. These data indicate that cell death and pathogen arrest are separate disease resistance responses in plants.**

INTRODUCTION

Rx-mediated resistance against potato virus X (PVX), like that controlled by many disease resistance (*R*) genes, can be described in terms of an elicitor–receptor model. According to this model, there are separate stages in the process involving pathogen recognition and the plant's response (Staskawicz et al., 1995). In *Rx*-mediated resistance, the recognition stage involves an interaction between *Rx* (Bendahmane et al., 1997), which is thought to encode the receptor, and the PVX coat protein (CP), which is the elicitor (Bendahmane et al., 1995). The response stage consists of mechanisms able to suppress accumulation of viruses, including those that are taxonomically unrelated to PVX (Köhm et al., 1993; Bendahmane et al., 1995).

Although the *Rx*-mediated mechanism is consistent with the elicitor–receptor model, the *Rx* response is distinct from that of other well-characterized *R* genes. The most striking feature of *Rx*-mediated resistance is the rapid arrest of PVX accumulation in the initially infected cell (Köhm et al., 1993). Unlike other disease resistance responses, this extreme resistance is not associated with a hypersensitive response (HR) at the site of inoculation. In addition, *Rx*-mediated resistance is active in protoplasts via mechanisms that either suppress virus replication or promote degradation of the viral RNA (Adams et al., 1986; Köhm et al., 1993; Bendahmane et al., 1995). In contrast, the HR type of resistance is not expressed in isolated protoplasts (Otsuki et al., 1972; Adams et al., 1985; Baulcombe et al., 1994). It is thought that expression of the HR type of viral resistance mechanisms requires cell-to-cell contact and is a tissue-related phenomenon (Adams et al., 1986).

With the exception of the *Rx* response, it is not clear whether an HR is an essential component of disease resistance mechanisms in plants. One view is that cell death removes the substrate for growth of biotrophic pathogens. Alternatively, the dying cells may be able to release signals that are themselves antibiotics or disinfectants (Lamb and Dixon, 1997). This latter view is supported by earlier studies on tobacco *N* gene–mediated resistance to tobacco mosaic virus (TMV). TMV particles could be found in cells surrounding the necrotic HR lesion, even when lesion expansion had stopped (Da Graça and Martin, 1976). Several candidates have been identified for the putative cell death–associated signals, including reactive oxygen species (Levine et al., 1994; Jabs et al., 1996) and nitric oxide (Delledonne et al., 1998). These signals have also been implicated in apoptotic cell death in animals (Schmidt and Walter, 1994).

In contrast, the phenotype resulting from the *dnd1* mutation in Arabidopsis indicates that cell death is separate and independent of the resistance mechanisms leading to suppression of the pathogen. The phenotype of the *dnd1* mutant is loss of the HR without loss of disease resistance (Yu et al., 1998). Also consistent with separation of HR and disease resistance are the effects of humidity and temperature that can inhibit the HR response without compromising resistance (Baulcombe et al., 1994; Hammond-Kosack et al., 1996). According to this view of independent resistance and HR, the cell death response may be to reinforce or stimulate the induction of defenses (Dangl et al., 1996; Alvarez et al., 1998) by activation of local and systemic acquired resistance (Staskawicz et al., 1995; Ryals et al., 1996).

To gain further insight into the relationship of virus resistance and cell death, we isolated *Rx* from tetraploid potato by using a map-based cloning strategy. We demonstrate that *Rx* of potato expressed as a transgene either in potato or in *Nicotiana* spp confers extreme resistance against PVX

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed. E-mail david.baulcombe@bbsrc.ac.uk; fax 44-0-1603-250024.

and that this resistance is not associated with cell death. However, *Rx* shows similarities with the nucleotide binding site, leucine-rich repeat (NBS-LRR) class of plant *R* genes conferring an HR. Using transient expression tests, we show that *Rx* has a potential to initiate cell death if the viral elicitor is expressed constitutively as a transgene rather than from the PVX genome. We further show that cell death does not normally occur when plants carrying *Rx* are challenged with PVX because the extreme resistance in the *Rx* response is epistatic to the HR.

RESULTS

Molecular Cloning of the *Rx* Gene

The *Rx* locus in potato cultivar Cara was genetically delimited within a bacterial artificial chromosome (BAC) clone (BAC77; Kanyuka et al., 1999) (Figure 1A). To confirm the presence of a functional *Rx* gene within BAC77, we developed a transient expression assay based on microprojectile bombardment (McCabe et al., 1988). The constructs used in this assay comprised a PVX vector under the control of the cauliflower mosaic virus (CaMV) 35S promoter that was inserted into the unique SrfI site of BAC77. This PVX vector was modified to carry a β-glucuronidase (*GUS*) reporter gene. It was also mutated in the open reading frame of the 25-kD movement protein so that *GUS* expression would report virus replication only in the bombarded cells expressing the candidate *Rx*. In the PVX-TK/BAC77 construct (Figure 1B), the PVX cDNA was from an *Rx*-avirulent strain. In PVX-KR/BAC77, the viral cDNA was from an *Rx* resistance-breaking (i.e., virulent) strain (Figure 1B). These two constructs were separately coated onto gold particles and then bombarded into the leaves of susceptible potato (*rx* genotype) and *N. benthamiana*. The GUS activity in virus-infected cells as a reporter of virus accumulation was assayed by histochemical staining of leaves 48 hr after bombardment.

In this assay, we expected to detect blue spots in leaves bombarded with the PVX-KR/BAC77 construct, which is indicative of high levels of GUS activity and PVX accumulation. However, if BAC77 contains *Rx*, the PVX-TK/BAC77 construct should have activated resistance so that GUS levels would be low or undetectable. The results of these biolistic transient assay tests for potato (data not shown) and *N. benthamiana* (Figure 1C) were completely consistent with these predictions: blue spots of GUS activity were obtained with the PVX-KR/BAC77 construct but not with PVX-TK/BAC77. Therefore, we concluded that BAC77 contains *Rx* and that *Rx* of potato is functional in heterologous plant species. In many of the subsequent biolistic tests, we exploited this second finding and used leaves of *N. benthamiana* plants because the microprojectile bombardment was more efficient than in potato.

To delimit *Rx* more precisely, we prepared five subclones of BAC77 containing overlapping DNA fragments (Figure 1A). These DNA fragments subsequently were cloned into plasmids carrying *Rx*-virulent or *Rx*-avirulent PVX constructs and subjected to biolistic assay, as described above for the full-length PVX/BAC77 constructs. Results of these tests showed that the functional *Rx* was in an 11-kb PvuII DNA fragment present in the BAC6 and BAC9 derivatives of BAC77 (Figures 1A and 1C).

The *Rx* Transgene Confers Extreme Resistance in Potato

To confirm the transient expression data, the insert DNA from BAC9 was stably introduced into potato cultivar Maris Bard (*rx* genotype) by Agrobacterium-mediated transformation. This 18-kb BAC9 insert was chosen for plant transformation instead of the smaller DNA insert in BAC6 to ensure that complete transcription regulatory sequences were present. The transgenic plants were tested for resistance to virulent and avirulent strains of PVX by mechanical inoculation. Seven of eight independent transgenic lines carrying insert DNA from BAC9 were resistant to *Rx*-avirulent PVX-TK and susceptible to virulent PVX-KR. This resistance was manifested as the absence of PVX-TK symptoms and complete lack of PVX-TK accumulation in inoculated and systemic leaves (data not shown).

We also tested the effect of the *Rx* transgene on PVX accumulation in protoplasts from two of these independent transgenic lines, M4 and M7, transformed with BAC9. For comparison, we also tested protoplasts from cultivar Cara (*Rx* genotype) and from cultivar Maris Bard (*rx* genotype). These protoplasts were inoculated with PVX-TK or PVX-KR, and the RNA was sampled at 24 hr after inoculation. Gel blot analysis of these RNAs confirmed that there was no accumulation of PVX-TK in protoplasts of cultivar Cara and of the two *Rx*-transformed lines. In contrast, PVX-KR accumulated at a high level in all protoplasts tested, as did PVX-TK in the protoplasts of cultivar Maris Bard (*rx* genotype; Figure 2). Thus, the viral resistance tests in plants and protoplasts confirmed that the transgenic *Rx*-mediated resistance was indistinguishable from the *Rx*-mediated phenotype in cultivar Cara. There was early arrest of PVX accumulation in single cells and an absence of the HR.

The *Rx* Transgene Confers Extreme Resistance in Heterologous Plant Species

Although we had established *Rx* function in *N. benthamiana* by using a transient assay (Figure 1C), this method would not have distinguished between extreme resistance and an HR. It remained possible that extreme resistance was a particular characteristic of *Rx* in potato. To investigate further the function of *Rx* in heterologous species, we produced

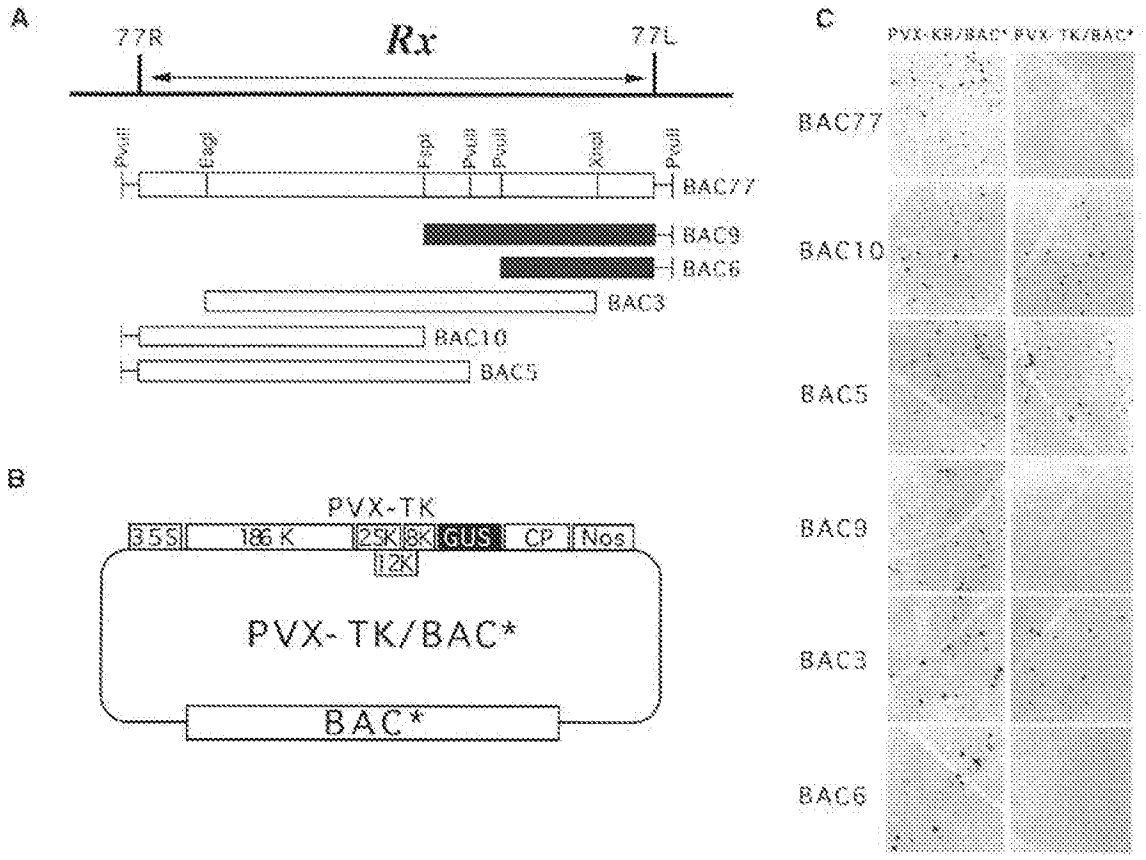


Figure 1. Identification of the *Rx* Gene by Using a Biolistic Transient Expression Assay.

(A) Genetic and physical map of the potato genomic region containing *Rx*, showing the BAC77 restriction map and the BAC subclones used in the transient expression assay. Left and right ends of BAC77 are indicated as 77L and 77R, respectively.

(B) Schematic structure of the BAC vectors used in the transient expression assay. PVX-TK/BAC* constructs carry insert DNA from BAC77 or from subclone derivatives described in **(A)** (BAC*) and the avirulent strain of PVX (PVX-TK) expressed from a CaMV 35S promoter. To monitor virus replication, we expressed the *GUS* gene from a duplicated PVX CP promoter. PVX-KR/BAC* is the same as PVX-TK/BAC*, except that PVX-KR/BAC* carries the *Rx* resistance-breaking strain of PVX (PVX-KR). Nos indicates the transcriptional terminator.

(C) Transient expression assay for *Rx* function. *N. benthamiana* leaf discs were bombarded with gold particles coated with either PVX-TK/BAC* or PVX-KR/BAC*. Virus replication was revealed by staining for GUS activity 2 days after bombardment. The potato insert DNA used in the bombardment is indicated at left. The lack of blue spots indicates *Rx* activity preventing virus accumulation.

transgenic *N. benthamiana* and *N. tabacum* carrying insert DNA of BAC9. Plants of 12 transgenic lines (six of each species) were rub inoculated with *Rx*-virulent and *Rx*-avirulent strains of PVX. RNA samples were taken from the inoculated and upper noninoculated leaves at 2 weeks after inoculation. RNA gel blot analysis of these samples revealed that PVX accumulation occurred only when a resistance-breaking strain of PVX was used as an inoculum. There was no detectable accumulation of the *Rx*-avirulent strain PVX-TK and no HR on the inoculated or systemic leaves (data not shown).

A more rigorous test for an HR associated with *Rx*-mediated resistance is to use graft inoculation (Figure 3A). All graft inoculations described below were reproduced in two independent experiments. Scions (upper part) of *N. benthamiana* carrying *Rx* were grafted to a stock (lower part) of a nontransgenic *N. benthamiana* plant that had been preinoculated with PVX-TK (10 independent grafts). As a control, we used *N. benthamiana* plants carrying the TMV resistance gene *N*. These plants were resistant against a TMV vector expressing the jellyfish green fluorescent protein (TMV-GFP). This *N*-mediated resistance was manifested at 4 days after inoculation as a localized HR in leaves inoculated with TMV-GFP (data not shown). We never observed systemic symptoms in these plants, indicating that *N*-mediated resistance restricted TMV to the inoculated leaf. In TMV-GFP graft-inoculated plants (10 independent grafts) from 10 days

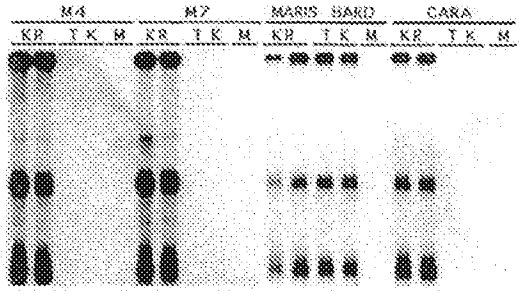


Figure 2. Gel Blot Analysis of PVX RNA Accumulation in Potato Protoplasts.

Protoplasts of potato cultivar Maris Bard (*rx* genotype; lanes MARIS BARD), potato cultivar Cara (*Rx* genotype; lanes CARA), or from two independent transgenic lines (M4 and M7) of potato Maris Bard containing the *Rx* transgene (BAC9 insert) were inoculated with either the virulent (KR) or the avirulent (TK) strains of PVX. M indicates mock-inoculated protoplast samples. Virus RNA accumulation was measured 24 hr after inoculation. Each lane of the gel was loaded with 2 μg of total RNA equivalent to 50,000 protoplasts. The hybridization probe was specific for the positive-strand RNA of PVX.

after grafting, the HR was manifested as a systemic HR (Figure 3B). Eventually this HR spread to cause death of the scion. The spreading HR was not observed in TMV-GFP graft-inoculated scions that did not carry *N* (Figure 3B). These controls demonstrated how the HR of graft-inoculated plants was no longer restricted to local necrotic lesions, as occurs in rub-inoculated leaves. Thus, graft inoculation exaggerates the HR.

In the graft-inoculated scions of a nontransgenic *N. benthamiana*, there were high levels (Figure 3C) and mosaic symptoms of PVX-TK, indicating that PVX could cross the graft union. However, the scions of transgenic *Rx* plants were symptomless, even after several weeks (Figure 3B). There was no evidence of an HR, even when leaves of the *Rx* scions were examined under a dissecting microscope or after trypan blue staining (Parker et al., 1993) for cell death (data not shown), and there was no accumulation of PVX-TK (Figure 3C).

We also conducted a double-graft experiment in which a scion of *N. benthamiana* (transgenic *Rx* genotype) was grafted between a healthy scion and one preinoculated with a PVX-TK stock of nontransgenic *N. benthamiana* (Figure 3A). We wanted to determine whether PVX-TK was able to cross a graft union and to pass through the vascular tissue of an *Rx* genotype plant. In 10 independent double grafts, RNA gel blot analysis showed PVX-TK accumulation in the inoculated stock and in the upper (nontransgenic) scion but not in the intermediate scion (Figure 3C). From these results, we conclude that *Rx*-mediated resistance did not suppress PVX-TK translocation through the phloem. These results also confirm that in the single-grafted plants, there would have been translocation of PVX-TK into the transgenic *Rx* scion and the potential for activation of an *Rx*-mediated HR. From the absence of an HR in these graft-inoculated plants, we conclude that there is no cell death associated with *Rx*-mediated resistance in *Nicotiana* spp.

The Rx Protein Is Similar to Products of *R* Genes Conferring the HR

The sequence of *Rx* was determined from an FspI-PvuII fragment of BAC77 (BAC9; 18,285 bp) and from analysis of *Rx* cDNA (see Methods and EMBL accession number AJ011801). Analysis of these sequences revealed that *Rx* has three exons and two introns of 234 and 111 bp (Figure 4A). The largest first exon contains the 5′ noncoding region of the mRNA and most of the coding region. The third exon contains only two nucleotides of coding sequence plus the TAG stop codon and the 3′ noncoding region of mRNA. The biolistic transient expression of this cDNA in *N. benthamiana* conferred resistance to PVX (data not shown).

Based on the cDNA sequence of *Rx*, we found a single open reading frame encoding a protein of 937 amino acid residues with a molecular weight of 107.5 kD (Figure 4B). Surprisingly, the primary structure of Rx is similar to that of the NBS-LRR (Jones and Jones, 1997) class of R proteins, in which the resistance is associated with an HR. The highest degree of similarity is between Rx and a subclass of NBS-LRR resistance proteins represented by RPS2, RPM1, and Prf (Jones and Jones, 1997). These Arabidopsis and tomato proteins contain a putative four-to-six heptad amphipathic leucine zipper motif at the N terminus (Jones and Jones, 1997). A putative leucine zipper is also present in the N-terminal region of Rx (domain I; Figure 4B). However, this domain in Rx does not fit the leucine zipper consensus as well as the corresponding motif in RPS2 (Mindrinos et al., 1994).

As in the other *R* gene products, the putative NBS domain (domain II; Figure 4B) of Rx comprises three motifs: kinase 1A or "P loop" (residues 168 to 180), kinase 2 (residues 237 to 247), and kinase 3a (residues 265 to 273). In Rx, the putative NBS is followed by a domain with unknown function that includes GLPL, CFLY, and MHD motifs. These motifs are characteristic of all NBS-LRR *R* gene products thus far identified (Hammond-Kosack and Jones, 1997; van der Biezen and Jones, 1998). The putative LRR domain of Rx (residues 473 to 868) comprises 14 to 16 imperfect copies of the LRR motif (Figure 4B). This motif shows a good match to the cytoplasmic LRR consensus sequence motif (Jones and Jones, 1997) and most closely resembles the LRR domain of the tomato Prf protein (Salmeron et al., 1996).

The C-terminal part of Rx (Figure 4B) contains three unique motifs with unknown functions: an amide-rich region (residues 869 to 893), a short basic region (residues 894 to 902), and an "acidic tail" region (residues 903 to 937). The acidic tail is encoded entirely within the short second exon of *Rx*. This domain is rich in aspartic and glutamic acid resi-

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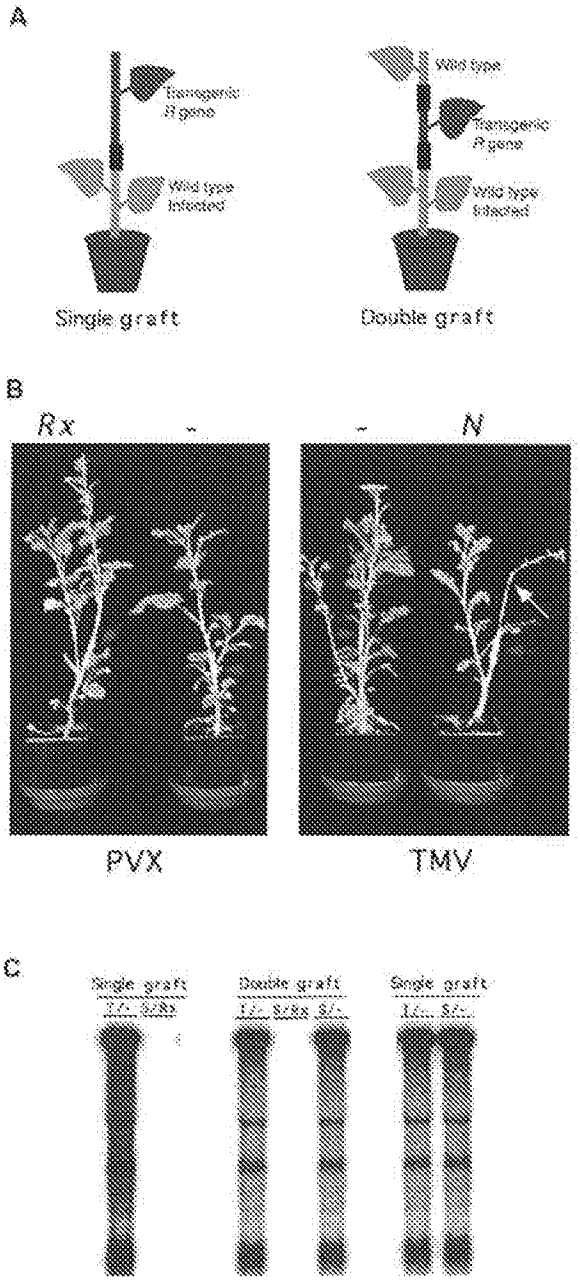


Figure 3. Cell Death Is Not Required for *Rx*-Mediated Resistance.

(A) Schematic representation of the grafting experiments indicating the virus-infected stock and the tested scion in the single and the double grafts, respectively.

(B) Grafted plants 4 weeks after grafting. The genotype of the root stock was wild-type *N. benthamiana*. The genotypes of the scions were wild-type *N. benthamiana* (indicated by a dash) or *N. benthamiana* transformed with *Rx* or the *N* gene, as indicated above the photographs. The viruses used in the infection of the root stocks were PVX-TK (referred to as PVX) or TMV-GFP (referred to as TMV), as indicated at bottom. The positions of the graft unions are indicated by red arrows. The TMV-induced systemic HR in the scion with *N* genotype is indicated by a white arrow.

(C) Analysis of PVX-TK accumulation in the root stock and the scion of single and double grafts. T and S indicate RNA extracted from the root stock and the scion, respectively. The genotype of the root stock and the scion is indicated by (–) for wild-type *N. benthamiana* or by Rx for *Rx*-transformed *N. benthamiana*. Each lane of the gel was loaded with 2 μg of total RNA. The hybridization probe was specific for the positive strand RNA of PVX.

dues and contains two copies of a 10-amino acid direct repeat (Figure 4B). These features are not present in any previously described products of *R* genes.

Constitutive Expression of the CP Induces *Rx*-Dependent Cell Death

From sequence analysis, *Rx* is structurally similar to *R* genes conferring an HR. In contrast, phenotypic analysis showed that *Rx*-mediated resistance is independent of an HR. These conflicting findings might indicate that subtleties in the Rx primary structure are required for the extreme resistance pathway. Alternatively, these findings could indicate that the *Rx*-mediated extreme resistance is a variation of the resistance mechanisms leading to HR and cell death. To address these possibilities, we examined the effects on *Rx*-mediated resistance when the expression of the viral elicitor (the PVX CP) (Bendahmane et al., 1995) was uncoupled from virus replication.

To uncouple CP expression from PVX replication, we expressed the CP gene from either the virulent or the avirulent strain of PVX under the control of the CaMV 35S constitutive promoter. To deliver these CP constructs into plants, we introduced them into binary pBin19 plasmid vector (Bevan, 1984) and transformed them into Agrobacterium (Figure 5A). The Agrobacterium cultures then were infiltrated into leaves of *Rx*-transformed *N. benthamiana* (line B18) in a procedure we refer to as agroinfiltration (Vaucheret, 1994).

When constructs encoding the elicitor CP (pBIN35-TK; Figure 5B) were agroinfiltrated into *Rx*-transformed *N. benthamiana* line B18, there was an HR. The necrosis first appeared within 48 hr after agroinfiltration and caused complete death of the infiltrated region by 72 hr (Figure 5B). We could rule out that this *Rx*-mediated HR was related to the Agrobacterium or to the infiltration method, because there was no HR when we used the construct pBIN35-KR (Figure 5B). This construct encoded the CP of an *Rx* resistance-breaking strain of PVX. Also, there was no HR when nontransgenic *N. benthamiana* plants were agroinfiltrated with either construct. When *Rx* genotype potato cultivar Cara or *Rx*-transformed potato and *N. tabacum* plants were used for

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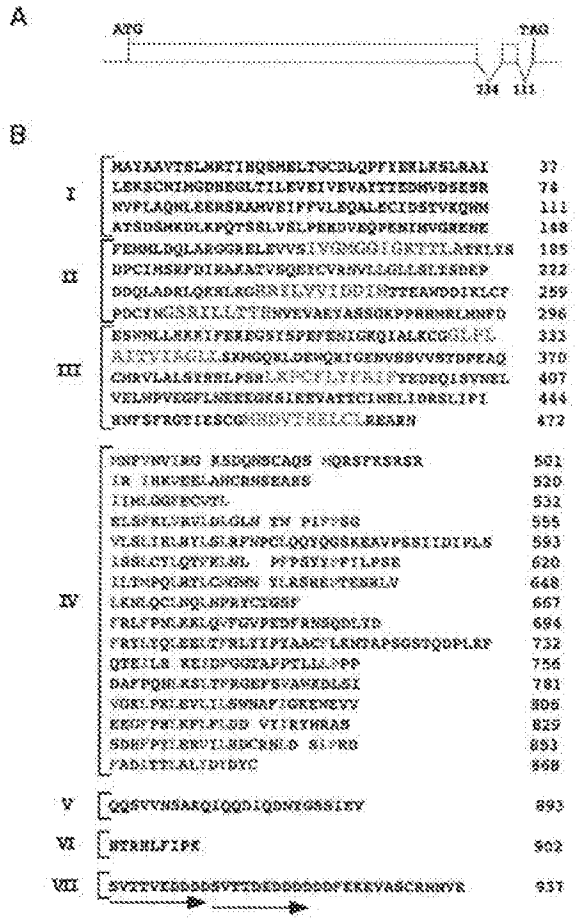


Figure 4. The *Rx* Coding Sequence and Primary Structure of the Rx Protein.

(A) Schematic diagram of the *Rx* coding sequence. Three exons are indicated by open rectangles. The positions of two introns of 234 and 111 bp in length are indicated by broken lines.

(B) Predicted Rx protein sequence. The amino acid sequence predicted from the DNA sequence of *Rx* (EMBL accession number AJ011801) has been divided into seven domains (I to VII), as described in the text. Three conserved kinase motifs of the NBS domain are shown in green. Conserved motifs with *R* genes are shown in brown (domain III). Residues of domain IV matching the consensus of the cytoplasmic LRR are highlighted as follows. Aliphatic residues L, I, M, V, or F are shown in red; conserved N, C, and T residues are in blue, and conserved P residues are indicated in green. Two direct repeats within domain VII (the "acidic tail") are shown by arrows.

similar experiments, the outcome was the same as when *N. benthamiana* line B18 was used; there was an HR, but only when the PVX-TK CP was expressed under control of the CaMV 35S promoter (data not shown). From these experiments, we conclude that there is the potential for an *Rx*-mediated HR. However, this potential is not realized when the PVX CP is expressed from the PVX genome during the viral infection cycle.

Extreme Resistance Is Epistatic to the HR

To further explore the relationship between extreme resistance and the HR, we analyzed the effect of *Rx*-mediated resistance on the *N* gene–mediated, HR-type resistance against TMV. These experiments used tobacco plants carrying the *N* gene either alone or in combination with transgenic *Rx*. The plants were challenged with recombinant isolates of TMV expressing the CP gene from either the *Rx*-virulent (PVX-KR) or the avirulent (PVX-TK) strain of PVX (Bendahmane et al., 1995; Figure 6A). The TMV-TK construct encodes the elicitors of both *N*- and *Rx*-mediated resistance (Padgett and Beachy, 1993; Padgett et al., 1997), whereas TMV-KR does not encode the elicitor of *Rx*-mediated resistance. Transcripts of the TMV-TK and TMV-KR cDNAs initially were inoculated onto *N. benthamiana*, and

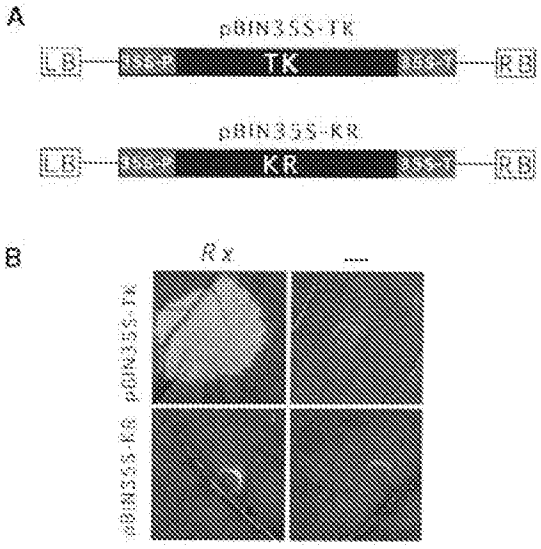


Figure 5. Constitutive CP Expression Induces *Rx*-Dependent Cell Death.

(A) Schematic representation of the T-DNA of pBIN35S-TK and pBIN35S-KR. pBIN35S-TK and pBIN35S-KR express the CP gene from the *Rx*-avirulent or the *Rx*-virulent strain of PVX, respectively. The box indicates the PVX CP open reading frame cloned between a 35S promoter (35S-P) and the transcriptional terminator (35S-T) of CaMV. LB and RB indicate the left and right borders of the T-DNA, respectively.

(B) Expression of PVX CP in *N. benthamiana*. The Agrobacterium strain carrying pBIN35S-TK or pBIN35S-KR (indicated at left) was infiltrated into wild-type (–) or *Rx*-transformed *N. benthamiana* (indicated at top). Leaves were photographed 3 days after infiltration.

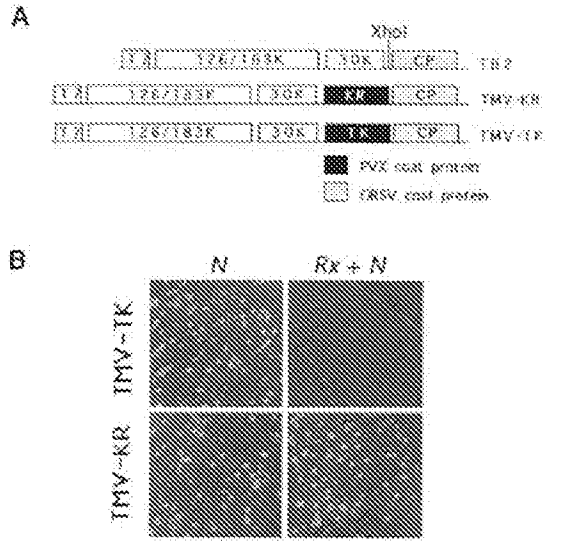


Figure 6. Extreme Resistance Conferred by the *Rx* Gene Is Epistatic to *N* Gene–Mediated Resistance.

(A) Genome organization of TMV in cDNA clones TB2, TMV-TK, and TMV-KR. The shaded region indicates odontoglossum ringspot virus (ORSV) CP. The black region indicates PVX CP. TMV-TK and TMV-KR express the CP gene from PVX-TK and PVX-KR, respectively.
(B) Transcripts of the TMV-TK and TMV-KR cDNAs initially were inoculated into *N. benthamiana*, and a sap extract was produced from the inoculated leaves 8 days after inoculation. This sap was then inoculated, without dilution, onto tobacco plants carrying the *N* gene either alone or in combination with *Rx*. The inoculated leaves were photographed 5 days after inoculation.

sap extracts were produced from the inoculated leaves at 8 days after inoculation. Tobacco plants carrying the *N* gene either alone or in combination with *Rx* were inoculated with these extracts containing high concentrations of the virus without dilution (Figure 6B). After 3 to 4 days, TMV-KR and TMV-TK induced an HR on *N. tabacum* expressing the *N* gene. There was also an HR when TMV-KR was inoculated onto plants of the *N, Rx* genotype. However, when these plants were inoculated with TMV-TK, there was no HR. These data indicate that *Rx*-mediated extreme resistance was activated before *N*-mediated resistance and therefore that extreme resistance is epistatic to an HR.

DISCUSSION

Here, we provide a molecular characterization of the *Rx* gene of potato and demonstrate that this gene controls separate virus resistance and cell death responses. The sequence of *Rx* revealed that the encoded protein has structures including those of the NBS, LRR, and other domains that are common to many *R* genes of plants (Jones and Jones, 1997; van der Biezen and Jones, 1998). It is surprising that Rx is similar to the leucine zipper class of NBS-LRR proteins. The other proteins in this class control resistance against bacteria (Mindrinos et al., 1994; Grant et al., 1995; Warren et al., 1998), nematodes (Milligan et al., 1998), and insects (Rossi et al., 1998). The product of *N*, which is the other known virus *R* gene, belongs to a separate class of NBS-LRR proteins in which the N-terminal domain has similarity to the Toll protein and the interleukin receptor (Whitham et al., 1994).

Although Rx has various R protein motifs, there are also acidic and amide motifs in the C-terminal domain (Figure 4) that are distinguishing features. These motifs are also present in paralogs of *Rx* on potato chromosome V as well as on chromosome XII adjacent to the cloned *Rx* locus (A. Bendahmane and K. Kanyuka, unpublished data). One of these paralogs is the *Rx2* locus present on chromosome V DNA introgressed from *Solanum acaule* (A. Bendahmane, unpublished results). This gene is functionally identical to but independent of the *Rx* locus described here (Querci et al., 1995). The *Gpa2* nematode resistance gene that is tightly linked to the *Rx* locus on chromosome XII (Rouppe van der Voort et al., 1997) may also be an *Rx* paralog.

The nature of the *Rx* response, as either extreme resistance or HR, was determined by the mode of expression of the CP elicitor. Transgenic CP elicited an HR, whereas CP produced from a virus caused extreme resistance (Figure 5). Our interpretation of this result is presented as a model in Figure 7. According to this model, when the elicitor CP was

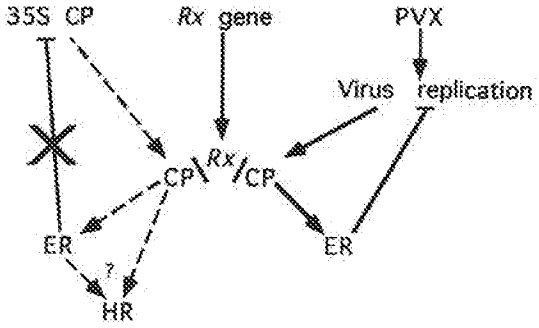


Figure 7. A Model for Extreme Resistance and the HR as Separable *Rx*-Mediated Responses.

Expression of the CP elicitor of *Rx*-mediated resistance from a transgene (left side; dashed arrows) leads to elicitation of extreme resistance (ER). Extreme resistance does not block expression of the transgene (X), and the continued CaMV 35S promotor–driven production of CP eventually leads to elicitation of the HR as a secondary response. Expression of the CP from a replicating virus (right side; solid arrows) also leads to elicitation of extreme resistance. However, in this situation, extreme resistance blocks virus accumulation and CP production, and there is no progression to the secondary HR.

expressed from a viral genome, there was early and rapid elicitation of extreme resistance. The consequent arrest of PVX accumulation would also have been associated with a block on other processes associated with the viral replication cycle, including further production of the CP (Figure 7). In contrast, when the CP was expressed from a transgene, the initial activation of extreme resistance would have had no effect on continued production of the CP. The continuing buildup of the elicitor CP would then lead to activation of the HR as a secondary resistance response (Figure 7).

Because *Rx* shares sequence similarity to many *R* genes (Figure 4), it is likely that variations of the model presented in Figure 7 are relevant to other disease resistance interactions in plants. For example, in *N*-mediated resistance against TMV, a situation could be envisaged that is intermediate between the extremes shown in Figure 7. The initial activation of *N*-mediated resistance may be slower than with *Rx*, and there would be delayed activation of the primary extreme resistance. In this example, the virus would continue to accumulate because it would have reached a high level and be replicating rapidly by the time the extreme resistance was activated. The continued accumulation of TMV would lead to an HR and necrotic lesions. This HR, being upstream of secondary defense induction, would reinforce the extreme resistance and eventually lead to a block on continued TMV accumulation.

A prediction of this model is that the same early responses would be activated in *N*- and *Rx*-mediated resistance but that they would be activated more rapidly, or be more pronounced, in the *Rx* background. These early responses will likely include an oxidative burst (Doke and Ohashi, 1988). Analysis of the *Rx* response in the background of superoxide dismutase overexpression would indicate whether production of active oxygen species is required for extreme resistance. It is unlikely that new gene expression is required for extreme resistance because high levels of actinomycin D do not block *Rx*-mediated resistance in protoplasts (Gilbert et al., 1998).

Other types of *R* gene responses may also have HR-dependent and HR-independent (similar to extreme resistance) components, as shown in Figure 7. The HR-independent response would be evident if resistance is activated early in the infection cycle. An HR would be produced if there was later activation of resistance. The resistance without the HR phenotype of the *dnd1* mutation could be explained in terms of this model. The mutation leads to activation of a generalized defense mechanism so that, in an *R* gene background, elicitation of the resistance mechanism may occur earlier in the infection cycle than in the wild-type plants. In a second example, the *Cf-9*-mediated resistance against the fungus *Cladosporium fulvum* in tomato is normally manifested as HR-independent arrest of hyphal growth (Jones et al., 1994). This phenotype is consistent with early activation of resistance in the fungal infection cycle. However, if the elicitor of *Cf-9*-mediated resistance is expressed from a virus vector, there is a systemic HR (Hammond-Kosack et al., 1995), indicating activation of the resistance late in the viral infection cycle.

The important general point from these various examples is the dynamic nature of resistance interactions in plants. Clearly, there is potential for quantitative and qualitative variation in resistance responses depending on the elicitor–receptor interaction. Factors affecting the response include timing, the level of interacting components, and the affinity between the elicitor and its receptor. According to this view, the outcomes of interactions between plants and pathogens represent variable points in a continuum of responses. At one end of the continuum, as with *Rx*, there is extreme resistance or HR-independent resistance when there is a high affinity between the elicitor and its receptor and when elicitor production is early in the infection cycle. HR-associated resistance may represent an intermediate response when the elicitor has a lower affinity for the receptor or when elicitor production is later in the infection cycle. A systemic HR may occur when there is a very-low-affinity interaction between the elicitor and the receptor. This response, representing the other end of the continuum of responses, may also occur when elicitor production is at a late stage in the infection cycle. In natural situations, the different types of responses likely represent stages in the oscillating evolutionary battle between disease-resistant plants and their pathogens.

METHODS

Viral cDNA Clones and in Vitro Transcription

The PVX-TK and PVX-KR constructs are cDNAs of potato virus X (PVX) isolate CP4. PVX-TK has codons ACA (specifying T) and AAA (specifying K) at positions 121 and 127 of the coat protein (CP) gene and is the same as wild-type CP4. The construct PVX-KR has AAA (K) and AGG (R) at the CP codons 121 and 127, respectively. These constructs were described previously (Goulden et al., 1993). The tobacco mosaic virus (TMV) constructs were derived from the TMV vector (TMV/odontoglossum ringspot virus; construct TB2) described previously (Donson et al., 1991). The TMV-TK and TMV-KR constructs were made by cloning PVX CP from PVX-TK and PVX-KR, respectively, in the XhoI site of TB2. Schematic structure of these constructs is described in Figure 6A. The TMV-GFP was made by cloning the green fluorescent protein (GFP) open reading frame in the XhoI site of TB2.

Plasmid Constructs for Biolistic Transient Expression Assay

PVX-TK/BAC77 and PVX-KR/BAC77 were made by digestion of the unique SrfI site in bacterial artificial chromosome BAC77 and ligation with the cDNAs of the *Rx*-avirulent or the *Rx*-virulent strain of PVX expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter. PVX in these constructs was defective in cell-to-cell movement and modified to express a β-glucuronidase (*GUS*) reporter gene. The cell-to-cell movement defect was due to a sequence deletion between nucleotides 4588 and 4945 of the 25-kD protein open reading frame (Angell et al., 1996). All the clones

derived from PVX-TK/BAC77 and PVX-KR/BAC77 were designated PVX-TK/BAC* and PVX-KR/BAC*, where the asterisks are used depending on the insert derivatives of BAC77 (see Figure 1).

Biolistic Transient Expression Assay

BAC plasmid DNAs were isolated using an alkaline lysis method (Leonardo and Sedivy, 1990) and purified on cesium chloride gradients (Sambrook et al., 1989). One hundred micrograms of plasmid DNA was precipitated onto 10 mg of 0.95-μm-diameter gold particles, as described previously (McCabe et al., 1988). Leaf discs from 6-week-old plants, which were placed onto Murashige and Skoog medium (Imperial, UK) containing 3% (w/v) sucrose, were bombarded with the gold particles coated with plasmid DNAs, as described previously (McCabe et al., 1988). The leaf discs were incubated at room temperature in the dark for 48 hr and then stained for GUS activity as described previously (Angell and Baulcombe, 1997).

Agrobacterium tumefaciens–Mediated Transient Expression (Agroinfiltration)

The CP genes from the avirulent or the virulent strain of PVX were inserted between the 35S promoter and the transcriptional terminator of CaMV and transferred into the binary vector pBin19 (Bevan, 1984) to create pBIN35S-TK and pBIN35S-KR, respectively (see Figure 5). These constructs were transformed into Agrobacterium strain C58C1 carrying the virulence helper plasmid pCH32 (Hamilton et al., 1996). pCH32 expresses VirG and VirE and was used to enhance T-DNA transfer. Agrobacterium cells were inoculated into 5 mL of L broth medium (Sambrook et al., 1989) supplemented with 50 μg/mL kanamycin and 5 μg/mL tetracycline and grown at 28°C overnight. L broth medium (50 mL) supplemented with 50 μg/mL kanamycin, 5 μg/mL tetracycline, 10 mM Mes, pH 5.6, and 20 μM acetosyringone was then inoculated with the 5-mL overnight cultures and grown at 28°C for 1 day. Cells were precipitated and resuspended to a final concentration of 0.5 OD_{600} in a solution containing 10 mM $MgCl_2$, 10 mM Mes, pH 5.6, and 10 mM acetosyringone. The cultures were incubated at room temperature for 2 hr before agroinfiltration. The agroinfiltration into Nicotiana benthamiana leaves was performed as described previously (Scofield et al., 1996; Tang et al., 1996).

Protoplasts Assay

Infectious viral RNA were electroporated into potato protoplasts, and RNA gel blot analysis was used to measure the accumulation of viral RNA, as described previously (Köhm et al., 1993). The probe used to detect PVX RNA was antisense RNA probe transcribed from the T7 promoter of pHB-RP plasmid linearized with ApaI and was described previously (Köhm et al., 1993).

Graft Inoculation

Graft inoculations involved the grafting of healthy scions to infected stocks. First, seedlings of *N. benthamiana* were sap inoculated with PVX strains. Later, when the plants exhibited symptoms on the systemic leaves, the apex of a healthy plant to be tested was grafted onto the infected stock. A diagonal cut was made in the stem of the third internode to insert the infected scion. The 2- to 6-cm scions were given a V shape at the end that would be inserted into the cut on the stock plant to ensure good contact between vascular systems. The graft was secured with Parafilm M (Sigma), and the whole plant was covered with a thin transparent plastic bag to avoid dehydration of the scion. The bag was removed 7 days after grafting. The presence of virus in the stock and the scion was determined 4 weeks after grafting by using RNA gel blot analysis. All graft inoculation experiments were repeated at least twice.

Plant Transformation

The FspI-PvuII fragment of BAC9 (18,285 bp) was cloned into the pSLJ7292 binary vector (Jones et al., 1992) digested with Ecl136II to create pSLJ9. This clone was introduced into Agrobacterium strain LBA4404. Transformation of *N. benthamiana* and *N. tabacum* was conducted by Agrobacterium-mediated leaf disc transformation (Horsch et al., 1985). Transformation of potato cultivar Maris Bard was performed as described previously (Gilbert et al., 1996). The transformation of *N. benthamiana* with the *N* disease resistance (*R*) gene was conducted by using Agrobacterium strain AGL1, which carries the binary vector pTG34 and was kindly provided by B. Baker (University of California, Berkeley; Whitham et al., 1994).

DNA Sequencing and Analysis

A shotgun cloning strategy was used for sequencing BAC9. Three aliquots of 15 μg of CsCl-purified DNA were sonicated for 30, 60, and 120 sec, respectively, by using Soniprep 150 (MSE, UK). DNA fragments in the range of 1 and 4 kb were gel purified, blunt ended using T4 DNA polymerase, and cloned into pGEM3Zf+ (Promega) digested with SmaI. Clones carrying potato insert DNA were selected by colony hybridization by using insert DNA from BAC9 as a probe. The sequencing reactions were performed using a dye terminator cycle sequencing reaction kit (Perkin-Elmer) and M13 universal forward and reverse primers. The sequencing reactions were resolved on an ABI377 automated sequencer (Applied Biosystems, La Jolla, CA). Sequence contigs were assembled using UNIX versions of the Staden programs package (R. Staden, Medical Research Council, Cambridge, UK). Homology searches were done using the BLAST program (Altschul et al., 1990). The genomic DNA sequence has EMBL accession number AJ011801.

Rapid Amplification of cDNA Ends

The 5′ and 3′ ends of the *Rx* cDNA were determined by rapid amplification of cDNA ends (RACE) by using the MARATHON cDNA amplification kit (Clontech Laboratories, Palo Alto, CA). To obtain specific RACE products, two consecutive rounds of amplification (35 cycles each) were necessary. Conditions for the first round of polymerase chain reaction (PCR) were as recommended by the manufacturer. PCR samples of the first amplification round were diluted 1:10 with distilled water, and a 1-μL aliquot was used as a template for the second round of PCR (15 sec at 94°C, 15 sec at 65°C, and 1 min at 72°C). Two sets of oligonucleotides were used in combination with the adapter primers (AP1 and AP2) of the kit: K3 (5′-ACTCGAGGT-CCTTATACTATCATGG-3′) and K4 (5′-GAACGAGTTATTCTTAGA-TTGCCG-3′) for the 3′ end of *Rx* and oligonucleotides K14 (5′-GCT-ACCTCTACGATTTCAACTTCCA-3′), and K15 (5′-GCAGGATTTCTC-CAGAATAGCTCTCA-3′) for the 5′ end of *Rx*. RACE products were

subcloned into the pGEM-T plasmid (Promega), and sequences of 10 independent 3′ and 5′ end clones were determined as given above. Sequences of 5′ and 3′ RACE PCR products overlapped with the sequence of the PCR product corresponding to the central region of the *Rx* cDNA amplified using primers asK4 (5′-GAGAGCTATTCTGGAGAAATCCTGC-3′) and asK15 (5′-GGCAATCTCTAAGAATAACTCGTTCA-3′).

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NOTE ADDED IN PROOF

It has now been established that the *Gpa2* nematode resistance gene is very similar to the *Rx* gene described here (**van der Vossen, E., van der Voort, J.R., Kanyuka, K., Bendahmane, A., Stiekema, W., Bakker, J., and Klein-Lankhorst, R.** (1999). Keystone Symposium Abstracts 88: Interactions and Intersections in Plant Signalling Pathways. (Abstract 324).

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The *Rx* Gene from Potato Controls Separate Virus Resistance and Cell Death Responses
Abdelhafid Bendahmane, Konstantin Kanyuka and David C. Baulcombe
Plant Cell 1999;11;781-791
DOI 10.1105/tpc.11.5.781

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A PROPOSAL FOR AN INTERNATIONAL NOMENCLATURE OF RACES OF *PHYTOPHTHORA INFESTANS* AND OF GENES CONTROLLING IMMUNITY IN *SOLANUM DEMISSUM* DERIVATIVES

W. BLACK [1]), C. MASTENBROEK [2]), W. R. MILLS [3]), and L. C. PETERSON [4])

Received 27 Aug. 1953

1. INTRODUCTION

A wide interest in physiologic races of *Phytophthora infestans* and in the genes for resistance derived from *Solanum demissum*, has resulted in the accumulation of data by investigators in many different countries of the world. Each investigator, using his own symbols for designating races and genes, has had difficulty in following the work of others and in determining how far the races and genes correspond. In an attempt to clarify this situation, the authors agreed to exchange differential host plants and to test the races of the parasite at their disposal. By this means they sought to compare the immunity genes present in the host series and the races of the parasite that had been isolated in Scotland, the Netherlands and the U.S.A. and to determine if a system of nomenclature suitable for international application could be formulated. This publication contains the results of these experiments and submits a proposal for the designation of genes and races that will facilitate the comparison and interpretation of results of experiments on an international basis.

2. EXPERIMENTS PERFORMED IN SCOTLAND

The three series of differential hosts were inoculated with eleven races of *P. infestans*. Only five of these races, viz. A, B^1, B^2, C and D were of local origin, having been isolated from field plots in Scotland. Of the remainder, race E was obtained from Tanganyika through the courtesy of Dr. C. B. WALLACE; race F was isolated in the course of experiments with race E and presumably arose from it; races G and H were obtained from Kenya through the courtesy of Dr. R. M. NATTRASS; and races I and J from Peru through the courtesy of Miss C. BAZAN DE SEGURA.

[1]) Chief Assistant, Scottish Plant Breeding Station, Edinburgh, Scotland.
[2]) Senior Phytopathologist, Plant Breeding Station Centraal Bureau, Hoofddorp, the Netherlands.
[3]) Associate Professor of Plant Pathology, Pennsylvania State College, State College, Pennsylvania, U.S.A. Authorized for publication on August 25, 1953, as paper No 1816 in the journal series of the Pennsylvania Agricultural Experiment Station, and as paper No 180 of the Department of Botany.
[4]) Associate Professor of Plant Pathology, Cornell University, Ithaca, New York, U.S.A.

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The tests were made by inoculating detached leaves of the host plants with spore suspensions of the different races of the parasite. The results obtained are recorded in Table 1. From this information it may be concluded that the three host series contain plants of similar genetic constitution; that the races, despite the different countries of origin may have some relationship to each other; and that the eleven races available do not represent a complete series of pathogenic types.

TABLE 1. REACTION OF THE THREE DIFFERENTIAL HOST SERIES TO BLACK'S COLLECTION OF RACES OF *P. infestans*

Differential hosts of			Races of *P. infestans*										
BLACK	MASTENBROEK	MILLS and PETERSON	A	B^1	B^2	C	D	E	F	G	H	I	J
S. tub.	*S. tub.*	*S. tub.*	s	s	s	s	s	s	s	s	s	s	s
1085(6)	43154–5	Essex	R	s	s	R	R	s	s	s	R	R	R
1512c(16)	44158–4	2LY–13	R	R	R	s	R	R	R	s	s	R	R
1253a(12)	4737–33		R	R	R	R	R	s	s	R	R	s	s
1506b	4431–5	AAB–2	R	R	s	s	s	R	s	R	R	s	R
	4651–2	GQT–1	R	R	R	R	R	R	R	s	R	R	R
	46174–30		R	R	s	R	R	R	s	R	R	R	R
	4414–2	CDF–9	R	R	R	s	R	R	R	R	R	R	R

R = resistant (immune). s = susceptible.

The probable interrelationships of these races are represented diagrammatically in Fig. 1, using as criteria the types of host plants they are able to attack, and the circumstantial evidence regarding their origin.

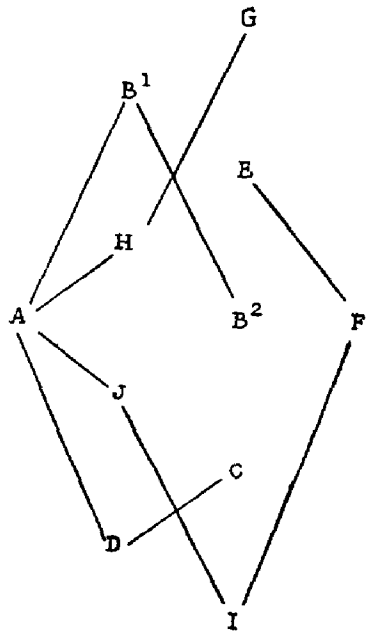


FIG. 1. DIAGRAM OF PROBABLE INTERRELATIONSHIPS OF RACES OF *P. infestans* EMPLOYED FOR TEST PURPOSES IN SCOTLAND

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3. EXPERIMENTS PERFORMED IN THE NETHERLANDS

The three series of differential hosts were inoculated with the eight races of *P. infestans* isolated in the Netherlands. Two experiments were made in 1951 with plants grown from detached sprouts and two experiments in 1952 with detached leaves on moist filter paper. Plants and leaves were sprayed with suspensions containing spores and swarmspores. The results were similar and are summarized in Table 2.

TABLE 2. REACTION OF THE THREE DIFFERENTIAL HOST SERIES TO MASTENBROEK'S COLLECTION OF RACES OF *P. infestans*

Differential hosts of			Races of *P. infestans*							
BLACK	MASTENBROEK	MILLS and PETERSON	N1	N2	N4	N5	N6	N7	N8	N9
S. tub.	*S. tub.*	*S. tub.*	s	s	s	s	s	s	s	s
1085(6)	43154–5	Essex	R	s	R	R	R	s	s	R
1506b	4431–5	AAB–2	R	R	s	R	s	s	s	s
1512c(16)	44158–4	2LY–13	R	R	R	s	s	R	s	s
	4414–2	CDF–9	R	R	R	R	s	R	s	s
	46174–30		R	R	R	R	R	s	s	R
	4651–2	GQT–1	R	R	R	R	R	R	s	R
1253a(12)	4737–33		R	R	R	R	R	R	R	s
	4739–58		R	R	R	R	R	R	R	R

From these experiments, the following diagram (Fig. 2) of relationships and differences between the races of *P. infestans* found in the Netherlands is outlined.

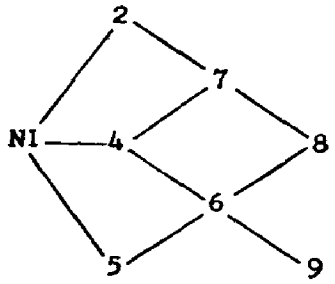


FIG. 2. DIAGRAM OF RELATIONSHIPS AND DIFFERENCES BETWEEN THE RACES OF *P. infestans* FOUND IN THE NETHERLANDS

4. EXPERIMENTS PERFORMED IN U.S.A.

The three series of differential hosts were inoculated with seven races, all of which have been isolated repeatedly from field plots in New York and Pennsylvania. Inoculations were made by atomizing swarmspores on actively growing, potted plants. The results obtained are shown in Table 3.

The pathogenic relationships of the races found in New York and Pennsylvania are expressed in Fig. 3.

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TABLE 3. REACTION OF THE THREE DIFFERENTIAL HOST SERIES TO MILLS' AND PETERSON'S COLLECTION OF RACES OF *P. infestans*

Differential hosts of			Races of *P. infestans*					
BLACK	MASTENBROEK	MILLS and PETERSON	A	B	C	D	BC	BD
S. tub.	*S. tub.*	*S. tub.*	s	s	s	s	s	s
1506b	4431–5	AAB–2	R	s	R	R	s	s
1512c(16)		2LY–13	R	R	s	R	s	R
1085(6)	43154–5	Essex	R	R	R	s	R	s
	4414–2	CDF–9	R	R	R	R	s	R
		3WM–19	R	R	R	R	R	s
	4651–2	GQT–1	R	R	R	R	R	R
1253a(12)			R	R	R	R	R	R

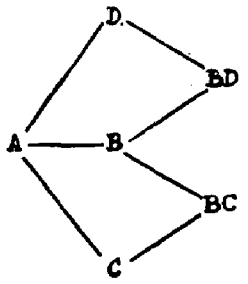


FIG. 3. DIAGRAM OF PATHOGENIC RELATIONSHIPS OF RACES OF *P. infestans* FOUND IN NEW YORK AND PENNSYLVANIA

5. COMPARISON OF RACES

In order to demonstrate the similarity of races employed in the three sets of experiments the results obtained in the different countries (Tables 1, 2 and 3) are combined in Table 4. It is clear from this Table that thirteen distinct races are now known to exist, that six of them are common to all three collections and of the remaining ones five are found in the collection of BLACK and two in that of MASTENBROEK. The races are arranged in a sequence based on the reactions they induce in the differential host plants and are given the proposed international designation in that order. The designation is that suggested by BLACK (2).

The probable relationships and differences between all these races are illustrated in Fig. 4. This diagram, which is based on Figs. 1, 2 and 3, is essentially similar to that of BLACK (2). The construction of this diagram has in fact the same general idea behind it as the nomenclature of MILLS and PETERSON (5, 6) and the diagram of MASTENBROEK (3, Fig.12). Apart from the thirteen known races and the three hypothetical ones it is possible that others, against which the known genes are ineffective, may arise.

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TABLE 4. A COMPARISON OF RACES OF *P. infestans* AND THE THREE DIFFERENTIAL HOST SERIES WITH A PROPOSED SCHEME OF INTERNATIONAL DESIGNATION OF RACES

Differential hosts of			Races of *P. infestans*												
BLACK	MASTENBROEK	MILLS and PETERSON	A N1 A	B^1 N2 D	H N5 C	J	D N4 B	G	E	B^2 N7 BD	C N6 BC	I	N8	F	N9
International designation of races			*0*	*1*	*2*	*3*	*4*	*1, 2*	*1, 3*	*1, 4*	*2, 4*	*3, 4*	*1, 2, 4*	*1, 3, 4*	*2, 3, 4*
S. tub.	*S. tub.*	*S. tub.*	s	s	s	s	s	s	s	s	s	s	s	s	s
1085(6)	43154–5	Essex	R	s	R	R	R	s	s	s	R	R	s	s	R
1512c(16)	44158–4	2LY–13	R	R	s	R	R	s	R	R	s	R	s	R	s
1253a(12)	4737 –33		R	R	R	s	R	R	s	R	R	s	R	s	s
1506b	4431 –5	AAB–2	R	R	R	R	s	R	R	s	s	s	s	s	s
	4651 –2	GQT–1	R	R	R	R	R	s	R	R	R	R	s	R	R
	4739 –58		R	R	R	R	R	R	s	R	R	R	R	s	R
	46174–30	3WM–19	R	R	R	R	R	R	R	s	R	R	s	s	R
	4414 –2	CDF–9	R	R	R	R	R	R	R	R	s	R	s	R	s

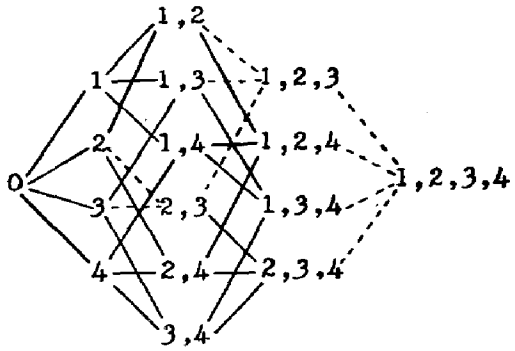


FIG. 4. DIAGRAM OF RELATIONSHIPS AND DIFFERENCES BETWEEN RACES OF *P. infestans*

6. COMPARISON OF GENES CONTROLLING IMMUNITY

It is apparent from the reactions observed and from the segregation ratios obtained by BLACK (1), MASTENBROEK (3, 4) and MILLS and PETERSON (5, 6), that four different genes are in operation and that these genes and the different combinations of them determine the range of resistant types involved. These genes were previously labelled differently by different authors, but it is now possible to compare them and to adopt a standard nomenclature suitable for international use. The authors propose that the nomenclature of BLACK be adopted. The four genes are therefore labelled R_1, R_2, R_3 and R_4, as shown in Table 5. Thus 1085 (6) (BLACK's gene R_1), 43154–5 (MASTENBROEK's gene R_9), and Essex (MILLS' and PETERSON's gene D) each carries the gene now designated as R_1.

The advantages in designating genes and races in this manner are (a) it probably allows for any extension that may be necessary in the future; and (b) it illustrates immediately the interrelationships between races and between genes and races without referring to diagrams or tables. For example, race *1, 2* (pronounced one two, not twelve) is capable of attacking genotypes R_1, R_2 and R_1R_2 but is unable to survive in the presence of genes R_3 or R_4. Thus susceptibility is only possible when the race has in its designation all the numerals present in the plant genotype. Accordingly

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genotype R_1R_2, being the natural host of race *1, 2*, is susceptible to races *1, 2; 1, 2, 3; 1, 2, 4* and *1, 2, 3, 4* only and immune from the rest.

TABLE 5. A COMPARISON OF GENES FOR RESISTANCE TO *P. infestans* IN THE THREE DIFFERENTIAL HOST SERIES, WITH A PROPOSED SCHEME OF INTERNATIONAL DESIGNATION OF GENES

International designation	Genes for resistance in the differential host series of			Races of *Phytophthora infestans*															
	BLACK	MASTENBROEK	MILLS and PETERSON	*0*	*1*	*2*	*3*	*4*	*1, 2*	*1, 3*	*1, 4*	*2, 3*[1]	*2, 4*	*3, 4*	*1, 2, 3*[1]	*1, 2, 4*	*1, 3, 4*	*2, 3, 4*	*1, 2, 3, 4*[1]
R_1	R_1	R9	D	R	s	R	R	R	s	s	s	R	R	R	s	s	s	R	s
R_2	R_2	R7	C	R	R	s	R	R	s	R	R	s	s	R	s	s	R	s	s
R_3	R_3	R8		R	R	R	s	R	R	s	R	s	R	s	s	R	s	s	s
R_4	R_4	R2, 5	B	R	R	R	R	s	R	R	s	R	s	s	R	s	s	s	s

[1]) Theoretical races; others known races.

7. SUMMARY

(1) The differential host series employed by the four authors were exchanged and the reactions to the races isolated or employed in the different countries were ascertained.

(2) The results obtained make it possible to standardize the designation of the races of the late blight fungus and of the genes for immunity derived from *Solanum demissum*.

(3) For both purposes the nomenclature of BLACK was chosen.

SAMENVATTING

Een voorstel tot een internationale benaming van physio's van Phytophthora infestans en van resistentiefactoren, afkomstig uit Solanum demissum

1. Teneinde na te gaan, in hoeverre de door de schrijvers geïsoleerde physio's van *P. infestans* en de thans bekende, uit *S. demissum* afkomstige factoren voor resistentie identiek zijn, werden toetssortimenten uitgewisseld en in de drie landen op hun vatbaarheid voor de daar aanwezige collecties van physio's onderzocht. De resultaten hiervan worden vermeld in de tabellen 1, 2 en 3. De verwantschap tussen de gebruikte physio's van elk der drie collecties blijkt uit de figuren 1, 2 en 3.

2. In tabel 4 is vermeld, welke physio's zeer waarschijnlijk aan elkaar identiek zijn. Een nieuwe, internationaal te gebruiken benaming wordt voorgesteld en opgenomen in de tabellen 4 en 5. Er blijken thans 13 physio's met verschillend spectrum bekend te zijn; zes er van komen in alle drie collecties voor, vijf alleen in die van BLACK, twee alleen in die van MASTENBROEK. De vermoedelijke verwantschap tussen deze dertien en nog drie onbekende, maar theoretisch te verwachten physio's (tabel 5) wordt weergegeven in fig. 4. De verwantschap tussen de bekende physio's is met getrokken lijnen, die tussen de bekende en de theoretisch te verwachten physio's met stippellijnen aangeduid.

3. Teneinde mogelijke verwarring op te heffen en het vergelijken van door de verschillende onderzoekers op dit terrein verkregen resultaten te vergemakkelijken,

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wordt een nieuwe, internationaal te gebruiken benaming voorgesteld, die gebaseerd is op de verwantschap tussen de physio's.

4. Uit tabel 5 blijkt, welke resistentiefactoren zeer waarschijnlijk aan elkaar identiek zijn en tegen welke physio's zij resistentie oproepen.

5. Ook voor de resistentiefactoren wordt het gebruik op internationale schaal van één benaming voorgesteld (tabel 5).

6. Zowel voor de physio's als voor de resistentiefactoren wordt de door BLACK (2) voorgestelde benaming gekozen. De theoretische achtergrond van de benaming der physio's is ook reeds aanwezig in de oude benaming van MILLS and PETERSON (5, 6) en in het schema van MASTENBROEK (3, fig.12).

7. De voordelen van de gekozen wijzen van benoeming zijn:

a. dat de vatbaarheid en de resistentie van een plant van bekende samenstelling t.a.v. de resistentiefactoren kan worden aangegeven, zonder dat een tabel of diagram geraadpleegd behoeft te worden. B.v. een plant in het bezit van factor R_3 is vatbaar voor de physio's *3; 1, 3; 2, 3; 3, 4; 1, 2, 3; 1, 3, 4; 2, 3, 4* en *1, 2, 3, 4*, dus voor alle physio's in welker benaming het cijfer 3 voorkomt. Tegen de overige physio's is de plant resistent. Een plant, in het bezit van de factoren R_1 en R_4, is vatbaar voor de physio's *1, 4; 1, 2, 4; 1, 3, 4* en *1, 2, 3, 4*, dus voor alle physio's, waarin *1, 4* voor komt. Voor alle overige physio's is zo'n plant onvatbaar (zie tabel 5).

b. dat, omgekeerd, onmiddellijk kan worden gezegd, welk physio voor welke combinaties van resistentiefactoren pathogeen is. B.v. physio *1, 2, 4* is pathogeen voor planten met combinaties van de factoren R_1, R_2 en R_4. Planten, waarin factor R_3 aanwezig is, zijn onvatbaar voor physio *1, 2, 4*.

c. dat dit systeem waarschijnlijk uitbreiding toelaat, wanneer dit nodig mocht zijn, zonder dat de benamingen veranderd behoeven te worden.

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RFLP Maps Based on a Common Set of Clones Reveal Modes of Chromosomal Evolution in Potato and Tomato

Merideth W. Bonierbale, Robert L. Plaisted and Steven D. Tanksley

Department of Plant Breeding and Biometry, Cornell University, Ithaca, New York 14853

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ABSTRACT

Potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum*) are members of the Solanaceae (nightshade family) and have the same basic chromosome number ($x = 12$). However, they cannot be cross-hybridized and, until now, it was unknown how conserved the gene order might be between these two species. We report herein the construction of a genetic linkage map of potato chromosomes based on genomic and cDNA clones from tomato. The potato map was drawn from segregation data derived from the interspecific cross *S. phureja* × (*S. tuberosum* × *S. chacoense*) ($2n = 2x = 24$), and consists of 135 markers defining 12 distinct linkage groups. Nearly all of the tomato probes tested hybridized to potato DNA, and in most cases, the copy number of the employed clones was the same in both species. Furthermore, all clones mapped to the same linkage group in both species. For nine chromosomes, the order of loci appears to be identical in the two species, while for the other three, intrachromosomal rearrangements are apparent, all of which appear to be paracentric inversions with one breakpoint at or near the centromere. These results are consistent with cytogenetic theory, previously untested in plants, which predicts that paracentric inversions will have the least negative effect on fitness and thus be the most likely form of chromosomal rearrangements to survive through evolutionary time. Linkage maps based on a common set of restriction fragment length polymorphism markers provide a basis for uniting the previously separate disciplines of tomato and potato genetics. Using these maps, it may now be possible to test theories about homologies or orthologies of other genes, including those coding for disease resistance and stress tolerances.

THE potato (*Solanum tuberosum* L.) is the most important food crop of the plant family Solanaceae, which contains some 3000 species including tomato (*Lycopersicon esculentum* Mill.), tobacco (*Nicotiana tabacum* L.), petunia (*Petunia hybrida* Hort. Vilm.-Andr.), eggplant (*Solanum melongena* L.), and garden pepper (*Capsicum annuum* L). The tuber-bearing Solanum are one relatively small group of a very large genus and include approximately 170 species. The basic chromosome number of potato, like most other solanaceous species, is $x = 12$ and wild forms range from diploids to hexaploids.

Tomato and potato are members of the same tribe (Solanaceae) (D'ARCY 1976), have nearly identical karyotypes and contain comparable monoploid amounts of nuclear DNA (0.7 pg). The fact that the two species cannot be cross-hybridized has prevented detailed comparative genetic studies and, by-in-large, genetics of the two species have remained distinct and nonoverlapping. In addition, the heterozygous (outbreeding), tetraploid nature of the cultivated potato, and lack of useful genetic markers, has hindered the construction of a genetic linkage map for this species.

The objectives of the research reported here were: (1) to construct a linkage map of potato based on restriction fragment length polymorphism (RFLP) markers, and (2) to determine the degree of similarity between potato and tomato chromosomes with respect to gene order. Genetically, tomato is one of the best-characterized plant species with well-populated linkage maps based on both morphological and molecular markers (TANKSLEY, MUTSCHLER and RICK 1987). By using previously mapped clones isolated from the tomato, we have been able to accomplish both of the stated goals. The construction of comparative maps based on a common set of clones provides a basis for uniting tomato and potato genetics, an accomplishment mutually advantageous for research in both species.

MATERIALS AND METHODS

Plant materials for segregation analysis: Parents and tuber progeny of an interspecific, diploid Solanum cross were obtained from C. QUIROS and D. DOUCHES, University of California, Davis. The pistillate parent of the cross (84S10) was an accession of the diploid species *Solanum phureja* Juz. et Buk. The staminate parent (T704) was an interspecific clone obtained by the hybridization of a dihaploid *Solanum tuberosum* ($2n = 2x = 24$) and a member of the diploid species *Solanum chacoense* Bitt. Attractive features of this population were its diploid constitution, prior characterization of isozyme loci (DOUCHES and QUIROS 1987), and the high likelihood that it would exhibit polymorphism at the DNA level. The mapping population consisted of 65 F_2

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progeny, among which segregation of alleles from the interspecific parent was monitored for genetic mapping. Plants were propagated clonally in the greenhouse at Cornell University. The *S. phureja* clone and the *S. tuberosum* × *S. chacoense* hybrid will be referred to hereafter as *phu* and *thr-chc*, respectively (HUAMAN and ROSS 1985).

DNA isolation, restriction digests, electrophoresis and blotting: Isolation of potato genomic DNA from leaf tissue was as described for tomato (BERNATZKY and TANKSLEY 1986a), except that sodium bisulfite was used instead of mercaptoethanol. Digestions with the following endonucleases (BRL) were performed according to the manufacturer's instructions using 2 units of enzyme per μg of DNA: *Eco*RI, *Eco*RV, *Hind*III, *Dra*I, *Xba*I, *Bst*NI, *Hae*III, *Taq*I, *Mbo*I, *Msp*I and *Hin*fI. Seven micrograms of digested potato DNA were loaded and separated on 0.9% agarose gels. Electrophoresis and Southern blotting were conducted as described by BERNATZKY and TANKSLEY (1986a). The only modifications of the above-mentioned protocols were the substitution of Genescreen plus (NEN) filters for Zetabind, and the use of capillary blots instead of dry blotting. On survey filters, the parents were represented in each of 11 pairs of restriction digests. Four to six replicated progeny filters, including one lane of digested tomato DNA (*L. esculentum* cv. VF36), 65 potato progeny, and one lane of each of the potato parental DNAs, were prepared from bulk digests with each enzyme.

DNA probes, labeling and hybridization: Both cDNA and genomic clones were used as probes for mapping the potato genome.

Construction of the cDNA and size-selected (0.5–3.0 kb) *Pst*I or *Eco*RI genomic libraries from tomato have been described elsewhere (BERNATZKY and TANKSLEY 1986a; TANKSLEY *et al.* 1987). The loci detected by hybridization of cDNA clones were designated *CD1*, *CD2*, etc., and those detected by the genomic clones are designated *TG1*, *TG2*, etc. Duplicate loci homologous to a single probe were suffixed with designations *A*, *B*, etc. Clones of several known genes were also used in the comparative mapping study: small subunit ribulose bisphosphate carboxylase (*Rbcs*), chlorophyll *a/b* binding protein (*Cab*), and the 45s major ribosomal repeat (*R45s*). The Rbcs and Cab clones were from tomato (PICHERSKY *et al.* 1985, 1986) and the ribosomal clone was from pea (JORGENSEN *et al.* 1982). The clones used in this study had previously been mapped to orthologous loci in tomato, and are distributed throughout 94% of the 1237 cM tomato RFLP map at intervals averaging 11 cM (TANKSLEY *et al.* 1987; Figure 1B).

Whole plasmids were either nick-translated or random-hexamer-labeled with ^{32}P-dCTP (RIGBY *et al.* 1977, FEINBERG and VOGELSTEIN 1983). Potato parental survey or progeny filters were hybridized overnight as previously described (BERNATZKY and TANKSLEY 1986b). Filters were washed to medium stringency (0.5 × SSC, 65°) and placed on X-ray film for 1–5 days.

Data from eleven isozyme markers and *Y*, a morphological marker determining tuber flesh color (HOWARD 1970), segregating in the same diploid population, were kindly supplied by DAVE DOUCHES. These data were included in the mapping analysis by their co-segregation with the DNA markers.

RFLP analysis and map construction: Enzyme-probe combinations were selected for which the *thr-chc* parent appeared to be heterozygous, while the other parent (*phu*) was either homozygous, or heterozygous but with different alleles. Potential heterozygosity with a given probe was inferred by the presence of more than one hybridizing restriction fragment per digestion on parental survey filters, and polymorphism between the parents required that at least one fragment be unique to the parent in question. Segregation of alleles from *thr-chc* were verified and scored in the progeny. Analysis of segregation data was performed on an IBM PC/AT computer using SPSS software. Recombination frequencies were converted to map units according to KOSAMBI (1944).

In some instances, heterozygous loci in the *phu* parent were also observed to segregate in the progeny, and a second set of mapping data was collected.

RESULTS

Description of the potato linkage map: A total of 134 segregating DNA and isozyme markers were assigned to 12 linkage groups in the potato genome. The high degree of conservation of chromosome content between tomato and potato (see following section) allowed the assignment of the potato linkage groups to chromosomes *1* through *12* by homeology to the tomato chromosomes (Figure 1A). Each of the chromosomes is marked by from 7 to 18 linked loci, with adjacent markers separated by 0–30 map units.

The linkage group *Idh1-Skdh1*, which DOUCHES and QUIROS (1987) reported to span a centromeric region of an unidentified potato chromosome, cosegregated in potato with several DNA markers from tomato chromosome *1*, placing it in the same map position in the two species. Linkage analysis also places six other isozyme loci—*Prx2*, *Pgm1*, *Pgm2*, *Got2* and *Pgi1*—in chromosomal locations corresponding with those previously reported for tomato. Three isozyme loci—*6Pgdh3*, *Dia1* and *Mdh3*—which have not yet been mapped in tomato were found to be tightly linked with RFLP markers on chromosomes *5* and *7* in potato. The chromosome *5* isozyme markers (*6Pgdh3* and *Dia1*) have been reported to span a centromeric region in potato (DOUCHES and QUIROS 1988), and one of them, *6Pgdh3* had previously been assigned to chromosome *5* in tomato by trisomic dosage analysis but not mapped to a specific position within the chromosome (TANKSLEY and KUEHN 1985).

Alleles of *Pgi1*, *Mdh3* and *Y* were segregating only from the *phu* parent, and the inclusion of these three loci in the potato map was made possible by their linkage with RFLP markers for which both parents were heterozygous. The markers mapped by segregation from *phu* are designated in Figure 1A with an asterisk.

Two markers, tomato genomic clone *TG218* and a third locus for CD21 (*CD21C*), which were not polymorphic in tomato were mapped in potato to chromosomes *7* and *8*, respectively. The locus *CD21C* extends potato chromosome *8* beyond the terminal DNA marker (*CD29*) on the tomato homeolog (Figure 1, A and B).

Comparison of the tomato and potato linkage maps: The tomato map, with which the potato map is being compared, was based on an F_2 population from

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FIGURE 1.—Comparative genetic maps of potato and tomato based on a common set of DNA clones. Two point linkage distances between adjacent markers are based on Kosambi (1944) map units. A, Potato. Asterisks indicate markers mapped by segregation of alleles from *S. phu*. B, Tomato. Hatched boxes represent intervals in which the order of markers is inverted between the two genomes. Brackets indicate approximate position of centromeres.

a cross between inbred accessions of *L. esculentum* and *L. pennellii* (Bernatzky and Tanksley 1986b, Tanksley *et al.* 1987). On the tomato map, the loci we have examined in potato span a total of 1189 cM, and are distributed throughout 94% of the tomato genome (Figure 1B).

Of the tomato cDNA and genomic clones examined, all but two hybridized to potato genomic DNA under conditions of medium stringency (0.5 × SSC, 65°). Thus, for nearly every tomato genomic sequence or cDNA tested it was possible to find a homologous counterpart in potato.

In addition to the homology of DNA sequences throughout the tomato and potato genomes, the linkage order of those sequences on the chromosomes is remarkably well conserved. A comparison of the arrangement of loci in tomato and potato reveals that all DNA sequences mapped in potato can be assigned to linkage groups corresponding to chromosomes *1–12* in tomato (Figure 1, A and B). Nine chromosomes (*1, 2, 3, 4, 6, 7, 8, 11* and *12*) appear to have been *unaltered by any detectable rearrangements. Any* rearrangements that may have occurred within the intervals between two adjacent markers examined would have gone undetected in our analysis; however, on the average, the markers were linked at fairly close intervals (*ca.* 11 cM).

Evolution by paracentric inversions: Three of the tomato/potato homeologs differ by what appear to be paracentric inversions (Figure 1, A and B). One end of each of the inversions detected coincides with the approximate position of the centromere in the tomato homeolog. On chromosomes *5* and *10*, terminal regions, encompassing approximately 20 cM and 68 cM, respectively, on the tomato map, were found to be inverted between the tomato and potato genomes. The inversion on chromosome *5* is marked by three loci, *CD31*, *CD67* and *CD41*, and that on chromosome *10* by five, *CD34*, *CD72*, *CD63*, *CD5* and *CD32B*. Both arms of chromosome *9* have sustained paracentric inversions—one interstitial and the other terminal.

The average length of the four putative inversions differentiating the potato and tomato genomes is 34 cM (based on the tomato map), and together these segments constitute 11% of the genome.

Reduced recombination in potato *vs.* tomato chromosomes: The orthologous loci mapped in tomato cover 1189 cM in tomato, whereas the same markers in potato define only 606 cM. A paired *T*-test using sets of intervals revealed that the reduced recombination in potato chromosomes compared with tomato is significant ($P < 0.001$). The reduction in recombination appears to have affected all chromosomes since each of the potato chromosomes has a reduced genetic length (1.4–3.6-fold shorter) compared with the corresponding tomato chromosome. The mean interval

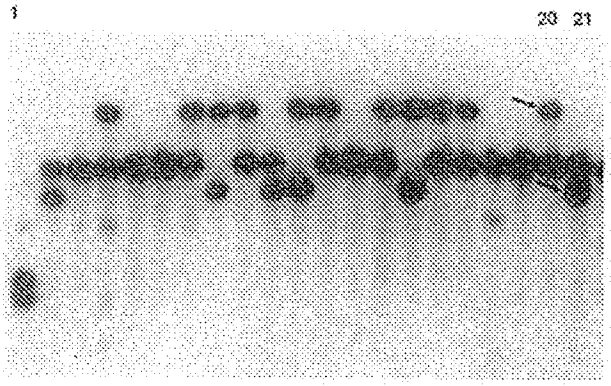


Figure 2.—Autoradiogram from hybridization of Southern blot of genomic DNA from segregating potato progeny (*Dra*I digest) with *TG65*. Lane 1, tomato; lanes 2–19, potato progeny; lane 20, T704 (*S.tbr-S.chc*); lane 21, 84S10 (*S.phu*). Arrows indicate restriction fragments unique to each parent.

length between adjacent markers in potato was 50% less than that found in tomato. However, in some regions of the potato chromosomes, recombination *was even more severely reduced. For example, certain* regions on chromosomes *1, 2, 3, 4, 5, 8* and *12* showed recombination in potato less than 25% of that observed in tomato. The proximal region on the long arm of chromosome *2* (between *CD1* and *CD45*) is an especially striking example since the interval contains 79 cM in tomato, but only 9 cM in potato.

The potato population was segregating for loci heterozygous in the *tbr-chc* parent and in some instances, also for loci heterozygous in the *phu* parent (Figure 2). As an independent assessment of recombination in the Solanum genome, linkage analysis was conducted for 12 randomly selected intervals bounded by markers segregating from the *phu* parent. These data, while not representing all loci, provided a basis for comparing recombination frequency among tomato, the interspecific potato parent, and the *phu* clone (Table 1). Of the intervals compared, only one varied significantly among the three populations. On chromosome *1*, the interval between *TG125* and *TG19* showed reduced recombination in the *tbr-chc* parent as compared with tomato—21 *vs.* >50 cM. However the rate of recombination in the *phu* parent for this interval (49 cM) was similar to that found in tomato.

Aberrant segregation ratios: Significant deviations from the expected 1:1 ratio were found at 28% of the loci segregating in the *tbr-chc* parent. The loci showing deviant ratios cluster on parts of chromosomes *1, 6, 7, 8* and *10* (Table 2, Figure 3). These regions were also among those found to exhibit reduced recombination relative to tomato.

The majority of the segregating loci (28/31 = 90%) scored from the *phu* parent fit the expected 1:1 ratio. Two of the three loci showing skewed segregation from this parent are tightly linked on chromosome *4*.

Restriction fragment length polymorphism: With

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TABLE 1

Comparison of recombination fraction in selected intervals among three mapping populations; tomato (*L. esc* × *L. pen* F_2), potato P1 = T704 (*S. tbr* × *S. chc*), and potato P2 = 84S16 (*S. phu*)

Interval	Chromosome	Recombination fraction		
		Tomato	Potato P1	Potato P2
TG19–TG53	*1*	0.14	0.20	0.25
TG19–TG125	*1*	0.50	0.21*	0.49
TG42–TG74	*3*	0.22	0.16	0.07
CD55–TG62	*4*	0.05	0.02	0.10
TG62–TG65	*4*	0.03	0.03	0.06
CD39–TG22	*4*	0.12	0.10	0.08
CD31–CD41	*5*	0.18	0.16	0.10
TG218–TG20	*7*	No data[a]	0.02	0.02
TG20–CD26	*7*	0.08	0.02	0.02
TG16–TG127	*8*	0.09	0.02	0.00
TG127–TG117	*8*	0.18	0.16	0.10
TG68–TG19	*12*	0.12	0.09	0.09

* $P < 0.05$.
[a] TG218 was not mapped (not polymorphic) in tomato.

TABLE 2

Loci in the potato genome with monogenic ratios deviating from the expected (1:1) Mendelian ratio ($P = 0.05$)

Chromosome	Locus	Ratio
1	*TG125*	41:24
1	*TG71*	38:22
1	*TG19*	38:20
1	*StdA1*	40:23
2	*CD43*	21:37
2	*CD35*	24:41
2	*TG34*	18:35
3	*TG129*	37:21
6	*CD14*	56:7
6	*CD67*	48:10
6	*TG25*	47:15
6	*CD25*	44:21
6	*TG59*	36:13
6	*TG119*	41:17
6	*CD42*	42:20
7	*TG20*	40:19
7	*TG128*	45:21
7	*CD54*	45:18
7	*TG61*	47:14
7	*TG13A*	43:13
8	*TG127*	23:43
8	*TG124*	22:44
8	*TG117A*	16:50
8	*Got4*	15:49
8	*TG45*	16:49
8	*CD21A*	16:50
8	*Pin*	16:50
10	*TG132*	19:37
10	*CD56*	24:41
10	*CD77*	24:42
10	*TG43*	24:31
10	*TG63*	32:39

one or more of the 11 restriction enzymes used in this study, potential polymorphic heterozygosity was found in the *tbr-chc* parent for 98% of the DNA clones examined, compared with 81% for the *phu* parent. The level of heterozygosity observed in the *phu* parent suggests that natural populations of this outcrossing species of Solanum are likely to maintain significant amount of restriction fragment length polymorphism. Restriction enzymes most successful in detecting polymorphism between the parents were *Eco*RI, *Eco*RV, *Hin*dIII, *Dra*I and *Xba*I.

Comparison of cDNA and genomic clones: cDNA clones and single copy genomic clones were nearly equal in the frequency with which they detected restriction fragment length polymorphism in the two potato parents. With all of the enzyme-probe combinations used, the cDNAs and the genomic clones detected polymorphism in 166/324 = 51%, and 342/572 = 60% of the cases, respectively.

Changes in genomic clone copy number and homology: In contrast to cDNA clones, two of the tomato single copy genomic clones were found to hybridize with multiple copies in the potato genome: clones *TG37* and *TG12*, which mark single loci on chromosomes *4* and *10* in tomato, each hybridized to repetitive elements and could not be mapped in potato (Figure 4A).

Two additional single copy genomic clones, *TG5* and *TG51* from tomato chromosomes *7* and *1*, could not be mapped in potato due to lack of sufficient homology with corresponding potato sequences (Figure 4B).

DISCUSSION

Chromosome evolution: Our knowledge about chromosome evolution in higher plants is based largely on meiotic analysis and comparisons of karyotypes. The former is necessarily restricted to interfertile species, as the analysis is performed on hybrid plants. The latter, while not limited to crossable species, has poor resolution, since changes in karyotypes can be brought about by many different processes and often these changes are not distinguishable by morphological comparisons of mitotic chromosomes.

The family Solanaceae is interesting in that it contains a highly diverse group of species, yet most share the same basic chromosome number (x = 12). Changes in basic chromosome number, especially dysploid decreases, are believed to be facilitated by chromosomal translocations (Brown 1972). We report here that, since the divergence of potato and tomato from their last common ancestor, the only detectable chromosomal changes have been paracentric inversions. The occurrence of only this type of rearrangement, which does not result in changes in karyotype, is consistent with cytological observations reporting the conservation of relative chromosomal arm lengths between the two species (Yeh and Peloquin 1965).

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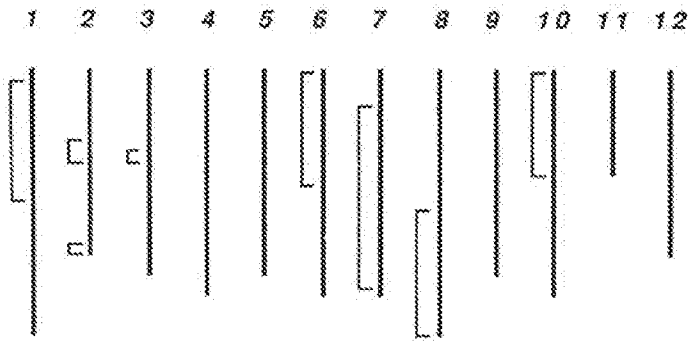


FIGURE 3.—Regions of the potato genome exhibiting aberrant monogenic ratios ($P < 0.05$).

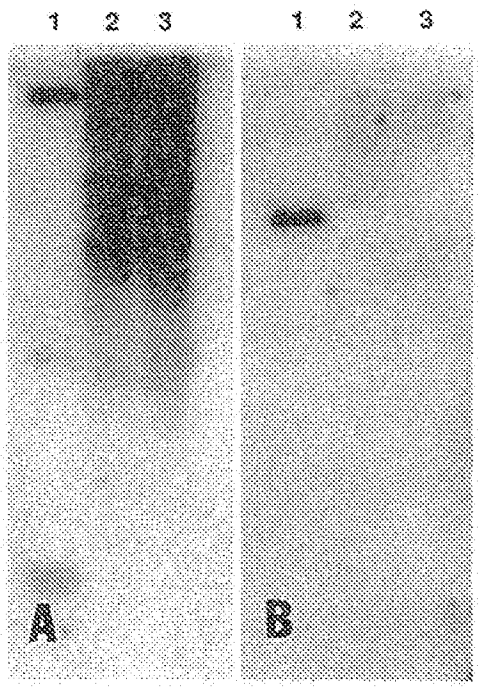


FIGURE 4.—Autoradiograms from hybridization of Southern blots of tomato and potato genomic DNA (*Eco*RV digests) with tomato clones. Lane 1, tomato; lane 2, potato $2n = 2x = 24$; lane 3, potato $2n = 2x = 48$. A, Clone *TG12* present in single copy in tomato and multiple copy in potato. B, Clone *TG5*: single copy in tomato and not homologous in potato.

The lack of translocations and pericentric inversions may be due to a selective disadvantage of these types of chromosomal rearrangement compared with paracentric inversions. A well-documented precedent for the predominance of paracentric over pericentric inversions is the phylogenetic work with Drosophila species (STONE 1955; STONE, GUEST and WILSON 1960; CARSON 1970). An observation explaining the survival of paracentrics in Drosophila has been proposed to exist in angiosperms as well (BROWN 1972; C. M. RICK, personal communication). By this mechanism, the specific orientation of cells in the linear tetrad of micro- or macrospores ensures that duplication- and deficiency-nuclei resulting from crossing over in the loop of inversion heterozygotes are not included in the gametes. Cytological evidence for paracentric inversions from bridges and fragments has been reported for several plant species (RICHARDSON 1936), but in the past, the resolution of chromosome mapping has not been fine enough to rule out the presence of pericentrics. Whatever the reasons for the predominance of paracentric inversions in the evolution of the tomato and potato, the predicted outcome has been the maintenance of chromosome number and arm ratio simultaneous with the reshuffling of gene order within chromosome arms.

Chromosome translocations are not a forbidden mutation in solanaceous species, since translocations can be induced and stably maintained in tomato (GILL *et al.* 1980) and are naturally occurring in the genus Capsicum (garden pepper) (PICKERSGILL, HEISER and MCNEILL 1978; TANKSLEY 1984). Comparative mapping of tomato and pepper chromosomes using RFLP markers has revealed significant linkage group rearrangement, some of which must have involved chromosomal translocations (TANKSLEY *et al.* 1988). Pepper ($2n = 2x = 24$), unlike potato, differs significantly from tomato in its genome size ($C = 2.76$, *vs.* $C = 0.74$ in tomato). It is not surprising that the fourfold change in DNA content which has occurred since the divergence of tomato and pepper was accompanied by a larger amount of genome rearrangement than is seen between tomato and potato.

In generalizing about the comparative chromosome arrangement of the two species examined in this study, one must proceed cautiously in light of the fact that polymorphism for variant chromosome structure may exist within as well as between populations of different species. Although in Drosophila the extent of chromosomal rearrangement within and among species does not necessarily parallel morphological diversity or taxonomical groupings (BOCK 1971; DOBZHANSKY 1972), it is clear that structural differences do play a fundamental role in the differentiation of species. Surveys of chromosomal polymorphism among species of Lycopersicon and Solanum could be accomplished by comparative genetic mapping, and would contribute to our understanding of the importance of this type of mutation in the evolution of fertility barriers in this important plant family.

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Recombination: The segregation data used to generate the tomato and potato maps presented here were derived from single segregating populations. Since meiotic recombination can vary among individuals within a species, it would be premature to conclude that potato chromosomes, in general, experience less crossing over than do their tomato counterparts. An additional confounding factor is that in both tomato and potato, interspecific crosses were used to determine map distances, and this is known to affect recombination (RICK 1969).

The outcome of our comparison of recombination in the *tbr-chc* versus the *phu* potato parents (Table 1) suggests that, at least in one instance, the lesser recombination seen in *tbr-chc* as compared to tomato is not a general phenomenon in potato, but was a function of the cross used for determining recombination.

DOUCHES and QUIROS (1987, 1988) also noted reduced recombination levels for several chromosome regions when they compared gene-centromere map distances in the interspecific hybrid used in this study (*tbr-chc*, T704) with those of "nonhybrid" diploid stocks of *S. tuberosum*. The *tbr-chc* parent is homozygous for the parallel spindles mutation (*ps*) which affords it the option of producing 2*n* gametes by first division restitution (FDR) (MOK and PELOQUIN 1975); but it does not express any of the recognized asynaptic or desynaptic mutant genes (reviewed in DOUCHES and QUIROS 1988) which have been shown to reduce recombination in other diploid Solanum stocks.

Although the hypothesis is difficult to test, it would be interesting to determine if areas of the potato genome that we and others (DOUCHES and QUIROS 1988) have identified as having extremely reduced recombination in interspecific populations might be indicative of structural polymorphism among *Solanum* species. If the interspecific potato parent used to construct the present map were heterozygous for inversions, one consequence would be genotype-specific (*tbr-chc* vs. *phu*) reduced recombination in distinct regions of the genome such as we have observed on chromosome *1* (see Table 1). The reduced recombination we observed between the genetic maps of tomato and potato could be due to the presence of unrecognized genes which influence crossing over or to a lack of homosequentiality of the genomes on which the potato map was based.

Nucleotide sequence homology of potato and tomato DNA: It is apparent from this study and from the work of ZAMIR and TANKSLEY (1988) that there is a high degree of conservation of most coding regions and single copy DNA sequences between tomato and potato, and that the linkage groups of the two species are well-conserved. In contrast, most highly repeated fraction of the two genomes has diverged to the point that there is little or no cross-hybridization. Only one of the three most highly repeated, non-ribosomal DNA repeats from tomato is found in potato (GANAL, LAPITAN and TANKSLEY 1988; SCHWEIZER *et al.* 1988). Some of the more highly repetitive, fast-evolving sequences are tandemly organized and might be expected to undergo rapid and concerted changes within isolated species (ARNHEIM 1983). However, others are highly interspersed throughout the genome (GANAL, LAPITAN and TANKSLEY 1988). It is apparent from this report that these interspersed sequences must also be evolving in a concerted manner, independently of gross chromosomal rearrangements.

Uniting potato and tomato genetics: Potato and tomato, being major crop species, have long histories of genetic and breeding research. Despite the fact that they are members of closely related genera, there has been little common ground between the two disciplines. Genetic maps based on a common set of DNA probes provide a basis for beginning to unite the two fields. Based on the comparative maps reported here, we now know which regions of the potato and tomato chromosomes are conserved, and where the differences occur. In tomato, more than 400 molecular markers have now been located on the genetic map (TANKSLEY *et al.* 1987; and unpublished data, this laboratory). Based on the position of the clones already mapped in potato, it is possible to predict the position of the additional 200 cloned markers from tomato. For practical purposes then, the potato map is comprised of the same number of loci mapped in tomato. Positions of unmapped isozyme loci in potato can also be predicted based on their position in the tomato map. The potato and tomato comparative maps have already provided a basis for determining the chromosomal localization of patatin-coding genes in both species (S. D. TANKSLEY, M. W. BONIERBALE and W. D. PARK, unpublished data).

Perhaps one of the most exciting outcomes of maps based on common clones will be the ability to ask questions about the homology and orthology of genes that have not yet been cloned and whose gene products are not known. For example, tomato and potato are common hosts to pathogens from a wide range of classes—including nematodes, viruses, bacteria and fungi (HOOKER 1981). Resistances to many shared pests have been identified among the genetic resources of both species, yet the relationship among resistance genes from the two species is unknown. Common map positions may be used to infer homology of resistance genes found in these two, heretofore, independent pools of resources, and theories concerning the inheritance and mechanisms of gene action proposed for one species may be suggested for the other.

Practical aspects of potato RFLP mapping: Prac-

tical applications of the reported linkage map to potato genetics and breeding will stem from the association of molecular markers with useful genes. The genetic resources of the potato are vast, and many sources of desirable traits have been identified in wild species (HANNEMAN and BAMBERG 1986), yet these resources have seldom been included in acceptable cultivars. Among the barriers which can impede the exploitation of wild species by conventional breeding methods are (1) the association of desired traits with unfavorable agronomic traits from unadapted sources, and (2) the complicated nature of the inheritance of certain desirable traits.

Genes controlling quantitatively inherited characters in tomato (OSBORN, ALEXANDER and FOBES 1987; TANKSLEY and HEWITT 1988), pepper (TANKSLEY and IGLESIAS-OLIVAS 1984), and maize (STUBER, EDWARDS and WENDEL 1987; EDWARDS, STUBER and WENDEL 1987) have been detected with molecular markers. The ability to detect, map, and monitor loci affecting both qualitative and quantitative traits by their linkage to molecular markers promises to increase the efficiency of their introgression into desirable genetic backgrounds. The mapping of genes of interest based on their linkage with RFLP markers may enhance selection in segregating populations by permitting a more exacting assessment of the genotype of individuals than is possible based on phenotype or progeny testing alone. Marker-enhanced selection may also be used to identify recombinant individuals which bear the genes to be introgressed with minimal amounts of flanking DNA (YOUNG and TANKSLEY 1988).

The fact that chromosome contents are conserved between potato and tomato suggests the possibility of producing chromosome substitution lines between the species. Reports have already been issued regarding somatic fusion of the two species (MELCHERS, SACRISTAN and HOLDER 1978). Chromosome substitution lines might be used as an intermediate step in the introgression of genes between the potato and tomato gene pools. Long stretches of homosequential chromosome segments could facilitate homologous recombination in meiosis of future chromosomal exchange lines that might be derived from somatic fusion products.

The heterozygous, polyploid constitution of the cultivated potato is probably the principal reason for the previous lack of a genetic linkage map. Although the current map was developed at the diploid level, the linear order of the loci detected is expected to be the same at the tetraploid level, and the map reported in this paper should therefore serve most genetic and breeding studies in the cultivated potato. A quality of RFLPs of particular importance to breeders of polyploid, cross-pollinating crops is their codominant nature. RFLPs will be a valuable tool for assessing allelic frequencies in individuals and monitoring genotypic shifts in populations. The selection of breeding parents with high plex levels for particular desirable alleles can be accomplished directly, and the ability of alternate breeding schemes to approach the goals of particular population improvement programs can be efficiently evaluated.

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J. E. Bradshaw · C. A. Hackett · R. C. Meyer
D. Milbourne · J. W. McNicol · M. S. Phillips
R. Waugh

Identification of AFLP and SSR markers associated with quantitative resistance to *Globodera pallida* (Stone) in tetraploid potato (*Solanum tuberosum* subsp. *tuberosum*) with a view to marker-assisted selection

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Abstract Seventy eight clones from the cross between SCRI clone 12601ab1 and cv Stirling were used to explore the possibility of genetical linkage analysis in tetraploid potato (*Solanum tuberosum* subsp. *tuberosum*). Clone 12601ab1 had quantitative resistance to *Globodera pallida* Pa2/3 derived from *S. tuberosum* subsp. *andigena*. The strategy adopted involved identifying single- (simplex) and double- (duplex) dose AFLP markers in the parents from segregation ratios that could be unambiguously identified in their offspring, detecting linkage between a marker and a putative quantitative trait locus (QTL) for resistance, and placing the QTL on the linkage map of markers. The numbers of scorable segregating markers were 162 simplex ones present only in 12601ab1, 87 present in Stirling, and 32 present in both; and 72 duplex markers present only in 12601ab1 and 45 present in Stirling. The total map length was 990.9 cM in 12601ab1 and 484.6 cM in Stirling. A QTL with a resistance allele present in double dose (QQqq) in 12601ab1 was inferred from the associations between resistance scores (square root of female counts) and two duplex markers linked in coupling, which, in turn, were linked in coupling to four simplex markers also associated with resistance, but to a lesser degree. The largest marker class difference was the one for the duplex marker P61M34 = 15. It accounted for 27.8% of the phenotypic variance in resistance scores, or approximately 30% of the genotypic variance. Subsequently, this duplex marker was found to be linked in coupling with a duplex SSR allele Stm3016 = a, whose locus was shown to be on chromosome IV in a diploid reference mapping population. The other QTLs for resistance segregating in the progeny were not identified for one or more of the following reasons: the markers did not cover the whole of the genome, there were unfavourable repulsion linkages between the QTLs and markers, or the gene effects were not large enough to be detected in an experiment of the size conducted. It is concluded that prospects appear good for detecting QTLs and using marker-assisted selection in a tetraploid potato breeding programme, provided that, in future, the population size is increased to over 250 and more SSR markers are used to complement the AFLPs; the same is likely to be true for other autotetraploid crops.

Key words Potato · *Solanum tuberosum* · Nematode resistance · *Globodera pallida* · Linkage in autotetraploids · Quantitative trait locus

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J. E. Bradshaw (✉) · R. C. Meyer · D. Milbourne
M. S. Phillips · R. Waugh
Scottish Crop Research Institute, Invergowrie,
Dundee DD2 5DA, UK

C. A. Hackett · J. W. McNicol
Biomathematics and Statistics Scotland,
Scottish Crop Research Institute, Invergowrie
Dundee DD2 5DA, UK

Introduction

The European cultivated potato (*Solanum tuberosum* subsp. *tuberosum*) is a tetraploid species (2n = 4*x* = 48) which displays tetrasomic inheritance. It was derived from a narrow genetic base of a few introductions of subsp. *andigena* from South America in the late 16th century, and possible further casual introductions in the 17th and 18th centuries (Bradshaw and Mackay 1994). As a consequence, it lacked genes for adequate levels of resistance to pathogens and pests such as late blight [*Phytophthora infestans* (Mont.) de Bary] and potato cyst nematodes (PCN) (*Globodera* spp.) which became problems once it had assumed its role as a staple food crop (Bradshaw and Mackay 1994).

During the 20th century, attempts have been made to remedy these deficiencies by the introgression of resistance genes into subsp. *tuberosum* from the wild and cultivated *Solanum* species of Central and South America (Hermsen 1994). However, a *Solanum demissum*-derived major gene resistance to late blight failed to give durable resistance (Malcolmson 1969) and the widespread deployment of the *H1* gene from subsp. *andigena* CPC1673 to control pathotype Ro1 (and Ro4) of the golden potato cyst nematode [*Globodera rostochiensis* (Woll.)] has inadvertently selected for the white potato cyst nematode [*Globodera pallida* (Stone)] (Bradshaw et al. 1996). More recently, cultivars with high levels of field resistance to late blight, such as Stirling and Torridon, have been bred from *S. tuberosum* germplasm derived from *S. demissum*, but they have not been widely grown (Wastie 1991; Bradshaw et al. 1995 a). Furthermore, high levels of quantitative resistance to *G. pallida* have also been incorporated into subsp. *tuberosum* from subsp. *andigena* (for example, SCRI clone 12601ab1), and to both nematode species from *Solanum vernei*, but only partial resistance has been achieved in successful cultivars such as Santé and Nadine (Bradshaw et al. 1996). The major gene resistances to *G. pallida* found to-date are only effective against some of the populations/pathotypes found in Europe; for example the *H2* gene for resistance to pathotype Pa1 from *Solanum multidissectum* (Dunnett 1961) and *Gpa2* for resistance to pathotype Pa2 from *S. tuberosum* subsp. *andigena* CPC1673 (Rouppe van der Voort et al. 1997).

Therefore, in Europe, there is a pressing need to combine the high levels of quantitative resistance to late blight and the white potato cyst nematode, available in subsp. *tuberosum*, with the commercially acceptable yields and qualities demanded by processors and supermarkets. Hence, it seemed timely to explore the possibility of genetical linkage analysis in tetraploid potato as a prerequisite to using molecular-marker-assisted selection to achieve this objective in a tetraploid breeding programme, without the need to spend time and effort on haploidisation, diploid analysis, and polyploidisation in order to avoid the complexities of tetrasomic inheritance. The efficient introgression of new resistances from wild species is a different issue, and is not the subject of this paper.

AFLPs (Vos et al. 1995) were chosen as the primary marker system because they allow a large number of segregating markers to be followed in a single experiment. They are based on the selective PCR amplifications of small restriction fragments (80–400 bp) of genomic DNA and are normally dominant markers. They have already been used in diploid potatoes for the construction of high-density linkage maps (Van Eck et al. 1995), the saturation mapping of potato chromosome VII around the *Gro1* gene for resistance to *G. rostochiensis* (Ballvora et al. 1995) and mapping the *Gpa2* gene for resistance to *G. pallida* (Rouppe van der Voort et al. 1997), as well as by Milbourne et al. (1997) for the analysis of genetical relationships in tetraploid potato cultivars. Subsequently, some of the highly polymorphic co-dominant SSR-based markers which are currently being developed for potato (Milbourne et al. 1998) were included to aid the chromosomal identification of a linkage group containing a putative quantitative trait locus (QTL) for resistance to *G. pallida*.

The cross between clone 12601ab1 and cv Stirling was chosen because these parents were known to have both high levels of resistance to *G. pallida* and *P. infestans*, respectively, and also high general combining abilities for these resistances (Bradshaw et al. 1995 b). The PCN results are presented and discussed in this paper; the blight results and more detailed theory on the construction of the marker linkage map can be found elsewhere (Hackett et al. 1998; Meyer et al. 1998).

Materials and methods

Plant material

The cross 12601ab1 × Stirling was one of a diallel set of crosses made in 1992 (Bradshaw et al. 1995 b). The reciprocal cross was made in 1993. Seedlings were raised from seed in a glasshouse in 10-cm-square pots during the summer of 1994 and tubers were harvested in mid-September from 331 plants (262 from the cross and 69 from its reciprocal). The 331 clones were maintained by growing two plants of each in 12.5-cm-square pots in a glasshouse in 1995, the spare tubers being used to raise plants for a foliage blight test. The maintenance plants were harvested in mid-September and the tubers kept in a dark store at 8°C until required. By mid-January 1996 they were just beginning to sprout. Two tubers of each clone were again planted for maintenance, leaving the remainder for use in testing for resistance to potato cyst nematodes (PCN).

PCN tests

There were sufficient tubers of 281 clones for them to be assessed for resistance to *G. pallida* Pa2/3 (Lindley) in a closed container test with four replicates, as described by Phillips et al. (1980). The test was set up on 13 and 14 February 1996 with one tuber per 60-ml Clearopac container unless the tubers were very small, when two were used. The numbers of females visible through the transparent walls of the containers were counted from 16 to 22 April 1996. Since small tubers of clone 12601ab1 and cv Stirling were not available at the same physiological age as those of the clones, they were assessed later in the year along with 18 clones covering the range of female counts found in the first test. This second test also comprised four replicates and was set up on 17 June 1996 and scored on 8 August 1996. The variance-stabilising square-root transformation was performed on the female counts before doing more detailed statistical analyses on the resulting PCN scores.

Mapping population and DNA extraction

A subset of 94 clones was randomly chosen for the initial mapping population from the first 211 of those available from the cross 12601ab1 × Stirling. Between 5 and 10 g of young, fully expanded

leaves were harvested from the plants grown in the glasshouse and freeze-dried. Total genomic DNA was extracted from the freeze-dried leaf material using the CTAB method of Saghai-Maroof et al. (1984). The quality and quantity of DNA were evaluated after ethidium-bromide staining of 0.8% agarose gels and UV transillumination.

AFLP assays

AFLP assays were performed using a modification of the protocol of Vos et al. (1995) as described by Milbourne et al. (1997). The 6-bp cutting enzymes *Eco*RI and *Pst*I were obtained from Boehringer Mannheim and the 4-bp cutting enzyme *Mse*I from New England Biolabs. T4 DNA ligase and T4 polynucleotide kinase came from GIBCO BRL. Ampli*Taq* and Ampli*Taq* LD DNA polymerase, as well as 1 × PCR buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl pH 8.3), were purchased from Perkin Elmer and the nucleotide-triphosphates from Pharmacia. Finally, AFLP primers and adapters were obtained from Genset, Paris. Autoradiograms were scored manually and independently by two people. Each band was treated as a locus with a dominant versus a recessive allele. Intensity differences were observed in segregating bands, but visual interpretation was not considered reliable enough to assign allelic dosage to a given clone.

SSR assays

Sufficient forward primer for 100 PCRs was 5′ end-labelled with γ^{32}P-ATP in a 20-µl reaction consisting of 0.3 µmol of primer, 30 µCi of 4500 Ci/mmol γ^{32}P-ATP (ICN), 30 U of T4 Polynucleotide Kinase (Gibco) and 1 × Gibco Forward Reaction Buffer (70 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 100 mM KCl, 1 mM 2-mercaptoethanol), incubated at 37°C for 1 h, after which the reaction was terminated by heating to 70°C for 10 min.

Ten-microliter PCRs consisted of 20 ng of genomic DNA, 1 × PCR buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl pH 8.3), 0.3 U of *Taq* polymerase (Boehringer-Mannheim), 0.3 µM of forward and reverse primers and 200 µM of dNTPs. The cycling conditions for PCR on a PE 9600 thermocycler or MJ Research DNA Engine were as follows: 94°C for 3 min, followed by 25 cycles of 94°C for 30 s, annealing temperature for 30 s, 72°C for 30 s, followed by 72°C for 5 min. Equal volumes of electrophoresis loading buffer (95% formamide, 10 mM EDTA, 0.5 mg/ml bromophenol blue and xylene cyanol) were added to the samples, which were then denatured at 95°C for 5 min, snap-cooled on ice, and subjected to polyacrylamide-gel electrophoresis on 6% acrylamide, 7 M Urea Easigel (Scotlab) in 1 × TBE (89 mM Tris-Borate, 2 mM EDTA). An M13 sequencing marker (prepared by performing the control reaction in the Sequenase V.2 sequencing kit from Amersham) was also run to estimate product sizes. Visualisation of results was achieved by exposure of fixed, dried gels to X-ray film.

Identification of segregation type

Markers occurring in just one parent were accepted as present in single- (simplex) or double-(duplex) dose if their observed segregation ratio was not significantly different from 1:1 or 5:1, respectively, as tested by a χ^2 goodness of fit with a 1% significance level. Markers occurring in both parents were likewise accepted as present in single-dose if their segregation ratio was not significantly different from 3:1, but this time at the 5% significance level to avoid overlap with markers giving the 11:1 segregation ratio expected from a duplex × simplex cross. These are the only segregation ratios that can be distinguished with the population size used (Bailey 1961). Although no double reduction (Fisher and Mather 1943) is assumed, its presence would not prevent the unambiguous identification of the three types of cross, Aaaa × aaaa, AAaa × aaaa and Aaaa × Aaaa, where A is the dominant marker present in either single- or double-dose. Histograms were plotted of the number of simplex and duplex AFLP bands from each parent carried by each clone in order to identify any selfs or anomalous results. As a consequence, 16 clones were eliminated from the experiment (see later), and the analyses repeated on the remaining 78 clones.

Linkage-map construction

Recombination frequencies and lod scores were calculated between pairs of markers as described by Hackett et al. (1998) and Meyer et al. (1998), using the statistical programme Genstat (Genstat 5 Committee 1993). These were then used for input to the linkage software, JoinMap 2.0 (Stam and Van Ooijen 1995) to obtain combined maps for each parent in which homologous groups were merged. The maps were printed with Drawmap (Van Ooijen 1994).

QTL analysis

For all segregating markers, a single-marker analysis was performed by determining the difference in mean PCN scores between the two marker classes, presence and absence of marker, and evaluating its significance by a *t*-test. All differences significant at the 5% level were noted, whilst recognising that some of them could have arisen by chance. Hence, a more appropriate threshold value for declaring a significant QTL effect was sought through the empirical method of Churchill and Doerge (1994). This was done for the markers segregating 1:1 and 5:1 from each parent and for those segregating 3:1, making five sets of analyses in total. In each analysis, the PCN scores for the 78 clones were shuffled, analysed for QTL effects at all markers in the set, the largest *t*-value stored, and the entire procedure repeated 1000 times. The stored *t*-values were then ordered and the 95% quantile taken as the estimated critical value at which the overall type-I error rate for the experiment was 0.05 or less. The differences between marker classes are functions of the frequency of recombination (i.e. recombinant chromatids) between the marker and the QTL and the genotypic values of the genotypes at the QTL (there could be up to eight different alleles segregating), but these can not be estimated from a simple single-marker analysis.

Results

AFLP marker analysis

Thirty nine AFLP primer combinations allowed 3173 loci to be surveyed and, of the 1066 markers which segregated in the population, 573 were considered scorable with the required accuracy. The percentage of informative loci was not significantly different between the *Eco*RI and *Pst*I templates, but the *Pst*I autoradiograms were much clearer and easier to score than the *Eco*RI gels due to a reduced number of bands per gel.

Although analysis of the parental source of markers in the 94 clones failed to detect any selfs, it did highlight a distinct group of 16 clones with an unusually low number of paternal products (Fig. 1). As there was no obvious experimental or genetical explanation for the origin of these distinct clones, they were omitted from

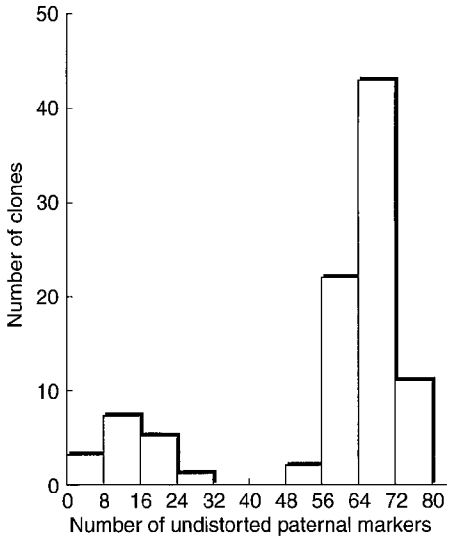


Fig. 1 Low number of paternal markers in 16 out of 94 clones from the cross 12601ab1 × Stirling

the subsequent marker, QTL, and linkage analyses which were, therefore, done on the remaining 78 clones. This left 559 scorable, segregating markers, of which 446 were present in one parent and absent in the other, while 113 were present in both parents.

Figure 2 shows the observed segregation ratios for the 446 markers which were present in one parent and absent in the other, and the theoretical distributions for 284 markers segregating 1:1 (53:25 is more likely to be a 1:1 than a 5:1 ratio) and 162 markers segregating 5:1 (54:24 is more likely to be a 5:1 than a 1:1 ratio). For a population size of 78, one would expect a clearcut separation of single- and double-dose markers, but this was not observed. Therefore, the linkage analyses were confined to markers that could be accepted as present in single- or double-dose on the criteria given earlier, whilst recognising that extreme but acceptable ratios appeared to be more numerous than expected by chance. These markers amounted to: 162 simplex markers present only in 12601ab1; 87 present in Stirling; and 32 present in both; and 72 duplex markers present only in 12601ab1 and 45 in Stirling.

AFLP linkage map

The map for 12601ab1 comprised 17 groups of four or more markers and 13 groups of just two or three markers with 35 out of 266 markers remaining unlinked (8/162 single-dose 1:1 markers, 10/72 double-dose 5:1 markers, and 17/32 single-dose 3:1 markers). The sizes of the linkage groups ranged up to 96.6 cM, with a total length of 990.9 cM.

The map for Stirling comprised nine groups of four or more markers and 17 groups of just two or three markers with 47 out of 164 markers remaining unlinked (12/87 single-dose 1:1 markers, 19/45 double-dose 5:1 markers, and 16/32 single-dose 3:1 markers). The sizes of the linkage groups ranged up to 73.0 cM, with a total length of 484.6 cM.

The parental maps contained seven bridging (3:1) markers which allowed seven maternal groups to be identified as homologous to seven paternal groups.

PCN scores

The histogram of PCN scores for the 78 clones is shown in Fig. 3. The differences between clones were statistically significant ($P < 0.001$), and an analysis of the components of variation (between clones and clones × replicates interaction) revealed that 93.7% of the observed variation was genetical. The correlation between the scores of the 18 clones in the first and in the second test was $r = 0.859$ ($P < 0.001$) and the regression equation, test 1 = 0.895 + 1.23 test 2, allowed the

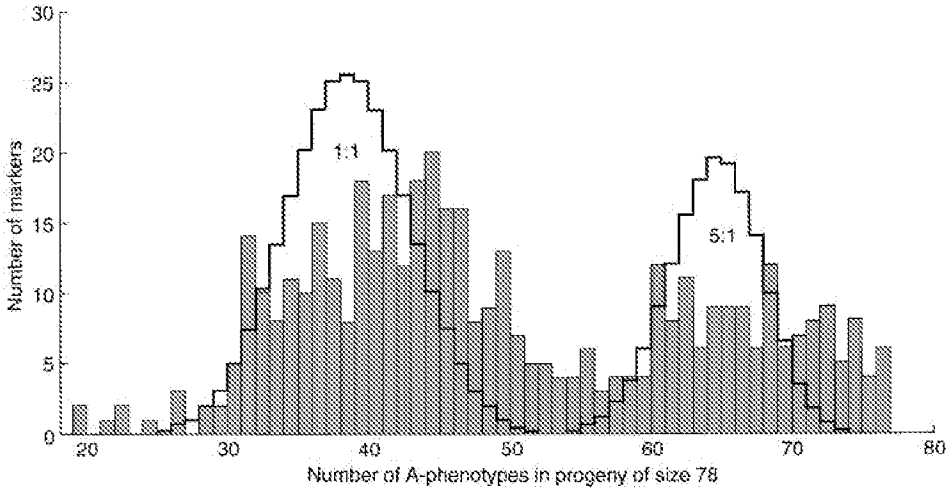


Fig. 2 Observed and expected (1:1 and 5:1) segregation ratios for 446 markers present in one parent in either single-(Aaaa) or double-dose (AAaa) and absent in the other (aaaa)

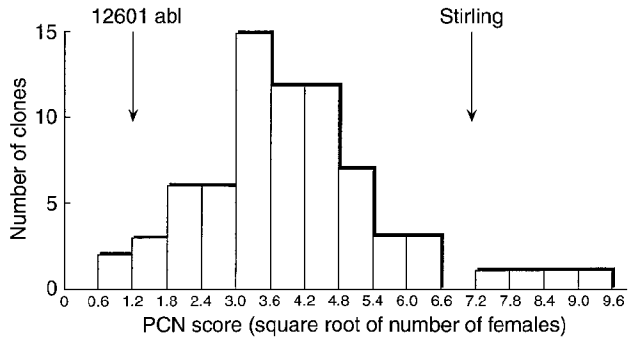


Fig. 3 PCN (*G. pallida*) scores of 78 clones from the cross 12601ab1 (resistant) × Stirling (susceptible)

parental values to be estimated as 1.203 for 12601ab1 and 7.102 for Stirling. These are also shown in the histogram.

QTL analysis

The single-marker analysis detected a number of associations between PCN scores and AFLP markers when the significance level was 5% ($t \approx 2.00$): 12 (1:1), 7 (5:1) and 1 (< 5:1) in 12601ab1, 12 (1:1), 4 (5:1) and 4 (> 5:1) in Stirling, and 3 (3:1). However, just 2 (5:1) associations in 12601ab1, together with the 1 (< 5:1) association, remained significant on using the more stringent *t*-value of 3.49 found by the method of Churchill and Doerge (1994).

Table 1 PCN resistance associated with AFLP markers and SSR marker Stm3016 on chromosome IV (b and c are homologous chromosomes)

Marker (−single dose, = double dose)	*t*-test	Percentage variance accounted for	PCN score	
			Marker present	Marker absent
IVb				
Stm3016 = a	− 4.38***	21.1	3.62	5.44
P46M37 − 5	− 2.47*	6.6	3.55	4.45
P61M34 = 15	− 5.36***	27.8	3.60	6.01
E34M61 − 27	-1.87^{NS}	3.4	3.69	4.43
E44M42 = 6	− 4.84***	23.8	3.63	5.86
E35M37 − 6	-1.90^{NS}	3.5	3.65	4.36
E39M42 − 22	− 2.11*	4.7	3.65	4.45
E38M39 − 37	-1.40^{NS}	1.3	3.71	4.24
E31M61 = 17	-0.34^{NS}	0.0	3.95	4.21
E35M121 − 2	-1.22^{NS}	0.7	3.77	4.24
E34M121 = 5	-0.15^{NS}	0.0	3.96	4.06
P40M34 − 11	− 2.28*	5.8	3.65	4.53
E46M37 = 9	-0.56^{NS}	0.0	3.94	4.27
P61M47 − 1	-1.16^{NS}	0.5	3.81	4.27
IVc				
Stm3016 = a	− 4.38***	21.1	3.62	5.44
P61M34 = 15	− 5.36***	27.8	3.60	6.01
E44M42 = 6	− 4.84***	23.8	3.63	5.86
P46M37 − 4	− 2.22*	5.2	3.55	4.37

***$P < 0.001$; **P 0.01–0.001; *P 0.05–0.01; NS, not significant

The two duplex markers associated with PCN resistance, P61M34 = 15 and E44M42 = 6, were closely linked in coupling (Table 1 and Fig. 4). Furthermore, they were also linked in coupling to four simplex (1:1)

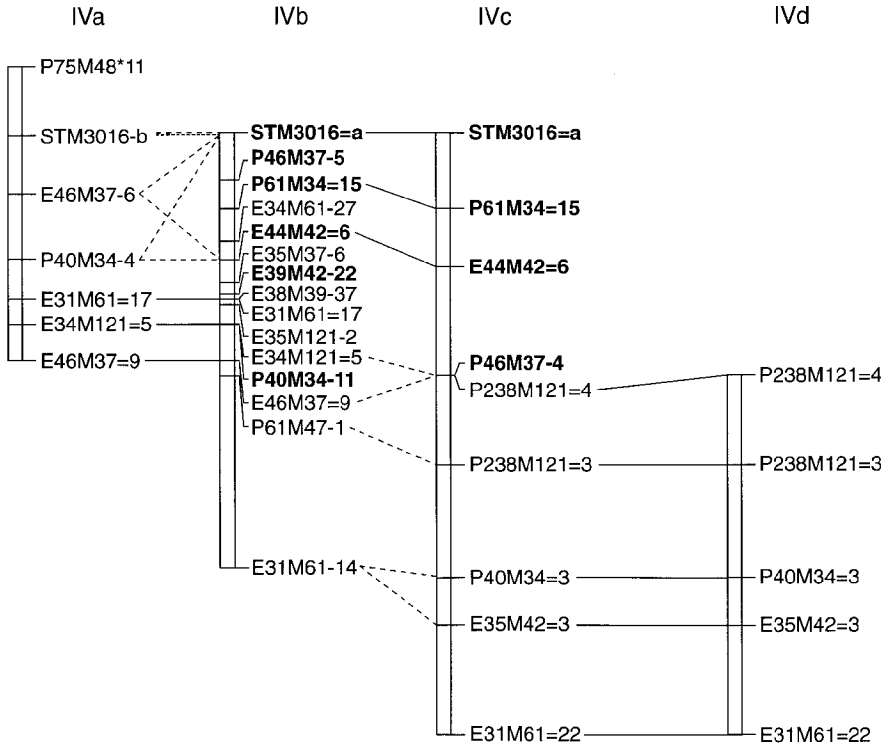


Fig. 4 AFLP markers (− simplex, = duplex, ∗ simplex also present in Stirling) and SSR locus (STM3016) on four homologues of chromosome IV (– – – repulsion linkage of simplex and duplex marker, —duplex marker, - - - alleles at SSR locus) in the female parent 12601ab1 and their association (*bold print*) with PCN resistance

markers which were associated with PCN resistance by the *t*-test. Three of these markers, P46M37–5, E39M42–22 and P40M34–11, were linked to each other in coupling, while the fourth (P46M37–4) was on a homologous chromosome. As all of these markers were associated with increased resistance, one can postulate that a QTL with an allele for PCN resistance in double-dose (QQqq) was present in the 12601ab1 parent and that the two copies of the allele were linked to the duplex and simplex markers in duplex/duplex and simplex/duplex coupling, respectively (AQ/AQ/aq/aq × aq/aq/aq/aq where A is P61M34 = 15).

It is not possible to obtain an accurate estimate of the magnitude of the effect of the resistance gene because the marker class difference ($\overline{\text{A---}} - \overline{\text{aaaa}}$) of 2.41 for marker P61M34 = 15 depends on four unknown parameters, the recombination frequency (r) and three genotypic values (μ):

$$\overline{\text{A---}} - \overline{\text{aaaa}} = \tfrac{1}{5}[(1 - 6r^2)\mu_{\text{QQqq}} + 4(1 - 3r + 3r^2)\mu_{\text{Qqqq}} - (5 - 12r + 6r^2)\mu_{\text{qqqq}}].$$

However, one can say that marker P61M34 = 15 accounted for 27.8% of the variance in PCN scores (Fig. 5).

The third marker associated with PCN resistance, E39M42 = 11, was not placed on the linkage map because it did not make the criterion for acceptance as segregating 5:1. It accounted for 14.7% of the variance in PCN scores, but one can not rule out the possibility that it may be linked to the markers just described.

The lack of associations between the PCN scores and the other markers in the linkage group (Table 1 and Fig. 4) can be attributed to unfavourable linkage relationships between the markers and the two copies of the resistance allele.

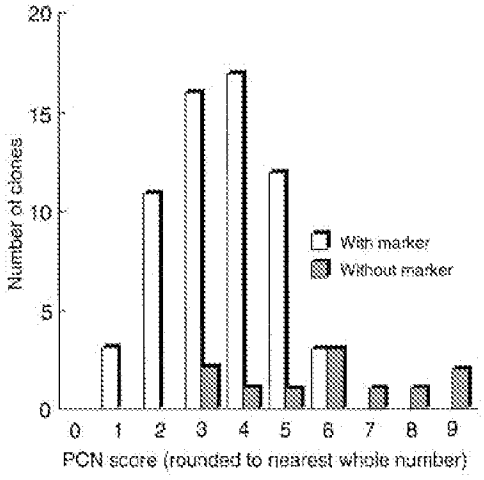


Fig. 5 PCN (*G. pallida*) scores (square root of number of females rounded to nearest whole number to give a 0 to 9 scale) of clones with and without the AFLP marker P61M34 = 15 which was present in a double dose in female parent 12601ab1 but was absent from the male parent Stirling

Chromosomal identification of the linkage group containing PCN resistance

Subsequently, newly developed SSR-based markers (Milbourne et al. 1998) were included to aid the chromosomal identification of the linkage group containing the putative QTL for resistance to *G. pallida*. Of 79 alleles from 36 loci, 34 segregated 1:1, 13 segregated 5:1, and 20 segregated 3:1. The single-marker analysis detected eight associations between PCN scores and these SSR alleles when the significance level was 5% ($t \approx 2.00$), but just one association remained significant with the more stringent *t*-value used for the AFLP markers. It involved allele Stm3016 = a which segregated 5:1 and was linked in coupling with the AFLP duplex marker P61M34 = 15 (Table 1 and Fig. 4). Furthermore, the Stm3016 locus was mapped in the diploid potato population of Gebhardt et al. (1991) and this allowed the linkage group to be identified as chromosome IV.

Discussion

The use of AFLPs allowed a large number of segregating markers to be followed in a single experiment. The occurrence of 117 markers displaying a 5:1 segregation ratio confirmed that *S. tuberosum* is a tetraploid which displays tetrasomic inheritance. The large number of markers also revealed 16 anomalous clones which would not have been detected by their phenotypes for a single trait, although they did resemble the female parent for PCN scores. More experimental work is needed to determine the origin of these clones as there is no obvious explanation in terms of selfing, contamination, or experimental error. A detailed study of meiosis in the parents and offspring is one possible line of inquiry. It was also clear from the segregation ratios of all of the markers present in one parent and absent in the other, that there were departures from the theoretical separation into two groups segregating 1:1 and 5:1. Again, further work is required to determine the causes but, assuming no misclassification of markers, three possibilities are worthy of investigation. Although potatoes have small chromosomes with few chiasmata per chromosome, double reduction is known to occur, an example being the inheritance of resistance to Potato Virus Y (Mendoza et al. 1996). Double reduction would be expected to reduce the mean number of A--- phenotypes in a progeny of size 78 (Fig. 2) from 39 towards 35.75 (simplex markers) and from 65 towards 60.67 (duplex markers), and also create a new peak at or above 74.75 (triplex markers), but one would still expect two non-overlapping distributions. The second possibility is departures from tetrasomic inheritance due to non-random pairing of homologous chromosomes, with the extreme being disomic inheritance. In

other words, a marker present in one parent in double-dose could segregate with a ratio between 5:1 (65:13) and 3:1 ($58\frac{1}{2}$:$19\frac{1}{2}$). This might occur in regions of the genome where genes from wild species have been introgressed into subsp. *tuberosum*. However, in order to get the apparent excess of markers between a 1:1 (39:39) and 3:1 ratio (Fig. 2), one would need linked duplicate genes (loci 1 and 2) under tetrasomic ($A_1A_2/a_1a_2/a_1a_2/a_1a_2 \times a_1a_2/a_1a_2/a_1a_2/a_1a_2$) or disomic inheritance ($A_1A_2/a_1a_2 \times a_1a_2/a_1a_2$), which is unlikely with AFLP markers. The third possibility is distorted segregation from the linkage of markers to genes affecting survival ability, a likely occurrence in an outbreeding crop like potato, which is known to suffer inbreeding depression. Distorted segregation ratios have already been reported in diploid genetical studies with potatoes (Bonierbale et al. 1988; Gebhardt et al. 1991). Clones and markers displaying anomalous results were eliminated from the further analyses in order to avoid unnecessary difficulties of interpretation.

The PCN scores displayed continuous variation (Fig. 3), as previously found by Dale and Phillips (1982) in a cross with this source of *G. pallida* resistance from subsp. *andigena* (Howard et al. 1970). One genetical component of this resistance has been identified in this present experiment, a gene in 12601ab1, in the duplex state, linked in coupling to marker P61M34 = 15, also in the duplex state, and accounting for 27.8% of the phenotypic variance in PCN scores, or approximately 30% of the genotypic variance. It is perhaps not surprising that the resistance gene was present in the duplex state as it could have been inherited from both the maternal and paternal grandparent of 12601ab1 {[*H1H3*(6) × P. Ivory] × [*H1H3*(32) × P. Ivory]}, where the *H1H3* clones were from crosses between the subsp. *andigena* source and subsp. *tuberosum*. It is also clear from Fig. 5 that this gene is necessary for high levels of resistance to *G. pallida*. Hence it might be worthwhile cloning and sequencing the AFLP fragment corresponding to the duplex marker P61M34 = 15 in order to develop an STS primer for use in marker-assisted selection and for confirming the chromosomal location of the gene on chromosome IV of the published diploid map of potato (Gebhardt et al. 1991), as already done by Meyer et al. (1998) for a blight resistance QTL. However, the SSR marker Stm3016 = a may prove even more suitable for marker-assisted selection.

As there is synteny between the 12 chromosomes of potato and tomato (Gebhardt et al. 1991; Tanksley et al. 1992; Milbourne et al. 1998), one can tentatively conclude that the *Gpa* QTL from 12601ab1 is on the same arm of chromosome IV (near GP221) as the *Hero* gene (near GP180) which gives a wide spectrum of resistance against *G. rostochiensis* in tomato (Ganal et al. 1995). Other genes for PCN resistance discovered to-date are as follows. Kreike et al. (1994) found that about 50% of the variation in quantitative resistance to *G. pallida* pathotypes Pa2/3 from *Solanum spegazzinii* could be explained by a major locus *Gpa* on chromosome V, whereas two minor loci on chromosomes IV and VII accounted for less than 10% each. The major dominant gene *Gpa2* for resistance to pathotype Pa2 of *G. pallida* has been mapped at the distal end of chromosome XII (Rouppe van der Voort et al. 1997). Dominant major genes for qualitative resistance to *G. rostochiensis* pathotype Ro1 have been mapped to chromosome V: *H1* from *S. tuberosum* ssp. *andigena* (Gebhardt et al. 1993; Pineda et al. 1993) and *GroV1* from *Solanum vernei* (Jacobs et al. 1996); and to chromosome VII: *Gro1* from *S. spegazzinii* (Barone et al. 1990). The *H1* gene was in fact present in single dose in 12601ab1 and was found to be linked in coupling to AFLP markers E40M42-1 and P238M61-1, at 21 ± 4.7 cM from the latter (data not presented). It remains to be seen if other chromosomes contain genes for resistance to potato cyst nematodes.

Although cv Stirling was not the source of PCN resistance in the population under study, clones more susceptible than Stirling are known (Bradshaw et al. 1995 b) and hence one can not rule out the possibility that some of the variation in PCN scores came from Stirling, although no associations with Stirling markers were clearly identified.

There are a number of possible reasons which alone, or in combination, could explain why the other QTLs which must have been segregating for PCN resistance were not identified in this experiment. They could have been missed because the markers did not cover the whole of the genome. The total tetraploid female map length of 990.9 cM is only about 25% of the genome, based on a known total diploid map length of 1120 cM (Jacobs et al. 1995). They could also have been missed because of unfavourable linkages between the QTLs and markers, for example, AQ/Aq/aQ/aq. Indeed, in Table 1 and Fig. 4, one can see the importance of favourable linkages for the detection of a QTL and markers suitable for marker-assisted selection in an autotetraploid. The production of dihaploids from 12601ab1 and QTL analysis in appropriate diploid crosses would make detection easier, but one would then encounter problems in deciding if any markers found linked to QTLs were suitable for marker-assisted selection in the tetraploid population because dihaploid AQ/aq could have come from the suitable linkages AQ/AQ/aq/aq and AQ/aq/aq/aq, the less suitable AQ/aQ/aq/aq, or the unsuitable AQ/aQ/Aq/aq and AQ/Aq/aq/aq. Similar problems would occur if 12601ab1 was crossed to tetraploid clones containing the marker A, but lacking PCN resistance. However, these problems may be overcome as more of the highly polymorphic co-dominant SSR-based markers which are currently being developed for potato become available (Milbourne et al. 1998). A third possibility is that gene effects were not large enough to be detected in an experiment of the size conducted. If, as found by the

permutation test, a t-value of 3.32 is required to declare a significant association with a marker segregating 1:1, then the smallest marker class difference that could have been detected with a population of 78 clones would be about 1.20, based on the standard error of 0.362 for the largest difference actually found of 0.98. For comparison, the difference in PCN scores between 12601ab1 and Stirling was 5.90. Doubling the population size would reduce the detectable difference from 1.20 to 0.85, and doubling it again would reduce it to 0.60. Hackett et al. (1998) concluded that a population size of at least 150 individuals should be used in future to develop a linkage map in an autotetraploid species and that a larger number, say 250, would provide a better chance of identifying homologous chromosomes. These population sizes would also seem sensible for detecting the major QTLs segregating in a tetraploid population.

Therefore, with an increased population size of 250 and SSR markers to complement AFLPs, prospects appear good for marker-assisted selection in a tetraploid breeding programme. Indeed, the necessary development work could be done as part of the second stage of the breeding strategy advocated by Bradshaw and Mackay (1994) for clonally propagated potatoes. Namely, having used progeny tests to identify the best crosses, 250 seedlings of each are raised from residual seed in a glasshouse for subsequent clonal evaluation and cultivar production. As selection is not practised in the glasshouse, such populations are ideal for phenotypic and marker evaluation with a view to marker-assisted selection in later clonal generations, in choosing parents for the next round of crossing, and in subsequent cycles of recurrent selection.

Finally, given these encouraging results, it would seem worthwhile developing the theory and practice of QTL analysis in autotetraploids by considering the effects of distorted ratios and double reduction in more detail, as well as the prospects for more sophisticated approaches to locating QTLs and determining their effects using marker means and individual observations in the marker regression and interval mapping techniques already developed in diploids (Kearsey and Pooni 1996).

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C. R. Brown · C.-P. Yang · H. Mojtahedi · G. S. Santo
R. Masuelli

RFLP analysis of resistance to Columbia root-knot nematode derived from *Solanum bulbocastanum* in a BC_2 population

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Abstract The mapping of resistance to *Meloidogyne chitwoodi* derived from *Solanum bulbocastanum* is reported. A population suitable for mapping was developed as follows. A somatic hybrid of nematode-resistant *S. bulbocastanum* and cultivated tetraploid potato was produced. This was backcrossed to tetraploid potato, and a single resistant BC_1 was selected and backcrossed again to the same recurrent tetraploid parent. The mapping population consisted of 64 BC_2 progeny scored for restriction fragment length polymorphic (RFLP) markers and 62 of these were evaluated for the reproductive efficiency of race 1 of *M. chitwoodi*. Forty-eight polymorphic RFLP markers, originally derived from tomato and mapped in diploid cultivated potato, were assigned to 12 chromosomes of *S. bulbocastanum*. Of the 62 progeny screened for nematode resistance, 18 were non-hosts and four were poor hosts. The rest were highly susceptible (good hosts). Analysis of the resistance (including non-hosts and poor hosts) as both a qualitative trait and as a meristic trait on which QTL analysis was applied supported the same genetic hypothesis. Genetic control was localized solely to factor(s) lying at one end of chromosome 11. The level of expression of resistance in the *S. bulbocastanum* parent and the resistant portion of the BC_2 was essentially the same. This fact, together with the highly significant LOD scores for one end of the chromosome-11 marker array, supports a genetic model equivalent to monogenic dominant control.

Key words Wild species · Introgression · *Meloidogyne chitwoodi* · Gene mapping · Potato resistance breeding · QTL

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C. R. Brown (✉) · C.-P. Yang
USDA/Agricultural Research Service, 24106 N. Bunn Rd., Prosser, WA 99350, USA

H. Mojtahedi · G. S. Santo
Washington State University, Irrigated Agriculture Research and Extension Center, 24106 N. Bunn Rd., Prosser, WA 99350, USA

R. Masuelli
Universidad Nacional de Cuyo,Chacra de Coria, Mendoza, Argentina

Introduction

Molecular markers have expanded the analytical capability of geneticists by providing virtually unlimited polymorphism spanning the entire genome. This is especially useful in the breeding of crops where associations of markers with phenotypes can be utilized in an analytical fashion to select progeny and parents for crossing (Tanksley 1993). The introgression of traits from wild relatives is one area where the slow rate of progress, and the high rate of failure, in trait transfer invites the use of polymorphic molecular markers to analyze donor trait introduction and the nature of its incorporation into the cultivated genome.

Columbia root-knot nematode (*Meloidogyne chitwoodi* Gold) is an important pest of potato (*Solanum tuberosum* L.) in the Pacific Northwest of the United States, and a potentially important pest in the Netherlands (Evans and Trudgill 1992). Several studies have revealed resistance to two of the three races of the nematode in wild tuber-bearing *Solanum* species (Brown et al. 1989, 1991, 1994), while extensive screening in cultivated potato has failed to find resistance (Hoyman 1974; Martin, unpublished). *Solanum bulbocastanum*, a Mexican diploid (2n = 24) which is difficult to cross with cultivated potato, was found to have resistant accessions (Brown et al. 1989). Cultivated tetraploid (2n = 48) *Solanum tuberosum* ssp *tuberosum* and diploid *S. bulbocastanum* were combined by protoplast fusion to produce hexaploid (2n = 72) somatic hybrids (Austin et al. 1993). Nematode-resistant F_1 hybrids were crossed to cultivated tetraploid potato and nematode resistant BC_1 introgressants were verified (Brown et al. 1994, 1995). The present study presents the localization of resistance to race 1 of *M. chitwoodi* on a restriction fragment length polymorphism (RFLP) map of *S. bulbocastanum* chromosomes assigned in a BC_2 progeny population segregating for nematode resistance and polymorphic markers.

Materials and methods

Pedigree

The derivation of the segregating population used to establish polymorphic markers and assemble the RFLP map is shown in Fig. 1. Nematode resistance was detected in several accessions of *S. bulbocastanum* (Brown et al. 1989). A single selected nematode-resistant clone of *S. bulbocastanum* was somatically hybridized with cultivated tetraploid potato 203900 obtained from NRSP-6, Potato Introduction Station, Sturgeon Bay, Wisconsin. A somatic hybrid, designated CBP233, found to be resistant to the nematode (Austin et al. 1993; Brown et al. 1994), was hybridized with a second cultivated tetraploid, A84118.3, obtained from Dr. J. Pavek, USDA/ARS, Aberdeen, Idaho. A single nematode-resistant BC_1 individual, DG 17, was hybridized to A84118.3 again to produce the BC_2 (Brown et al. 1995). Markers were scored and resistance was measured within this population of BC_2 progeny.

DNA extraction and hybridization with probes

Fresh leaves, 20–35 g, from greenhouse-grown plants were macerated in a blender with ice-cold buffer and DNA extracted, according to Bonierbale et al. (1988). Ten micrograms of purified potato nuclear DNA was digested separately with 30–50 units of the restriction enzymes (RE) *Dra*I, *Eco*RV, *Bam*HI, *Pst*I, *Xba*I, *Hin*dIII, or *Eco*RI following manufacturers' recommendations. Digested DNA was run at 80 mA in 0.9% agarose gels for 14–16 h in NEB (neutral electrophoresis buffer, Tris, 10 mM; EDTA, 1 mM; sodium acetate, 1.25 mM; adjusted to pH = 8.1). The gel was de-purinated with 0.25 N HCl (aq) for 10 min and rinsed with distilled water. DNA was transferred to Amersham Hybond-N-plus (RPN.203N) with 0.4 N NaOH (aq) overnight using capillary action. After quick plasmid preparations were performed (Birnboim and Doly 1979; Kraft et al. 1988) plasmids containing the RFLP probes as inserts were oligo-labelled using the Multiprime DNA labelling system (RPN.1601Y, Amersham) with ^{32}P-dCTP. Hybridization was carried out at 65 °C overnight and the filter washed to high stringency starting at 2 × SSC plus 0.1% SDS for 30 min and completed at 0.5 × SSC plus 0.025% SDS.

Mapping

Markers were obtained from Dr. S. D. Tanksley, Department of Biometry and Plant Breeding, Cornell University, Ithaca, New York. These consisted of cDNA clones from the tomato genome previously mapped to locations in a diploid cultivated potato × wild potato F_1 progeny (Tanksley et al. 1992). Markers were selected on the basis of providing coverage of the 12 homologues of potato. This allowed an initial identification of the chromosome(s), and regions thereon, that co-segregate with nematode resistance. As a first step, Southern analysis was performed on batches of genomic DNA of the resistant *S. bulbocastanum* parent and the susceptible cultivated potato parents restricted with different REs, using the cDNA markers as probes. Polymorphism was identified for particular marker × RE combinations, with an emphasis on the size fragments unique to *S. bulbocastanum*.

Linkages and map distances were calculated using the computer program MAPMAKER/EXP (Lander et al. 1987). Determination of LOD scores for quantitative data of nematode resistance was calculated using MAPMAKER/QTL (Paterson et al. 1988).

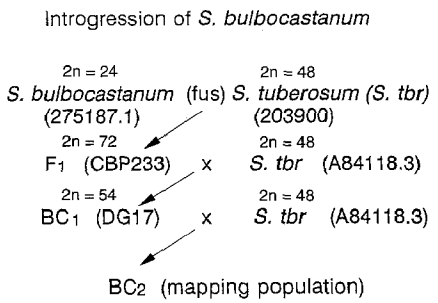


Fig. 1 Derivation of the BC_2 mapping population

Nematode inoculation

Rooted cuttings of 62 BC_2 progeny, the resistant *S. bulbocastanum* and susceptible recurrent parents 203900 and A84118.3, approximately 7 cm tall, were transplanted into plastic pots (10 cm diameter) containing loamy sand (84% sand, 10% silt, and 6% clay) and were fumigated with methyl bromide. The nematode population used in this experiment, WAMc1 (*M. chitwoodi*, race 1), was maintained at the Washington State University-Irrigated Agriculture Research and Extension Center, Prosser, Wash., collection (Pinkerton et al. 1987). Inoculum was derived from single-egg mass cultures, and prepared by collecting eggs after shaking infested roots of tomato (*Lycopersicon esculentum* Mill cv Columbia) in 0.5% NaOCl (Hussey and Barker 1973). At transplanting, an aliquot containing 5000 eggs was pipetted into depressions in the soil around the base of the plants of five replicated pots arranged in a completely randomized design. Potato (cv Russet Burbank), tomato (cv Columbia), alfalfa (*Medicago sativa* L., cv Thor), and wheat (*Triticum aestivum* L., cv Gaines) were included as experimental entries. Potato and tomato, suitable hosts for *M. chitwoodi*, were used as susceptible standards. Alfalfa, a non-host for race 1, was included to detect cross-contamination by race 2 of *M. chitwoodi*, for which it is a suitable host; while wheat, a non-host for *M. hapla*, was used to check against this possible contaminant. Plants were grown with regular watering and fertilization at 24 ± 3 °C for 55 days, and eggs were extracted and counted (Hussey and Barker 1973).

Chromosome counts

Root tips for chromosome counts were taken from cuttings rooted in vermiculite. Root tips measuring 3 mm in length were immersed in distilled water at 1 °C for 24 h. They were then placed in fixative (3:1, 95% ethanol: glacial acetic acid) for 24 h and transferred to 70% ethanol for long-term storage at 4 °C. Root tips were placed in 1 N HCl (aq) at 55 °C for 8 min, washed with tap water, placed in a drop of acetocarmine (45% acetic acid with 2% w/v carmine), macerated, and heated gently over an alcohol flame before and after a cover slip was placed over the tissue which was smeared by tapping the coverslip sharply with the eraser tip of a pencil. Chromosome counts were made at 1000 ×.

Results

Development of the map

The RFLP markers had been previously selected to provide broad coverage of the linkage groups. Forty-eight markers were placed on 12 linkage groups (Fig. 2). Four markers were not associated with any groups and two others were closely linked to each other but did not lie on any of the established linkage groups. Four of the forty eight markers were established in linkage groups that differed from their assignment in the diploid potato linkage map (Tanksley et al. 1992). The marker TG20 changed from chromosome 7 to 1; TG176 from chromosome 8 to 1; TG71 from chromosome 1 to 4; and TG275 from chromosome 6

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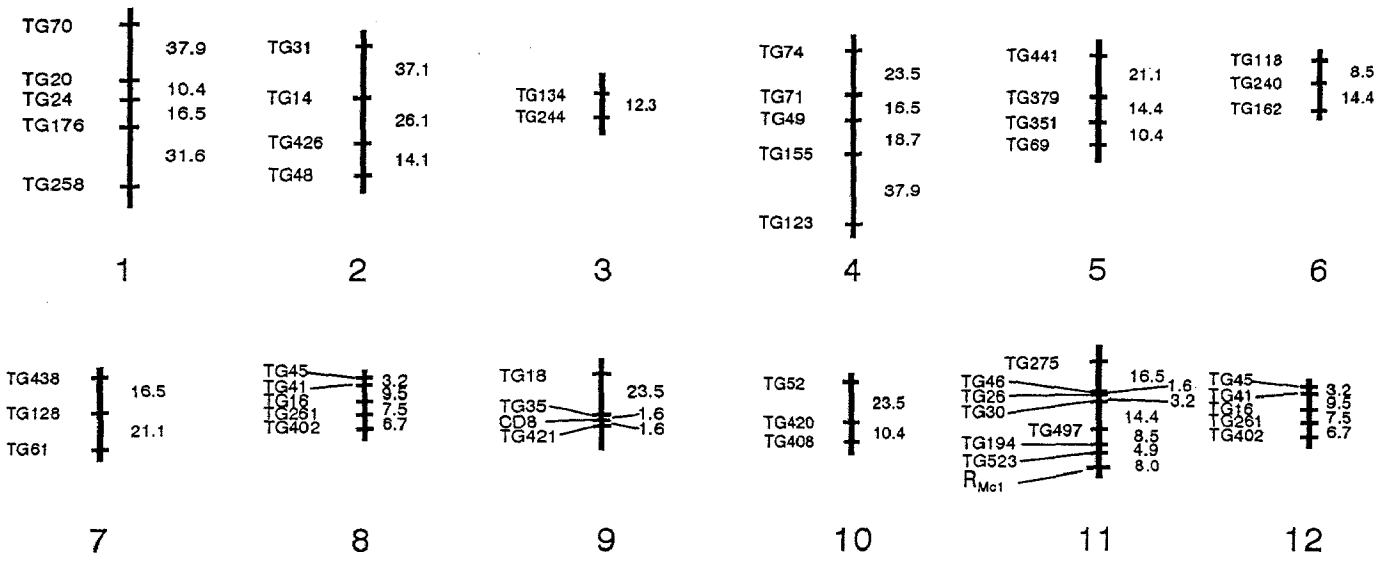


Fig. 2 RFLP map of *S. bulbocastanum* with the assignment of the putative *M. chitwoodi* resistance locus, R_{Mc1}, to chromosome 11

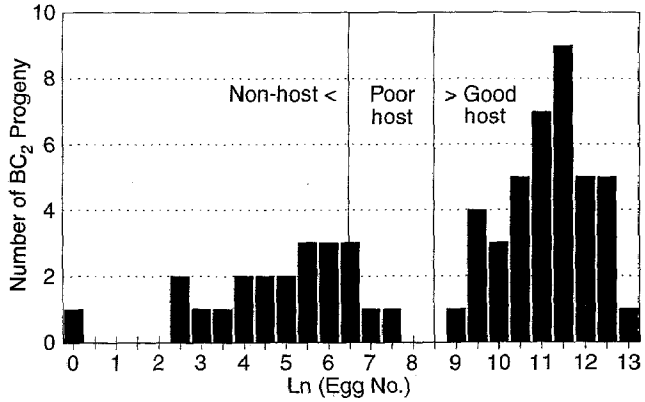


Fig. 3 Distribution of Ln (no. eggs) for 62 BC_2 progeny. Host suitability regions are indicated

to 11. This might indicate a disruption of synteny between *S. bulbocastanum* (Series Bulbocastana) and cultivated potato (Series Tuberosa; *S. bulbocastanum* has in fact been found to be a genetic outlier in several studies of genetic relationship (Bonierbale et al. 1990; Debener et al. 1990). The homosequentiality between *S. bulbocastanum* and cultivated potato and/or tomato is, however, sufficient to serve as a guide for the selection of markers.

The F_1 somatic hybrid was determined to have a 2n = 72 constitution. The BC_1 parent selected as the parent of BC_2 had 2n = 54.

Pattern of segregation of nematode resistance

Reproductive efficiency (R_f=final population/initial population) depends on the fecundity of a proportion of the primary inoculum and the intermediate generations that successfully infest the host and reach reproductive maturity. Reproductive efficiency is one measure of resistance of a host crop to species of *Meloidogyne* (Oostenbrink 1966; Sasser et al. 1984). This measurement was chosen as the resistance parameter in the present study. Host status is generally divided into three categories on the basis of R_f values as follows: $R_f > 1.0$, suitable host; $0.1 < R_f < 1.0$, poor host; $R_f < 0.1$, non-host (Sasser et al. 1984).

The population of 62 BC_2 individuals separated into three groups. Eighteen progeny were classified as non-hosts ($R_f < 0.1$) and four as poor hosts ($0.1 < R_f < 1.0$), while the remainder were classified as good hosts ($R_f > 1.0$). The distribution of the mean natural logarithm of nematode counts of the progeny is shown in Fig. 3. The data were used as a meristic trait for quantitative trait analysis. For convenience, the graph is divided into the three host suitability categories discussed above.

The treatment of Ln (no. of eggs) as a quantitative trait locus (QTL) is presented in Fig. 4. LOD scores were only significant for certain markers on chromosome 11. Resistance factor(s) appear to be located unambiguously on the left-hand portion of the map of chromosome 11 as depicted in Fig. 4. Resistance was also treated as a discrete trait where BC_2 progeny exhibiting non-host and poor-host responses were classified as resistant. A putative nematode resistance locus, R_{Mc1}, maps 8 cM distal to TG523 (Fig. 2).

The chromosome number of the BC_1 parent was less than would be expected from a totally balanced megasporogensis. Masuelli et al. (1995) examined the microsporogenesis of the hexaploid F_1 hybrid, CBP233, used in the present backcross study and found pollen mother cells exhibiting either regular or completely disrupted meiosis with unequal partitioning of chromosomes to tetrads. While CBP233 was hexaploid, 2n = 72, DG17 had 2n = 54. It would appear that DG17 was a product of irregular meiosis. However, it is likely that the chromosomes lost were those of the *S. tuberosum* genome as DG17 was selected

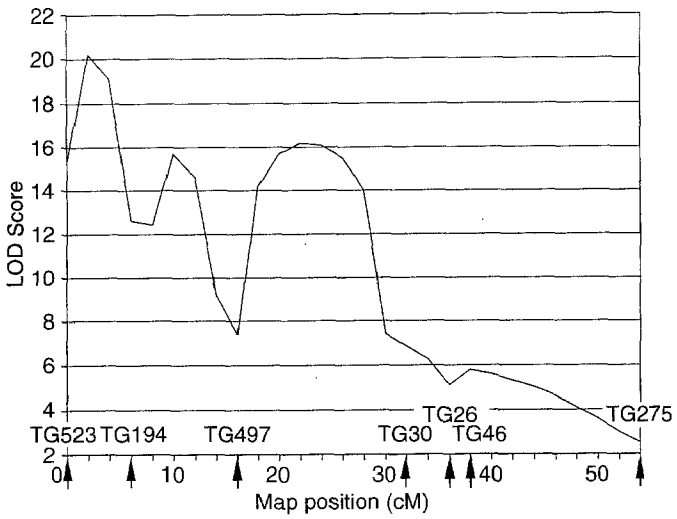


Fig. 4 LOD scores for QTL analysis of Ln (no. eggs) of BC_2. Chromosome 11 was the only linkage group with significant scores

not only because it was resistant, but also because it possessed a full complement of *S. bulbocastanum* markers.

The level of resistance appears to be undiminished between the resistant *S. bulbocastanum* parent and resistant BC_2 segregants. One-third of the progeny are resistant, while the QTL analysis attributes genetic control to one portion of chromosome 11, distal to marker TG523, and to no other location on the map. The model of inheritance is consistent with a completely dominant monogene. However, this analysis cannot exclude the possibility that several linked loci are reponsible for resistance.

All of the somatic hybrids produced from the original fusions were resistant to the nematode. The plants arose from a single callus and may have been derived from a single heterofusion cell (Austin et al. 1993). Four of the 20 BC_1 progeny evaluated were resistant to the nematode (Brown et al. 1995), while 35% of the BC_2 progeny were resistant. The QTL analysis shows an unambiguous attribution of genetic control to a particular region of chromosome 11. Considering that the inheritance is consistent with monogenic dominant genetic control and the segregation pattern in the BC_1 and BC_2 generations, it would appear that the single selected clone of *S. bulbocastanum* was heterozygous for this locus. The polysomic composition of the populations and the deviation from a 1:1 segregation in both the BC_1 and BC_2 generations would not by themselves have permitted such a conclusion. The RFLP map, combined with resistance segregation data, has served, therefore, as a valuable elucidative analytical tool.

Discussion

Potato has been the beneficiary of extensive mapping studies. Bonierbale et al. (1988) and Gebhardt et al. (1989, 1991) described genetic maps of potato and tomato, based on RFLP markers, and the uses to which the maps could be put to enhance breeding efficiency. Debener et al. (1990) and Bonierbale et al. (1990) used RFLPs to determine phylogenetic relationships between distinct species of *Solanum*. This has led to the knowledge that *S. bulbocastanum* is distinct from other species of wild and cultivated potato. Molecular markers have proven useful in the mapping of a number of other traits in cultivated potato. Kreike et al. (1993) reported the association of two regions on chromosomes 10 and 11 with quantitative resistance to *Globodera rostochiensis* pathotype Ro1. Resistance to *G. rostochiensis* (Golden nematode) derived from *S. tuberosum* ssp. *andigena* germ plasm accession CPC 1673 has been mapped to chromosome 5 using RFLP markers, and closely linked markers have been identified (Gebhardt et al. 1993; Pineda et al. 1993). The genetic control of trichome characters originating from *Solanum berthaultii* that provide insect resistance when backcrossed into cultivated potato has been attributed to several different chromosome regions as QTLs (Bonierbale et al. 1994). Molecular mapping of QTLs affecting tuber dormancy has assigned control to several chromosomes of potato with a predominant effect ascribed to chromosome 7 (Freyre et al. 1994).

Mapping of traits has also been important in other crop species. The effect of introgressed chromosome segments of the wild species *Lycopersicon chmielewskii* on the expression of quality traits and yield in cultivated tomato has been determined by Azanza et al. (1994). Significant contributions were attributed to wild donor chromosomes 7 and 10. Random amplified polymorphic DNA (RAPD) markers have been found that are linked to the single gene in *Beta vulgaris*, originating from *Beta patellaris*, controlling resistance to the Beet cyst nematode, *Heterodera schachtii* (Uphoff and Wricke 1992). RAPD-based markers have been identified that flank this gene, $Hs1^{pat-1}$ (Salentjin et al. 1995).

The *Mi* gene, controlling resistance to *Meloidogyne incognita*, derived from *Lycopersicon peruvianum* and introgressed into *L. esculentum*, has been mapped to chromosome 6 of tomato and closely linked markers have been identified (Williamson et al. 1994). The level of homosequentiality between the tomato and potato genomes is high (Gebhardt et al. 1991; Tanksley et al. 1992). It is interesting to ask the question whether the resistance factors described here are related to the *Mi* gene of tomato. The determination that the location of genetic control of resistance to *M. chitwoodi* is located on chromosome 11 in *S. bulbocastanum* argues for an unrelated gene or gene(s) in this wild species rather than the expression of an homoeologous Solanaceous or potato-related "*Mi*" factor. The placement of resistance on a genetic map should facilitate the identification of more closely linked markers that will accelerate the laborious process of selecting resistance to *M. chitwoodi* in segregating potato breeding populations.

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Characteristics of Resistance to Columbia Root-knot Nematode Introgressed from several Mexican and North American Wild Potato Species

Charles R. Brown and Hassan Mojtahedi
USDA/ARS, 24106 N. Bunn Rd.
Prosser, Washington, USA, 99350

G.S. Santo
WSU-IAREC
Prosser, Washington, USA, 99350

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Abstract

The Columbia root-knot nematode (CRN), *Meloidogyne chitwoodi* is a serious pest of potato in the Pacific Northwest of the US and in the Netherlands. At present, control is achieved by chemical fumigation, a costly practice. Resistance has been found in several wild species endemic to Mexico and the United States. This type of resistance is expressed as lower root infestation rates and a barrier to the successful establishment of a feeding site, giant cell, and reproduction. In resistant roots, the juveniles remain in a vermiform stage. It appears that localized cell death accompanies the resistance reaction, suggesting the functioning of an R-gene. The inheritance and chromosomal location was identified for two sources of resistance, *Solanum bulbocastanum*, a diploid, and *S. hougasii*, a hexaploid, as the upper arm of chromosome 11. The localization of resistance to the same chromosome suggests synteny and the possible presence of the primitive B genome of *S. bulbocastanum* in *S. hougasii*. Recent surveys have confirmed that two out of 12 plant introduction accessions of *S. fendleri* tested were 100% resistant to race 1 of CRN. Resistance derived from *S. bulbocastanum* was introduced into cultivated potato by protoplast fusion, and a traditional backcrossing program has produced advanced breeding clones with root-knot resistance, good horticultural type, acceptable fry color and long tuber shape.

INTRODUCTION

The Columbia root-knot nematode (CRN), *Meloidogyne chitwoodi* (Golden et al., 1980) is a serious pest of potato in the Pacific Northwest (PNW) of the US and the Netherlands (Evans and Trudgill, 1992; Santo, 1994). The damage threshold is very low, with Santo et al. (1981) having estimated that one nematode per 250 cm^3 soil at planting may lead to crop failure. In the PNW of US, a potato consignment with 10 or more percent tubers blemished due to CRN may be considered by the processors as unmarketable. Fig. 1 shows the damage apparent on an unpeeled tuber of Russet Burbank and the internal blemish that results from juveniles invading the tuber and producing egg masses which appear as brown spots. A tuber is considered a cull if six or more CRN spots are scored, and a strict control of this nematode before planting of potato is essential for crop safety. At present, control is heavily dependent on chemical fumigants that cost up to $700 US per hectare. Clearly enhanced resistance is highly desirable.

There was no resistance known in advanced cultivars, necessitating recourse to wild relatives. Resistance was discovered first in a wild Mexican species *Solanum bulbocastanum* (Brown et al., 1989). As this species is very difficult to hybridize sexually with cultivated potato, somatic hybrids were produced utilizing protoplast fusion (Austin et al., 1993). Subsequent breeding studies reported the recurrent backcrossing and selection of resistance to CRN (Brown et al., 1994, 1995, 1996). Resistance was also found in *S. hougasii* (Brown et al., 1999) and *S. fendleri* (Brown and Mojtahedi, unpublished). The purpose of this report is to describe genetic and breeding aspects of the incorporation of this resistance from these distinct wild species sources.

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MATERIALS AND METHODS

Genetic Materials and Methods

Wild species accessions of *S. bulbocastanum*, *S. hougasii*, and *S. fendleri* were obtained from the USDA/ARS Potato Introduction Station, Sturgeon Bay Wisconsin. Restriction Fragment Length Polymorphisms (RFLP) were analyzed in mapping populations of *S. bulbocastanum* (BC_2) and *S. hougasii* (BC_1) in Southern blots with ^{32}P-radiolabeled probes according to the methods of Bonierbale et al. (1988). In addition, Randomly Amplified Polymorphic DNA (RAPD) markers were established on the linkage map and the frequency assayed in the BC_2 (mapping population) and BC_3 (introgression population) of the *S. bulbocastanum* sources of resistance (Quiros et al., 1993). Linkage groups were first established using the RFLP markers using the tomato map as a guideline (Tanksley et al., 1992). RAPD markers were added to this infrastructure. The comparison of frequency of alien marker between BC_2 and BC_3 was carried out only considering RAPD markers. Mapping algorithms were performed by the program "MAPMAKER" (Lander et al., 1987)

Nematode Inoculation

Resistance to the nematode was determined by inoculation of 5000 eggs of CRN, race 1 (Isolate WAMc1, maintained at the Washington State University, Irrigated Research and Extension Center, Prosser, WA, US). The purity of the inoculum was verified by testing with carrot (cv 'Chantenay Red Core'), a suitable host for race 1 but a non-host for race 2, alfalfa (cv 'Thor'), a suitable host for race 2. Pepper (cv 'California Wonder'), a non-host for CRN, and suitable host for the Northern root-knot nematode, *M. hapla*, was inoculated to test for contamination by this species. Testing on susceptible standards of potato (cv 'Russet Burbank') and tomato (cv 'Rutgers') confirmed the viability of the inoculum. Resistance was expressed as reproductive factor (Rf = final population / initial inoculum), where non-host, poor host and good host status were indicated by $Rf \leq 0.1$, $0.1 < Rf < 1.0$, and $Rf \geq 1.0$, respectively (Oostenbrink, 1966).

Field tests were conducted at the Roza Unit of the Washington State University-Irrigated Agriculture Research and Extension Center in a field where CRN, race 1 has been maintained at high levels for 10 years by alternate year potato and field corn rotations. Clones were planted on May 15 and harvested 4 months later on Sept 15, 2001. Ten tubers each were harvested from six replications of five hill plots, peeled and scored on a scale of 0 to 6, corresponding to nematode sites scored on a peeled tuber of 0, 1 to 3, 4-5, 6-9, 10+, 50+, 100+, respectively (Mojtahedi et al., 1993)

RESULTS AND DISCUSSION

The hybrid derived from somatic fusion between *S. bulbocastanum* and cultivated potato was resistant to the nematode and female fertile, although pollen infertile. It served as the beginning of a backcross program that has proceeded through various generations. The progeny of the BC_2 served as mapping population permitting the positioning of both RFLP and RAPD markers on the 12 linkage groups of potato. The RFLP map established the location of $R_{Mc1(blb)}$ on the upper arm of chromsome 11 distal to the centromere. The BC_3 generation was produced by backcrossing five resistance BC_2 progeny with various cultivate tetraploid recurrent parents. The BC_2 and BC_3 population sizes were N = 63 and 60, respectively. The RAPD's useful in tagging *S. bulbocastanum* were assessed in both BC_2 and BC_3 in order to measure the transmission of alien genome assayed by markers spread over the 12 chromosomes in a quantitative fashion and examine the feasibility of selecting nematode resistant BC_3 progeny with a minimum of extraneous alien genome. The comparison averaged over all the progeny was 44 percent in the BC_2 and 25% in BC_3. The markers on chromosome 8 were present in high proportion in both the BC_2 and BC_3 perhaps indicating that a meiotic aberration occurring in the somatic F_1 hybrid led to a duplex configuration of chromosome 8 from *S. bulbocastanum* in the BC_1 and the consequent high frequency of transmission to the BC_2 seen here. The high transmission to

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BC_3 is less easily explained, but might have resulted from some of the five founders being duplex also. The selection of resistant BC_2 as progenitors of the BC_3 also meant a selection for chromosome 11 markers, which are also elevated relative to the rest of the genome in the BC_3 (Fig. 2). A second and different presentation of frequencies is shown in Fig. 3. Here the BC_2 and BC_3 are presented according to the percent of the population falling into different intervals of percent of alien genome averaged over all markers. Interestingly, approximately 7.8 percent of the population has less than 25 percent of the alien genome markers present in BC_2 while 30.3 percent were below this in the BC_3. This information was used to select resistant BC_3 progeny with the least alien genome possible as progenitors of the succeeding backcross population.

A group of BC_4 clones were selected as single-hill, and 12-hill plots in a successive years of field evaluation without nematode resistance assessment. They were planted in a field infested with CRN, race 1 assessed for tuber damage and tested in pots inoculations for Rf value. The results are shown in Fig. 4. Three of the clones depicted to the left, PA98N2-1, PA98N4-2, and PA98N13-3 carry $R_{Mc1(blb)}$, while this factor has not been transmitted to the four clones on the right side of the graph. The nematode reproduction is prolific in the susceptible clones and almost nil in the resistant clones as indicated by the Rf values. Similarly, nematode damage is dichotomous with the resistant clones showing very low values, which would be acceptable as undamaged to processors while the susceptible clones would certainly be classified as culls. These results also indicate that expression of resistance is at a satisfactory level in the BC_4 generation where the genetic background contains the least proportion of alien genome up to this point in the breeding program. The BC_3 progeny PO94A10-3 was genotyped by RAPD markers as a resistant individual containing 14% of the alien genome markers. It is an oblong-shaped tuber with russet skin and a total yield approaching that of the standard cultivar Russet Burbank, although it has poor frying qualities and has a propensity to severe cracking. The resistant clone PA98N13-3 is a derivative of BC_3 progeny PO94A10-3 that was genotyped by RAPD markers as a resistant individual containing 14% of the alien genome markers. It has improved tuber type, high yield and good frying qualities.

Germplasm surveys revealed that *S. hougasii*, a Mexican wild species, also harbored resistance to CRN (Brown et al., 1991). A single hybrid individual was obtained by sexual methods and the subsequent BC_1 generation was used as a mapping population. RFLP's derived from the tomato map Tanksley et al. (1992) were used to localize the resistance. It was found that resistance derived from *S. hougasii* is syntenic to that derived from *S. bulbocastanum*. The marker order is shown in Fig. 5 on chromosome 11 in relation to the nematode resistance loci of both species. Certain markers could not be used in both mapping populations as the panel of restriction enzymes revealed polymorphism in one population but not the other. The synteny suggests that the allohexaploid *S. hougasii* may contain as one of its homeologous genomes, a B-like genome similar to that of *S. bulbocastanum*. The genome of *S. bulbocastanum* is putatively distantly related to the A genome of cultivated potato as evidenced in phyletic studies based on molecular markers and by the strong reproductive isolation exhibited by *S. bulbocastanum* when crossed to most *Solanum* spp. (Debener et al., 1990).

The discovery of resistance to CRN in *S. fendleri* (Janssen et al., 1997) was followed up by our own survey of a core collection consisting of 12 accessions covering the range of distribution of the species. Two of the collections showed non-host level resistance to CRN, race 1 (Fig. 6). The susceptible accessions showed uniformly high Rf values in all the sexual progeny within the seedlot, while every seedling in the resistant seedlots was at the non-host level. All accessions were, however, susceptible to CRN, race 2. The two resistant accessions stem from collections made in two separate mountain ranges in Southeastern Arizona, (i.e., within US territorial borders). Sexual hybrids with resistant *S. fendleri* accessions were made by crossing with cultivated diploid accessions and were screened for resistance. All hybrids were non-hosts, indicating resistance is expressed in a dominant fashion (data not shown). Since these hybrids are triploids, they will be doubled somatically in tissue culture in order to provide a fertile bridging hybrid

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that should be intercrossable with cultivated tetraploid potatoes.

CONCLUSIONS

The identification of resistance to CRN in three wild *Solanum* species suggests a common evolutionary origin of these resistances. Synteny between genomic positions in *S. bulbocastanum* and *S. hougasii* adds further support to this. Further studies with the source of resistance from *S. fendleri* may extend this pattern. The inheritance as monogenes and the characteristic nearly complete inhibition of nematode feeding and development in the roots suggest R-genes. Continued examination of these sources of resistance and measuring of the resistance response in different environmental circumstances may reveal distinguishing characteristics in the future that will guide future use in breeding programs to produce resistant potato cultivars. Resistant cultivars will help to reduce the amount of soil fumigation that will be employed in CRN infested fields.

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Figures

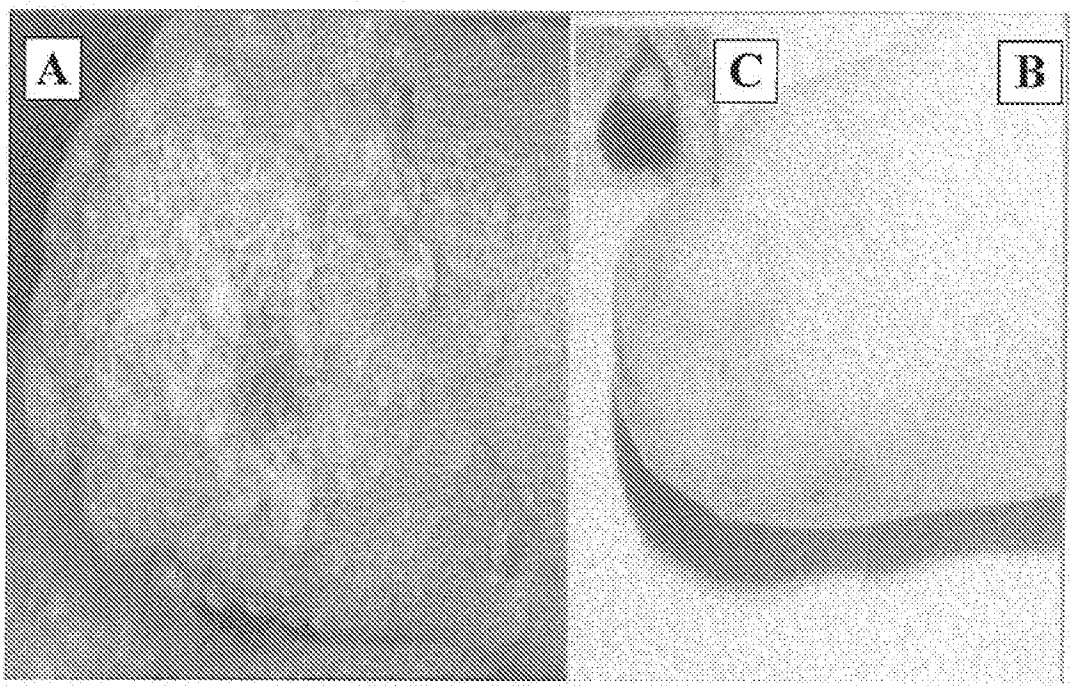


Fig.1. External and internal damage caused by the Columbia root-knot nematode, *Meloidogyne chitwoodi*. In unpeeled cultivar Russet Burbank galling is apparent on tuber surface (A). Infection sites indicate locations of females with egg masses on peeled tuber (B). Tubers with a few infection sites are unmarketable. Female with egg mass dissected from tuber tissue, 60 x (C).

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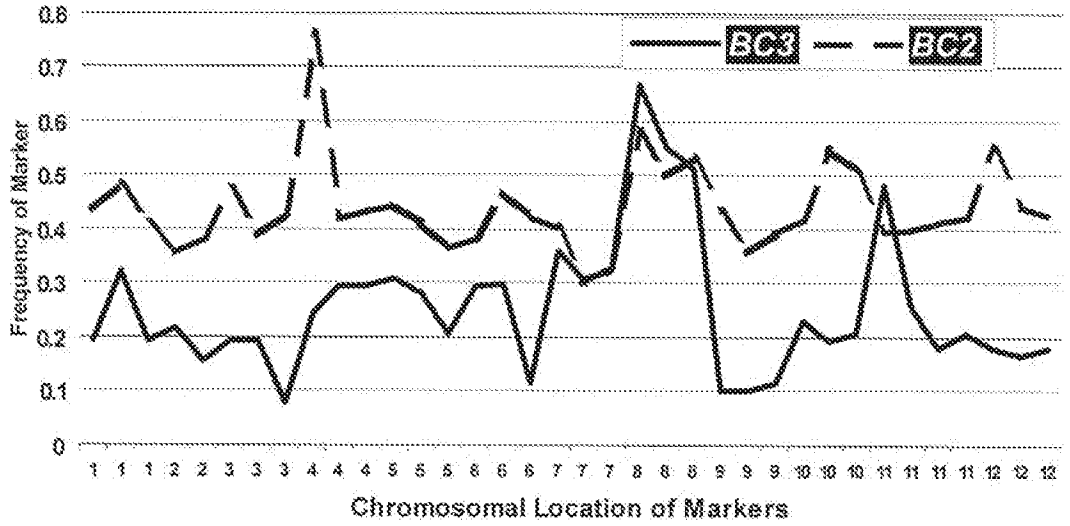


Fig. 2. Distribution of alien genome in BC_2 and BC_3 generations of tetraploid populations introgressing the Columbia root-knot nematode, *Meloidogyne chitwoodi* race 1 resistance from *Solanum bulbocastanum*. Genetic markers are RAPD's localized on chromosomes and specific to the alien genome (i.e., *S. bulbocastanum*).

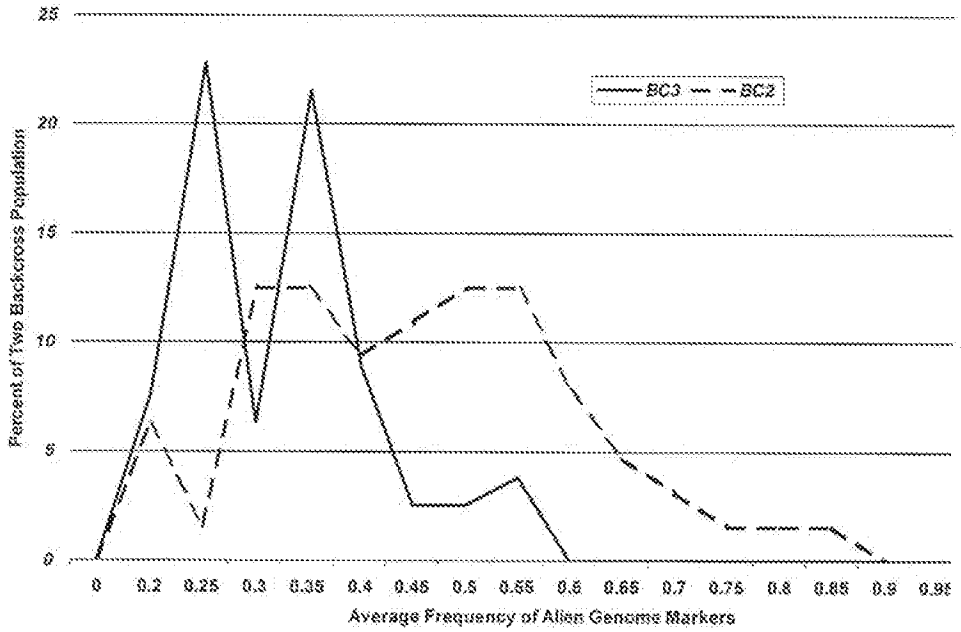


Fig. 3. Distribution of progeny in BC_2 and BC_3 with different content of alien genome marked by chromosome-assigned alien-genome-specific RAPDs.

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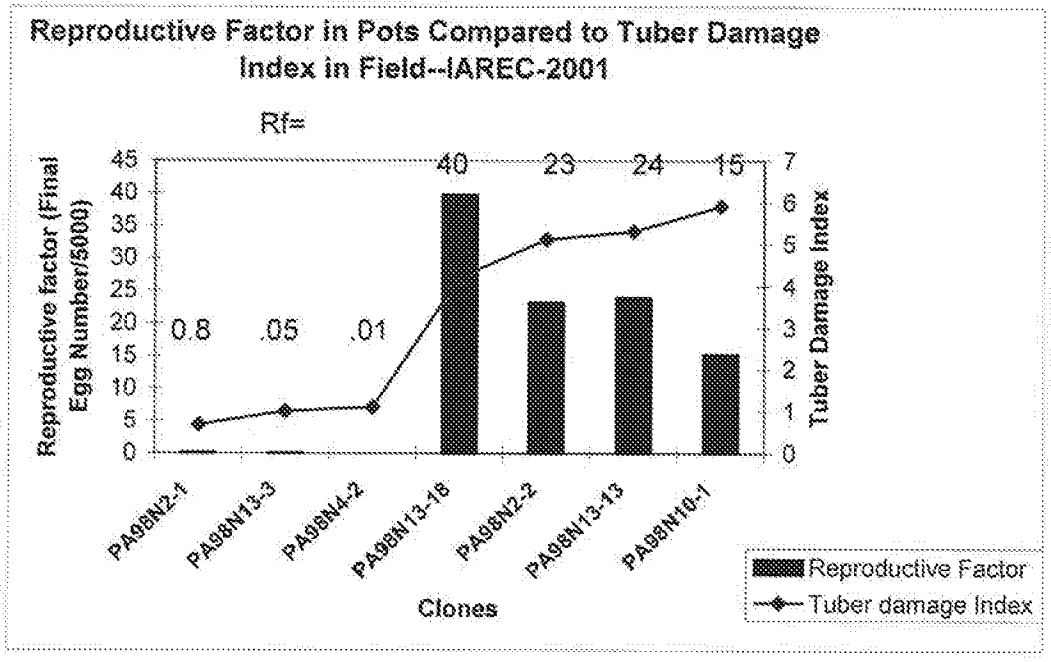


Fig. 4. Relationship of Rf values [Rf = (final egg count / egg count of inoculum)] in pot tests and performance of clones with and without the $R_{Mc1(blb)}$ in the BC_3 of introgression program to introduce resistance to the Columbia root-knot nematode, *Meloidogyne chitwoodi* race 1 derived from *Solanum bulbocastanum*.

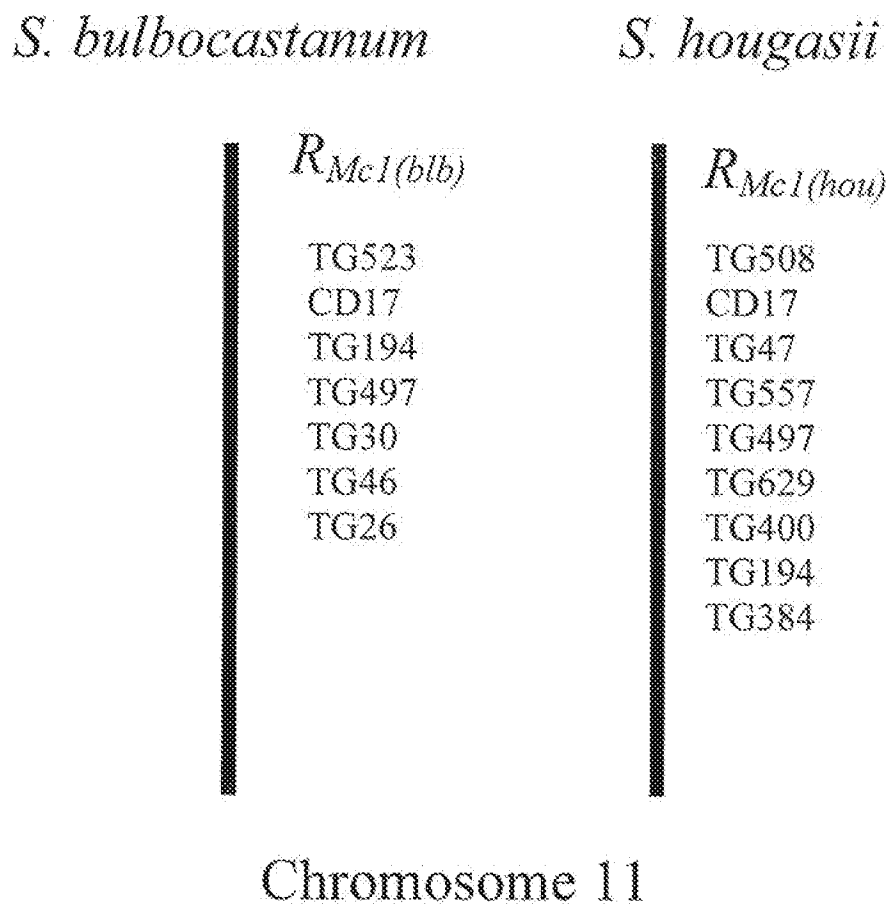


Fig. 5. Mapping order of RFLP markers for the position of resistance genes on chromosome 11 of *Solanum bulbocastanum* and *S. hougasii*.

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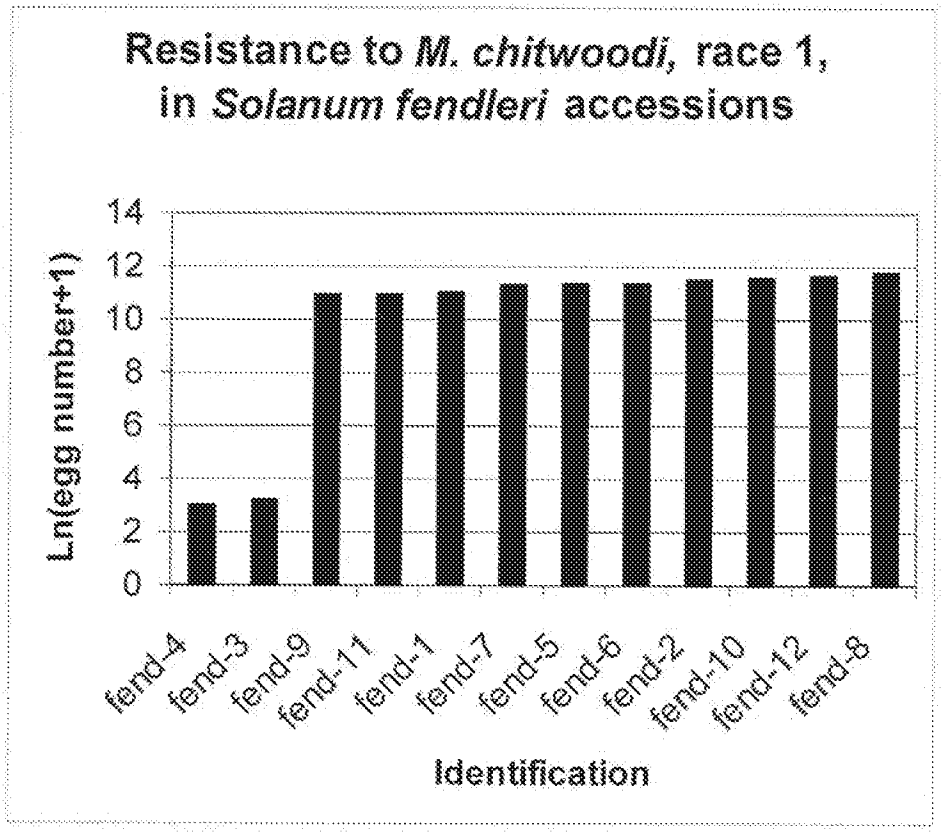


Fig. 6. The natural logarithm of egg counts of pot tests of 12 Plant Introduction accessions of *Solanum fendleri*, representing a core collection. The seedlings of Fen 3 and Fen 4 were uniformly non-hosts (Rf < 0.1) for introduce resistance to the Columbia root-knot nematode, *Meloidogyne chitwoodi* race 1, while all other PI's were uniformly good hosts. (Rf > 1.0).

Segregation of Total Carotenoid in High Level Potato Germplasm and Its Relationship to Beta-Carotene Hydroxylase Polymorphism

C. R. Brown*[1], T. S. Kim[2], Z. Ganga[3], K. Haynes[4], D. De Jong[2], M. Jahn[2], I. Paran[5], and W. De Jong[2]

[1]USDA/ARS, Prosser, WA, USA
[2]Dept of Plant Breeding, Cornell University, Ithaca, NY, USA
[3]Aroostook Research Farm, University of Maine, Presque Isle, ME, USA
[4]USDA/ARS, Beltsville, MD, USA
[5]The Volcani Center, Bet Dagan, Israel
*Corresponding author: Tel: 509-786-9252; Fax: 509-786-9277; Email: cbrown@pars.ars.usda.gov

ABSTRACT

High carotenoid potato may have particular value for human health due to the antioxidant properties and the therapeutic value for eye health in patients at risk for macular degeneration. Carotenoid concentrations were determined among the progeny of a cross between two high carotenoid lines derived from diploid *Papa Amarilla* germplasm from South America. The total carotenoid content ranged from 82 to 2686 µg / 100 g fresh weight (FW). The higher values greatly exceeded the mid-parent value of the cross. An index for yellow tuber flesh color was determined for a subset of the progeny. A cleaved amplified polymorphic sequence (CAPS) assay was developed to distinguish the alleles of beta-carotene hydroxylase (*bch*) in the two high-carotenoid parents. A *bch* allele (denoted B) common to the high carotenoid parents co-segregated with yellow flesh in the progeny of a white-flesh x yellow-flesh cross, making *bch* an excellent candidate for the classical *Y* locus, required for yellow tuber flesh. The same allele was also present in all other yellow-fleshed potato clones tested. Genotype at *bch* explained a portion of the variation of total carotenoid (R^2 = 0.42). Clones homozygous for the B allele (BB) contained, on average, slightly more carotenoid than heterozygous Bb clones, which in turn had much more carotenoid than homozygous bb clones, suggesting a partially dominant gene model. Similarly, bb flesh was significantly less yellow than Bb and BB, the latter two being quite close. Total carotenoid varied considerably between progeny in the Bb and BB genotype categories, suggesting that variation at one or more additional loci have a significant effect on total carotenoid levels. Since the total carotenoid levels in many *Papa Amarilla* cultivars and progeny are much higher than those in white- and yellow-fleshed tetraploid cultivars, it may be possible to breed for high carotenoid values in tetraploid germplasm by introducing one or more genes derived from *Papa Amarilla* germplasm.

RESUMEN

Las papas con alto contenido de carotenoides podrían tener particular valor en la salud humana debido a las propiedades antioxidantes y al valor terapéutico para la salud ocular, en pacientes con riesgo de degeneración macular. La concentración de carotenoides fue determinada en la progenie de un cruzamiento entre dos lineas con carotenoides, derivadas de germoplasma del diploide Papa Amarilla de Sudamérica. El contenido total de carotenoides varió de 82 a 2686 microgramos por 100g de peso fresco. Los mayores valores totales excedieron al valor del progenitor medio de los cruzamientos. Para un subconjunto de la progenie, se determinó el índice del color amarillo de la pulpa. Se desarrolló una prueba de división de secuencia polimórfica amplificada (CAPS) para distinguir alelos de beta caroteno hidroxilasa (*bch*) en dos de los progenitores

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con alto contenido carotenoideo. Un alelo *bch* (designado B), común en progenitores de alto caroteno co-segregó a pulpa amarilla en la progenie de un cruzamiento de pulpa blanca x pulpa amarilla, haciendo *bch* un excelente candidato para el clásico locus *Y*, requerido para pulpa amarilla del tubérculo. El mismo alelo estuvo también presente en todos los otros clones de pulpa amarilla probados. La explicación de la variación de una porción del total de carotenoide (R^2 = 0.42) la dio el genotipo *bch*. Clones homocigotas para el alelo B (BB) contenían en promedio, ligeramente mas carotenoide que los clones heterocigotas Bb, los cuales a su vez tienen mucho mas carotenoide que los clones homocigotas bb, sugiriendo un modelo de gen parcialmente dominante. Similarmente, la pulpa de bb fue significativamente menos amarilla que Bb y BB, siendo las dos últimas muy parecidas. El total de carotenoide varió considerablemente entre progenies en las categorías de genotipo Bb y BB, sugiriendo que la variación en uno o más loci, tienen efecto significativo sobre los niveles totales de carotenoide. Desde que el total del nivel de carotenoide en muchos cultivares y progenies de Papa Amarilla son mucho mas altos que en los cultivares tetraploides de pulpa blanca y amarilla, sería posible hacer cruzamientos para mayores valores de carotenoide en el germoplasma tetraploide, introduciendo uno o mas genes del germoplasma derivado de Papa Amarilla.

INTRODUCTION

Potato (*Solanum* spp.) tubers vary widely with respect to the types and concentrations of carotenoids they contain. White-fleshed clones contain low levels of carotenoid, while yellow-fleshed varieties contain more. The yellow flesh trait is considered to be controlled by a single locus (*Y/y*) mapped to chromosome 3 (Bonierbale et al. 1988). Even so, yellow-fleshed varieties vary greatly in degree of yellow coloration and concentration of carotenoid (Brown et al. 1993, 2005; Lu et al. 2001). The carotenoids in tubers are primarily xanthophylls; tubers contain only a trace of beta-carotene. The presence and concentrations of xanthophylls vary in different potato genotypes, with lutein predominating. Zeaxanthin, violaxanthin, and others have been reported in various studies (Iwanzik et al. 1983; Brown et al. 1993; Lu et al. 2001; Nesterenko and Sink 2003). The highest levels of tuber carotenoid are found in potato varieties of the Andes of South America, which are collectively known as *Papa Amarilla* (yellow potato). *Papa Amarilla* is represented by diploid cultivated potatoes *Solanum tuberosum* L. in the Groups Phureja and Goniocalyx. A number of reports of carotenoid content have contributed to the knowledge base in the last 60 years. These are summarized in Table 1. The first reports indicated that white-fleshed potato contained less than 100 µg / 100 g fresh weight (FW), while yellow-fleshed tubers contained more. Intensely yellow flesh was accompanied by levels reaching 560 µg. However, it is only in the last 15 years that very high levels of carotenoid have been recognized. Brown et al. (1993) reported wide variation in segregating genotypes derived from *Papa Amarilla* germplasm, with some clones exceeding 2000 µg. Several other studies have reported such high levels (Lu et al. 2001; Brown et al. 2005). Haynes et al. (1994) found that yellow color was more intense in smaller tubers. In a report by Brown et al. (1993), the existence of a so-called orange allele, *O*, was postulated for the *Y/y* locus. The effect of *O* was to confer accumulation of a high level of zeaxanthin in the tuber flesh. Heritability of yellow intensity (YIE313) was determined by Haynes (2000) to be extremely high (h^2 = 0.99), which also agreed with a well-expressed and highly heritable and possibly simple genetic component. Transmission of high expression of carotenoid by crossing the *O* allele into non-*Papa Amarilla* germplasm was not successful (unpublished breeding results) in producing progeny with high levels, forcing the search for better explanations of genetic control. The identification of possible candidate genes for the potato *Y/y* locus, based on mapping carotenoid pathway genes in other Solanaceae provides an opportunity to explore candidate gene polymorphism in popu-

TABLE 1—*Citations of total carotenoid content in potato flesh expressed as µg / 100 g fresh weight (FW).*

Concentrations (µg / 100 g FW) of total carotenoids in flesh	Citation
14-187	Caldwell et al. 1945
60	Brunstetter and Wiseman 1947
199-560	Kasim 1967
102-219	Tevini et al. 1984, 1986
27-329	Iwanzik et al. 1983
Segregation > 2000 (*Papa amarilla*)	Brown et al. 1993
97-536	Hale 2003
1435-2200	Lu et al. 2001
48-879	Nesterenko and Sink 2003
40-795	Brown et al. 2005

lations showing a range of carotenoids accumulation (Thorup et al. 2000; Hirshberg 2001). The present report provides additional information that incrementally improves our understanding of the genetic control of carotenoid level.

MATERIALS AND METHODS

Genetic Materials

The majority of the potato genotypes analyzed here were from a progeny between a cross between two high carotenoid lines (*Yema de Huevo* and 91E22). *Yema de Huevo* was obtained from Dr. Nelson Estrada, INIA, Tibaitatá, Colombia. The clone 91E22 was selected from crosses between clones originally obtained from populations derived from Groups Phureja and Stenotomum provided by Dr. Frank Haynes, Department of Horticulture, North Carolina State University, Raleigh, NC (Brown et al. 1993). True seed derived from the cross was soaked for 24 h in 1500 mg L^{-1} gibberellic acid (Sigma, St. Louis). Seed was sown in soil (Sunshine Mix, No. 1, Sun Gro Horticulture Distributing, Bellevue, WA), seedlings were transplanted to 20.3-cm-diameter plastic pots and plants were grown to maturity in a greenhouse. Tubers derived from this were planted after breaking dormancy. These tubers were grown in the same manner as the seedlings described above; tubers were harvested and stored at 4 C in 85% relative humidity. One month after storage, tubers were removed from storage and carotenoids were extracted from them. Two replicates (derived from separate pots) of each genotype were processed for carteonoid extraction and three replicates (from separate pots) were evaluated for yellow intensity. A related family was constructed by crossing 91E22 (as a male) with 07248-02. The latter clone was kindly provided by H. De Jong (Agriculture and Agri-Food Canada), and has only *Solanum tuberosum* group *tuberosum* in its immediate ancestry.

Beta-carotene Hydroxylase Cloning and Development of CAPS Assay

Potato EST accessions BI179773 and BM111217 are similar in sequence to the tomato beta carotene hydroxylase gene located on chromosome 3. Primers BCH-F1 (5'CCAGTTCTGTTCTTCTCTCCG) and BCH-R1 (5'TCAAAAGGTCCTTCTCTTGGTCTA) are based on these ESTs and were used to amplify genomic DNA isolated from 91E22, with the following thermal profile: 94 C for 2 min, followed by 35 cycles of (94 C, 15 s; 55 C, 15 s; 72 C, 60 s). This and other amplifications below were carried out in a volume of 50 µL and contained 200 nM of each primer, 200 µM dNTPs, 10 mM Tris-Cl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.05% Nonidet-P40, 50-200 ng of template DNA, and 2.5 units of *Taq* DNA polymerase. The resulting PCR products were ligated into vector pGEM-T following the manufacturer's (Promega) instructions. Seven clones were obtained and sequenced, revealing two alleles that could be differentiated by virtue of a polymorphic *Taq* I restriction site. To monitor the segregation of these alleles in progeny, PCR was performed with primers BCH-F2 (5'CATGACATAGTTTGAATTTTGAGTC) and BCH-R2 (5'CGTTTGGCGCTGCCGTAAGTT), with the following thermal profile: 94 C for 2 min, followed by 35 cycles of (94 C, 15 s; 55 C, 15 s; 72 C, 30 s). Amplification products were then digested with *Taq* I.

Genomic DNA of cultivars and breeding clones was also evaluated with the CAPS assay. The following yellow-fleshed cultivars were evaluated: Amandine, Austrian Crescent, Banana, Bintje, Carola, Cynthia, French Fingerling, German Butterball, Keuka Gold, Ozette, Peanut, Penta, Regina, Rose Finn Apple, Rote Eersteling, Sandy, Sieglinde, Sylvia, Yagana, Yellow Finn, and Yukon Gold. The following white-fleshed clones were evaluated: AC Candy Cane, Allegany, Bake King, Cara, Chieftain, Chippewa, Dakota Rose, Dark Red Norland, Genesee, Green Mountain, IdaRose, Katahdin, Kennebec, Lenape, Marcy, Monticello, NY99, NY115, NY118, NY120, NYL235-4 (Prince Hairy), Purple 5, Red Pearl, Redsen, Rideau, Sebago, Stirling, and Superior. Primers YellowF1 (5'TAATTACTCCATTCTTTTGCT) and BCH-F2 (5'ATTTACATGACATAGTTTGAATTTTG) were used for allele-specific PCR, using the following thermal profile: 94 C for 2 min, follow by 45 cycles of 94 C, 15 s; 50 C, 30 s; and 72 C, 120s.

Carotenoid Extraction and Quantitation

Total carotenoid was extracted and quantitated using the methods reported by Brown et al. (2005), which contained minor modifications of procedures presented in Breemen (2001). Briefly, carotenoid was extracted in a chloroform: methanol phase separation, retaining the chloroform phase accumulated in two separations, drying and re-dissolving in methanol. Concentration was determined by optical density spectrophotometrically at 450 nm wavelength using the extinction coefficient for zeaxanthin in methanol. Total carotenoid was expressed as µg / 100 g FW.

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Yellow Index

The yellow index was measured using a Colorimeter (Minolta Chroma Meter Cr-200, Minolta Corporation, Ramsey, NJ). Tubers were sliced midway between the apical and stem end, patted dry on a paper towel, and immediately evaluated by placing the aperture of the colorimeter against the cut surface. A yellow index (YIE313) (American Society for Testing Materials 1991; Haynes et al. 1994) was derived from the three chromaticity values, Y, y, and z, using the algorithm YI E313 = 100 × [(1-(0.8467[Y/y(1-x-y)])/y]. Three replications per progeny were measured.

Statistical Analysis

The segregation of *bch* alleles was tested by chi-square analysis against the model of a 1:2:1 ratio of b/b:B/b:B/B. One-way analysis of variance, single degree of freedom orthogonal comparisons, and population standard deviation were performed according to Steel and Torrie (1980). Regression analysis of *bch* genotype vs carotenoid content was performed using the regression procedure in SAS (version 9.1.3, Cary, NC) program. Regression analysis was performed of total carotenoid regressed on the yellow index using Microsoft Excel Trendline Function testing linear and curvilinear options. The function with the highest coefficient of determination was selected.

RESULTS

Thorup et al. (2000) have previously suggested that the potato *Y* locus on chromosome 3 may correspond to *bch*. To test this hypothesis further, both alleles of *bch* were sequenced from diploid 91E22. One *bch* allele, which we designate B, contained a single *Taq* I restriction site and gave a diagnostic product 233 bp in length after amplification and subsequent digestion with *Taq* I (Figure 1). The other allele, which we designate b, contained two *Taq* I restriction sites and yielded a diagnostic fragment 147 bp in length with our CAPS assay (Figure 1). Allele B was found to absolutely co-segregate with yellow tuber flesh color in 155 progeny (82 yellow: 73 white) of a cross between 91E22 and a white-fleshed *S. tuberosum* diploid, 07248-02 (Figure 1). The carotenoid content of 10 randomly selected yellow-fleshed clones of this cross averaged 389 µg / 100 g FW (range 236-620), while 10 white-fleshed clones averaged 153 µg / 100 g FW (range 107-204).

When the same CAPS assay was used to examine an additional 21 yellow- and 28 white-fleshed varieties and breeding clones, the fragment diagnostic for allele B was detected in all of the yellow-fleshed clones tested (Figure 2A and data not shown). The same restriction fragment was clearly absent in many white fleshed cultivars, e.g., Allegany and Superior (Figure 2A). Interpretation was nevertheless more difficult in other white-fleshed cultivars, e.g., Green Mountain and Katahdin, as the latter displayed a faint doublet of DNA fragments that migrated similarly to the 233 bp fragment of allele B (Figure 2A). Sequencing portions of *bch* from a cultivar (Bake King) that exhibited the doublet indicated that the doublet is most likely an artifact that results from recombination between alleles during PCR. One Bake King allele was found to contain two *Taq* I sites, while another allele contained none. Recombination between these alleles would be expected to yield two novel products, each with a single *Taq* I site. Consistent with the recombination hypothesis, the doublet was only observed in potato clones that contained both no-cut and two-cut *bch* alleles (Figure 2A and data not shown).

To further test for the presence of a unique allele of *bch* associated with yellow flesh color, but without complications arising from recombination, a pair of primers specific to allele B was also developed. Amplification with these allele-specific primers yielded an identically sized 397 bp product from all 21 yellow-fleshed cultivars and breeding clones tested (Figure 2B and data not shown). The same primers amplified no product from any of the 28 white-fleshed clones tested (Figure 2B and data not shown). These results provide strong circumstantial evidence that the *Y* locus codes for beta-carotene hydroxylase, although formal proof of this relationship will require complementation. Alternatively, it is possible that *bch* allele B and the dominant allele at the *Y* locus are tightly linked.

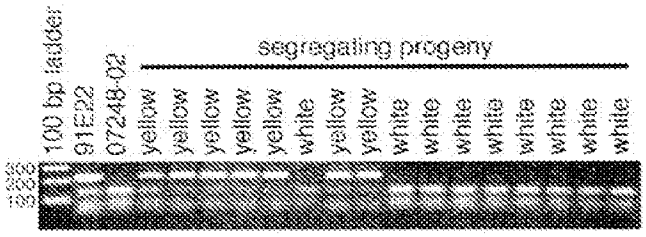


FIGURE 1.
Agarose gel showing that an allele of *bch* co-segregates with yellow tuber flesh in the progeny of cross between 91E22 (yellow flesh) and 07248-02 (white flesh). A fragment of *bch* was amplified by PCR and then digested with restriction enzyme *Taq* I. Products were separated on an agarose gel. A unique 233 bp fragment diagnostic of an allele we designate "B" can be seen in 91E22 and all progeny with yellow flesh.

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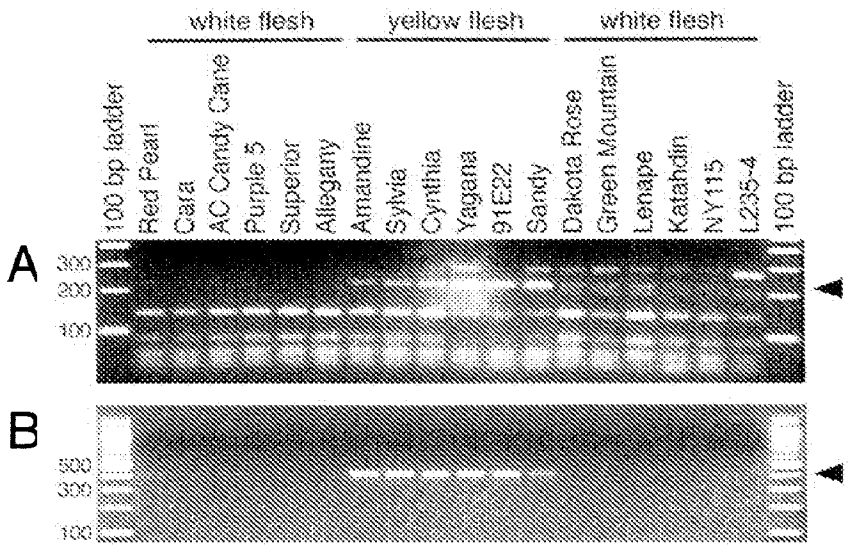


FIGURE 2.
Panel A. The CAPS assay illustrated in Figure 1 was applied to yellow- and white-fleshed varieties and breeding clones. The diagnostic allele B was detected in all yellow-fleshed clones. Allele B was clearly absent from the white-fleshed clones shown to the left, but it was difficult to determine if allele B was present in the white-fleshed clones shown to the right (partial results presented). Panel B. Primers designed to specifically amplify allele B were applied to the same clones as in panel A. These primers directed amplification of the expected 397 bp product only from yellow-fleshed clones (partial results shown).



FIGURE 3.
The parents in the cross 91E22 × *Yema de Huevo* were heterozygous at the *bch* locus, each containing alleles B (one *Taq* I site) and b (two *Taq* I sites). The progeny conform to expectations of a 1:2:1 ratio of BB:Bb:bb from a Bb × Bb cross (partial results shown).

Exceptionally wide variation in tuber flesh color and carotenoid content was observed in the progeny of a cross between diploid parents 91E22 and *Yema de Huevo*. To further assess the relationship between *bch* and yellow flesh color, as well as between *bch* and carotenoid content, the co-dominant *bch* CAPS marker described above was used to evaluate the parents and progeny. As shown in Figure 3, both 91E22 and *Yema de Huevo* were heterozygous at the *bch* locus, each containing alleles B (one *Taq* I site) and b (two *Taq* I sites). The progeny of this cross are expected to segregate 1:2:1 for BB:Bb:bb. Actual numbers were 38:58:21 ($\chi^2 = 4.9$, $0.05 < P < 0.1$). Thus the genetic model of 1:2:1 segregation was not rejected. Total carotenoid values of the progeny span a large range, extending from 82 to 2686 µg / 100 g FW. This distribution, including the parental values (see arrows), are shown in Figure 4. The parents 91E22 and *Yema de Huevo* had carotenoid levels of 794 and 932, respectively. Some progeny far exceed the value of both parents, transgressive segregation is pronounced. The total carotenoid values of 12 progeny (13.5% of the population) were more than one standard deviation ($\sigma_{population}$ = 554 µg / 100 g FW) above the mid-parent (MP) value (MP = 863 µg / 100 g FW). The total carotenoids of three progeny exceeded the mid-parent value by two standard deviations.

The progeny are shown in Figure 5 grouped according to *bch* genotype in order of ascending total carotenoid value. There were significant differences between genotypes. Orthogonal comparisons planned *a priori* (Table 2) found significant differences in carotenoid content between all groups (i.e., b/b [$\bar{x}$ = 286] < B/b [$\bar{x}$ = 1059] < B/B [$\bar{x}$ = 1233]).

Tubers of 44 progeny, Yukon Gold (yellow flesh) and Russet Burbank (white flesh) were assessed for yellow index and carotenoid content. The yellow index (YIE313) showed a natural logarithmic relationship with carotenoid content (Figure 6). The colorimeter failed to measure greater yellowness above about 1000 µg. This

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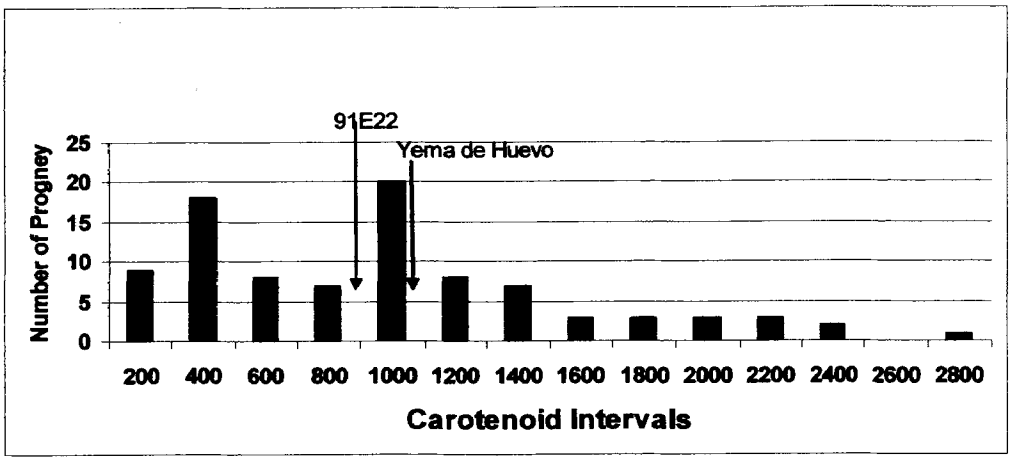


FIGURE 4.
Distribution of total carotenoid in 89 progeny of the cross 91E22 × *Yema de Huevo*. The range of the distribution suggests transgressive segregation.

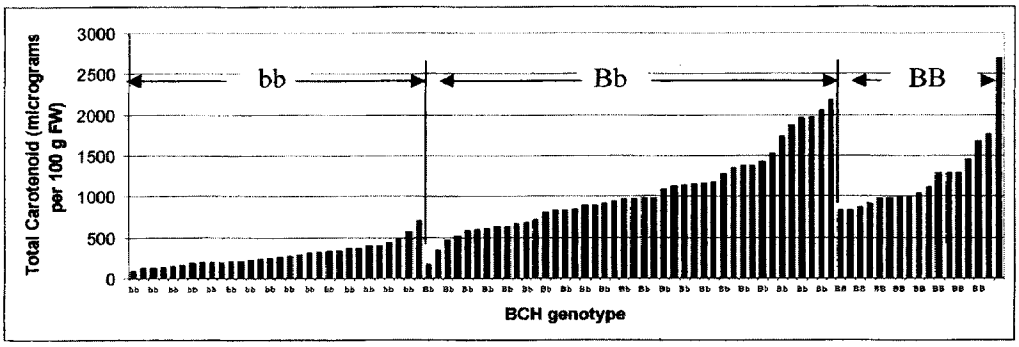


FIGURE 5.
Distribution of total carotenoids grouped by beta-carotene hydroxylase (*bch*) genotype. Variation in total carotenoids within the *bch* genotypes is very large. Orthogonal comparisons found significant differences between the groups bb, Bb, and BB.

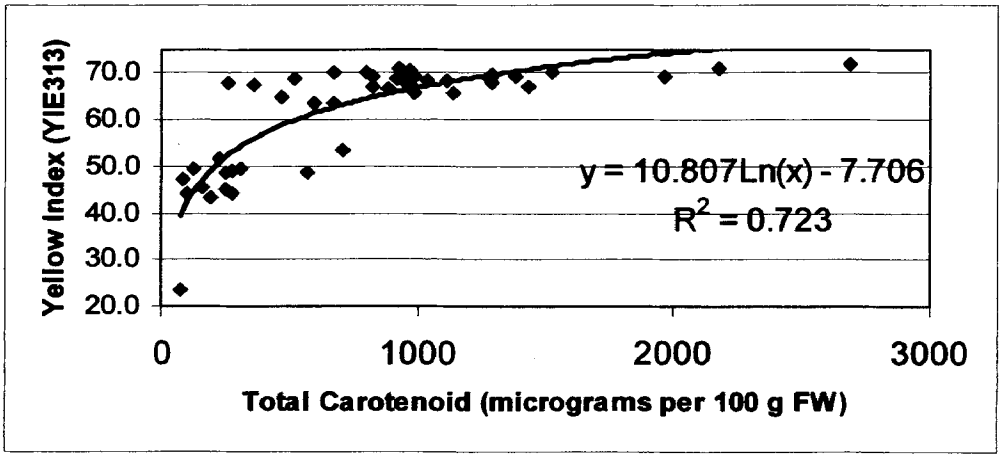


FIGURE 6.
Scatter plot of yellow index YIE313 in relation to total carotenoids. A natural logarithmic function more closely aligns with the data points than linear regression. The colorimeter failed to measure greater yellowness above approximately 1000 µg / 100 g FW.

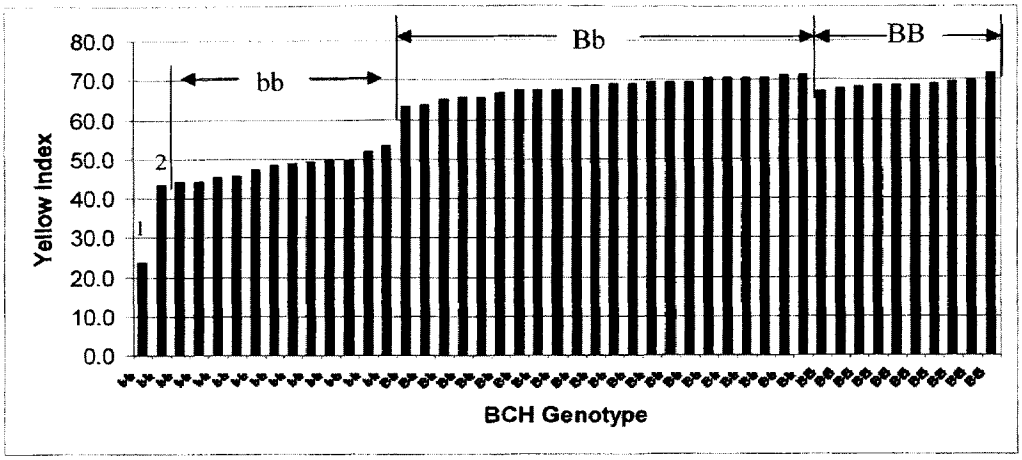


FIGURE 7.
Arrangement of yellow index (YIE313) values grouped according to beta-carotene hydroxylase genotype values. Orthogonal comparisons showed significant differences between all groups, although mean yellow indexes of Bb and BB are similar. Bars labeled 1 and 2 are Russet Burbank and Yukon Gold, respectively.

TABLE 2—*ANOVA and orthogonal comparsions of bb, Bb, and BB groupings of beta-carotene hydroxylase genotypes for total carotenoids (89 genotypes) and yellow index (44 genotypes). Progeny were derived from the cross of 91E22* × Yema de Huevo.

	Total Carotenoids		Yellow Index	
Source	df	Significance	df	Significance
Genotypes	88	$P < 0.001$	45	$P < 0.0001$
bb vs Bb	1	$P < 0.001$	1	$P < 0.0001$
Bb vs BB	1	$P < 0.001$	1	$P < 0.05$
bb vs BB	1	$P < 0.001$	1	$P < 0.001$
Error	89		92	

suggests that neither visual evaluation nor measurement by colorimetry will efficiently identify the highest levels (e.g., above 2000 µg) of carotenoid or differentiate them from the intermediate levels (1000 to 2000 µg). Extraction followed by optical quantitation will be necessary to select for the highest levels. The yellow index measurements of the progeny are presented grouped according to the *bch* genotype and ordered in ascending magnitude within group (Figure 7). Once again there were significant differences between genotypes. Three orthogonal comparisons found significant differences in the mean yellow index between the means of the bb, Bb and BB groupings (Table 2). The mean yellow index of bb (48.1) was significantly less than the means of Bb and BB genotype groupings (68.2 and 69.2, respectively).

DISCUSSION

Although *bch* polymorphism provides a loose explanation of the total carotenoid levels in the progeny of the cross studied here, the degree of variation within *bch* genotypes suggests that important contributions of other gene(s) will also be found for this population. Other polymorphisms, possibly within carotenoid biosynthetic genes like phytoene synthase and zeaxanthin epoxidase, need to be explored to obtain greater predictability and a more substantial association.

The highest levels of total carotenoid reported in the literature are all from germplasm derived from taxa in the Groups Phureja, Stenotomum and Goniocalyx that have been maintained in relatively closed populations at the diploid level without introduction of genes from other cultivated germplasm pools (Brown et al. 1993, 2005; Lu et al. 2001; Nesterenko and Sink 2003). These populations thus provide the most promising source for novel alleles that can be used to increase carotenoid content in cultivated tetraploid germplasm.

Römer et al. (2002) produced an increase of zeaxanthin in yellow-fleshed potato by transformation of sense and antisense constructs of zeaxanthin epoxidase. This inhibited conversion of zeaxanthin into violaxanthin. Increases in zeaxanthin over wild type ranged between four- and 130-fold. The highest levels of zeaxanthin reached 40 µg / g dry weight (approximately 1000 µg / 100 g FW). In a study of candidate gene expression of high (*Solanum phureja*) and low (*S. tuberosum*) carotenoids content, there was an inverse relationship between level of

zeaxanthin epoxidase transcript and carotenoids content during several stages of tuberization. Alternatively, relatively higher expression of RNA transcripts of certain enzymes in the carotenoid synthesis network in the early stages of tuberization (swelling stolons and developing tubers), was found for lycopene-ϵ-cyclase and isopentenyl pyrophosphate isomerase in *S. phureja* (*Papa Amarilla*) type compared to *S. tuberosum* cvs Pentland Javelin and Desiree (Morris et al. 2004). Identification of polymorphic alleles of genes encoding enzymes in the carotenoid pathway is the first step to elucidation of contribution to the high carotenoid phenotype characteristic in *Papa Amarilla*. The ability to transfer the high carotenoid trait from South American *Papa Amarilla* germplasm to cultivated North American and European tetraploid cultivars may require assembling a number of exotic alleles including the B allele of *bch* identified in this study.

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RESEARCH PAPER

Induction of vacuolar invertase inhibitor mRNA in potato tubers contributes to cold-induced sweetening resistance and includes spliced hybrid mRNA variants

David A. Brummell[1,*], Ronan K. Y. Chen[1], John C. Harris[1,†], Huaibi Zhang[1], Cyril Hamiaux[2], Andrew V. Kralicek[2] and Marian J. McKenzie[1]

[1] The New Zealand Institute for Plant & Food Research Limited, Food Industry Science Centre, Private Bag 11600, Palmerston North 4442, New Zealand

[2] The New Zealand Institute for Plant & Food Research Limited, Mount Albert Research Centre, Private Bag 92169, Auckland 1142, New Zealand

[†] Present address: Australian Centre for Plant Functional Genomics, Waite Campus, University of Adelaide, PMB1, Glen Osmond, SA 5064, Australia

[*] To whom correspondence should be addressed. E-mail: david.brummell@plantandfood.co.nz

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Abstract

Cold storage of tubers of potato (*Solanum tuberosum* L.) compromises tuber quality in many cultivars by the accumulation of hexose sugars in a process called cold-induced sweetening. This is caused by the breakdown of starch to sucrose, which is cleaved to glucose and fructose by vacuolar acid invertase. During processing of affected tubers, the high temperatures involved in baking and frying cause the Maillard reaction between reducing sugars and free amino acids, resulting in the accumulation of acrylamide. cDNA clones with deduced proteins homologous to known invertase inhibitors were isolated and the two most abundant forms, termed INH1 and INH2, were shown to possess apoplastic and vacuolar localization, respectively. The *INH2* gene showed developmentally regulated alternative splicing, so, in addition to the *INH2*α transcript encoding the full-length protein, two hybrid mRNAs (*INH2*βA* and *INH2*β**B*) that encoded deduced vacuolar invertase inhibitors with divergent C-termini were detected, the result of mRNA splicing of an upstream region of *INH2* to a downstream region of *INH1*. Hybrid RNAs are common in animals, where they may add to the diversity of the proteome, but are rarely described in plants. During cold storage, *INH2*α and the hybrid *INH2*β mRNAs accumulated to higher abundance in cultivars resistant to cold-induced sweetening than in susceptible cultivars. Increased amounts of invertase inhibitor may contribute to the suppression of acid invertase activity and prevent cleavage of sucrose. Evidence for increased RNA splicing activity was detected in several resistant lines, a mechanism that in some circumstances may generate a range of proteins with additional functional capacity to aid adaptability.**

Key words: Cold-induced sweetening, cold stress, hybrid RNA, invertase inhibitor, RNA splicing, *Solanum tuberosum*.

Introduction

Due to their sessile nature, plants must adapt their metabolism to cope with abiotic stresses such as drought, high salinity, and low temperature. These three environmental stresses are related to some extent, and there are genes expressed in response to all three challenges as well as those specific to a particular stress (Kreps *et al.*, 2002; Seki *et al.*, 2002; Rabbani *et al.*, 2003). Some of these stress-responsive genes encode regulatory proteins, whereas others protect cells by causing the accumulation of metabolic proteins and cellular protectants including sugars (Yamaguchi-Shinozaki and Shinozaki, 2006). These stress-induced responses enable the plant to adapt its physiology and survive.

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Potato is now the third most important food crop in the world, and the most important non-grain food crop (FAOSTAT, 2007). Metabolic stability of potato tubers during long-term cold storage is one of the prime trait targets for breeding programmes worldwide. Potatoes need to be cool-stored throughout the year to maintain uninterrupted supply to industry, and storage at low temperature (<8 °C) is beneficial because it reduces bacterial soft rots, decreases water and dry matter loss, and prevents sprouting without the need to add sprout inhibitor chemicals (Sowokinos, 2001). However, in many cultivars, low temperature storage causes glucose and fructose to accumulate at substantial levels (Sowokinos, 2001; Blenkinsop *et al.*, 2004). This is termed cold-induced sweetening, and is one of the most serious problems facing the processing industry. When affected potatoes are processed into crisps or chips, the high temperature associated with frying or baking causes these reducing sugars to react with free amino acids by the Maillard reaction, resulting in an unacceptable blackening and unpalatability of the product (Kumar *et al.*, 2004) and accumulation of the potent neurotoxin and probable carcinogen acrylamide (Mottram *et al.*, 2002; Stadler *et al.*, 2002). Variability in resistance to cold-induced sweetening between cultivars is presumably due to a composite of differences in mRNA expression patterns and post-transcriptional events.

Cold-induced sweetening occurs due to an imbalance between the rate of breakdown of starch and metabolism of the resulting sucrose, some of which enters the vacuole where it can be irreversibly cleaved by acid invertase to the hexose sugars glucose and fructose (Sowokinos, 2001; Blenkinsop *et al.*, 2004). The pathway from starch breakdown to hexose sugars is complex (Malone *et al.*, 2006), and several enzymes are potentially involved. Cold storage has been found to cause increases in mRNA abundance or enzyme activity of forms of β-amylase, UDP-glucose pyrophosphorylase, sucrose phosphate synthase, and acid invertase (Zrenner *et al.*, 1993; Hill *et al.*, 1996; Nielsen *et al.*, 1997; Reimholz *et al.*, 1997; McKenzie *et al.*, 2005). Transgenic manipulations have also suggested roles in cold-induced hexose accumulation for UDP-glucose pyrophosphorylase (Borovkov *et al.*, 1996), sucrose phosphate synthase (Krause *et al.*, 1998), sucrose phosphatase (Chen *et al.*, 2008), glucan-water dikinase, and phosphorylase-L (Rommens *et al.*, 2006). However, the activity of acid invertase is the most significant factor in determining the accumulation of hexose sugars, and correlations between acid invertase activity and the hexose:sucrose ratio have been made in cultivars exhibiting varying resistance to cold-induced sweetening (Richardson *et al.*, 1990; Zrenner *et al.*, 1996; Matsuura-Endo *et al.*, 2004; McKenzie *et al.*, 2005). Indeed, transgenic suppression of acid invertase prevents hexose accumulation and cold-induced sweetening of stored tubers (Zrenner *et al.*, 1996; Bhaskar *et al.*, 2010).

Invertase activity may be controlled post-translationally *in planta* by invertase inhibitors, which have long been known to be present in potato tubers (Schwimmer *et al.*, 1961; Pressey, 1966, 1967; Pressey and Shaw, 1966). Storage of susceptible potato tubers results in a cold-induced increase in acid invertase activity over ~3 weeks, followed by a decline that appears to be due to accumulation of the invertase inhibitor protein (Pressey and Shaw, 1966). Overexpression of a tobacco (*Nicotiana tabacum* L.) vacuolar invertase inhibitor in transgenic potato tubers strongly reduced acid invertase activity and the formation of reducing sugars (Greiner *et al.*, 1999). These observations suggest that endogenous potato invertase inhibitors, if present in particular cultivars, could potentially be important in determining the extent of cold-induced sweetening.

Invertase inhibitors are small (~17 kDa) proteins that bind to invertases, forming an inactive complex (Pressey, 1967; Bracho and Whitaker, 1990*a*, *b*; Rausch and Greiner, 2004). Invertase inhibitors are closely related to pectin methylesterase inhibitor proteins (Hothorn *et al.*, 2004*a*, *b*) and are of two types, found in the apoplast and vacuole, respectively. The sequences of invertase inhibitors have been determined from several species including tobacco, *Arabidopsis*, soybean, sweetpotato, maize, rice, and tomato (Greiner *et al.*, 1998, 1999; Bate *et al.*, 2004; Link *et al.*, 2004; Rausch and Greiner, 2004; Reca *et al.*, 2008; Jin *et al.*, 2009), but not so far from potato. The aim of this work was to characterize the invertase inhibitor genes of potato, and to examine the expression of the members of this gene family both in a range of tissues and in the mature tuber during cold stress. Invertase inhibitor mRNA accumulation was assessed in a range of lines to determine if invertase inhibitor gene expression correlates with resistance to cold-induced sweetening.

Materials and methods

Plant material

Flowers were collected from greenhouse-grown plants and separated into sepal, petal, stamen, and gynoecium. Leaves and stems were collected from young plants. Tubers were harvested from immature developing plants and staged by size, then peeled and central tissues collected. All tissues were immediately frozen in liquid nitrogen and stored at –80 °C until required. For cold storage studies, mature tubers of breeding lines 1021/1 (resistant to cold-induced sweetening), 937/3 (sensitive to cold-induced sweetening), and selected lines from a mapping population were harvested from field-grown plants after the above-ground vegetative organs had died, and stored in the dark at ambient temperature to equilibrate for 1 week. After this time (T0), tubers were randomly sorted into groups and stored for 2 or 4 weeks at either 4 °C to develop cold-induced sweetening or at 10 °C as a control. At the end of the storage period, tubers were peeled and central regions chopped into cubes of ~5 mm then snap-frozen in liquid nitrogen and stored at –80 °C until required.

cDNA cloning and sequence analysis

Total RNA was prepared using the hot borate method (Wan and Wilkins, 1994) from tubers of 937/3 and 1021/1 that had been stored for 2 weeks at 4 °C as above. mRNA was selected using a Dynabeads mRNA purification kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. A cDNA library for each cultivar was constructed using 5 μg of poly(A)$^+$ RNA and a ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions. To generate

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a template for probe synthesis, degenerate primers INHdF and INHdR (Supplementary Table S1 available at *JXB* online) were designed to conserved amino acid domains in tomato and tobacco invertase inhibitors, and used to PCR-amplify a band of ~300 bp from potato tuber cDNA, which was ligated into pBluescript and some clones sequenced. A clone with homology to known invertase inhibitors was used as a template for preparing a labelled probe by the random prime method (Feinberg and Vogelstein, 1983). The labelled, purified probe was used to screen both cDNA libraries at moderate temperature (57 °C) according to the manufacturer's instructions. Positive plaques were subjected to a second round of purification followed by *in vivo* excision and sequencing. Sequences were aligned and compared with existing invertase inhibitors using CLUSTALW2 (Larkin *et al.*, 2007), and the major forms designated *INH1* and *INH2*. The unusual C-terminus of the deduced protein of *INH2* did not show conservation with a similar sequence from tobacco (GenBank accession no. Y12806), so the downstream region of the cDNA was further analysed by 3′-RACE (rapid amplification of cDNA ends) using nested primers INH2-O and INH2-I (Supplementary Table S1), oligo $d(T)_{17}$ primer, and potato tuber cDNA as a template. This confirmed the sequence originally found (termed *INH2β*) and led to the discovery of an additional form, termed *INH2α*. Full-length clones of *INH2α* and *INH2β* were amplified from cDNA or genomic DNA as below. Signal peptide prediction was carried out using SignalP 3.0 (http://www.cbs.dtu.dk./services/SignalP/).

To examine the diversity of *INH2β* forms present in cDNA, primers were designed to the non-coding flanking regions of the cDNA for *INH2β* (sense primer INH2F and antisense primer INH2R2; Supplementary Table S1). cDNA was synthesized from tuber RNA of both 937/3 and 1021/1 using SuperScript reverse transcriptase (Invitrogen) and oligo $d(T)_{17}$ according to the manufacturer's instructions. PCR amplification using the primer pair, cDNA template, and PCR Extender proofreading *Taq* DNA polymerase (5 Prime Co., Gaithersburg, MD, USA) resulted in a band of ~800 bp, which was ligated into pBluescript and six clones sequenced for each cultivar.

Genomic characterization

Genomic DNA was prepared from young leaves of cultivars 937/3 and 1021/1 using a urea method. For investigation of the allelic diversity of *INH1* and *INH2*, primers were designed to the non-coding flanking regions of the cDNA for *INH1* (sense primer INH1F2 and antisense primer INH1R4) and for *INH2* (sense primer INH2F and antisense primer INH2R; Supplementary Table S1). Genomic DNA was PCR-amplified using the above primer pairs and *Pwo* (Roche, Auckland, New Zealand), TripleMaster (Eppendorff, Hamburg, Germany), or HiFidelity (Qiagen, Valencia, CA, USA) proofreading DNA polymerase. Two clones (for *INH1*) or at least 12 clones per genotype (for *INH2*) were sequenced on both strands.

For DNA gel blot analysis, aliquots of genomic DNA of the tetraploid cultivar 1021/1 and the diploid cultivar 3T were exhaustively digested with the indicated restriction enzymes, and either 20 μg (1021/1) or 12 μg (3T) of DNA was loaded per lane onto a 1% agarose gel. After separation of the DNA fragments by electrophoresis, the gel was blotted to Hybond-XL membrane (Amersham, Auckland, New Zealand) overnight. Short probe templates (~150 bp) were prepared from flanking non-coding or divergent coding regions that had previously been amplified by PCR from plasmids (for INH1, sense primer INH1F2 and antisense primer INH1R2; for INH2, sense primer INH2F4 and antisense primer INH2R4). These templates were labelled by PCR as previously described (Brummell *et al.*, 1999), using the antisense primer, [α-^{32}P]dATP, unlabelled nucleotides (dCTP, dGTP, dTTP), and *Taq* polymerase with 10 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min. The denatured probes were hybridized with the gel blot in Church and Gilbert (1984) solution at 65 °C overnight, then washed several times in 0.5× SSC/0.1% SDS at 65 °C and exposed to Kodak (Rochester, NY, USA) Biomax-MS film.

Subcellular protein localization using green fluorescent protein (GFP) fusions

For INH1, primers INH1GFPF and INH1GFPR (Supplementary Table S1) were used to PCR-amplify a fragment encoding the N-terminal 32 amino acids, and for INH2 primers INH2GFPF and INH2GFPR were used to amplify a fragment encoding the N-terminal 52 amino acids. In each case, an *Eco*RI site upstream of the translation start codon and a downstream *Bam*HI site were used to ligate the fragment into the CaMV35S-GFP expression vector. The in-frame *Bam*HI site added a glycine and a serine residue to the predicted protein between the INH fragment and the GFP. Plasmid DNA (1 μg) of the *35S:INH1Nterm:GFP:ocs* and *35S:INH2Nterm:GFP:ocs* constructs or *35S:GFP:ocs* alone (as a control) was mixed with 1 μm gold particles and bombarded into the adaxial epidermis of bulb of onion (*Allium cepa*) or white petals of lisianthus (*Eustoma grandiflorum*) as described (Shang *et al.*, 2007). Tissues were incubated on hormone-free Murashige and Skoog medium at 25 °C in the dark for 36 h, and transformed cells were examined under a Leica laser scanning confocal fluorescence microscope or an Olympus fluorescence microscope.

mRNA analysis

Total RNA prepared as above (10 μg per lane) was separated by electophoresis in 1.2% agarose denaturing formaldehyde gels, then blotted to Hybond-XL membrane (Amersham) overnight. DNA templates and labelled probes were produced as for genomic DNA gel blot analysis, and hybridized with the RNA gel blots in Church and Gilbert (1984) solution at 65 °C overnight. Blots were washed in 0.5× SSC/0.1% SDS at 65 °C and exposed to Kodak Biomax-MS film.

For quantitative real-time PCR analysis, DNA was eliminated from RNA samples by treatment with DNase I (Invitrogen). Aliquots of RNA (1 μg) were converted to cDNA using oligo $d(T)_{20}$, dNTPs, and SuperScript III reverse transcriptase (Invitrogen). Products were diluted 25-fold in water prior to use and aliquots of 2.5 μl used per reaction (10 μl final volume) in the presence of 0.5 μM clone-specific primers (Supplementary Table S1) and Light Cycler 480 SYBR Green I master mix (Roche). Primer pairs used were: INH1, INH1RTF+INH1RTR; INH2α, INH2RTF+INH2αRTR; INH2β, INH2RTF+INH2βRTR; and actin, ActRTF+ActRTR (Supplementary Table S1). Real-time PCR was performed in triplicate on a Rotor-Gene RG-3000 (Corbett Research, Sydney, Australia) following the manufacturer's instructions. Experiments were repeated using a minimum of two independent RNA preparations. Data acquisition and analysis were carried out using Rotor-Gene Series 6000 software version 1.7 (Corbett Research). Actin was used as the reference gene for calculation of relative transcript abundance, using the Relative Quantitation feature of the software. Melt analysis and visualization of the final products on agarose gels were used to ensure that products were a single band.

Sugar analysis

Frozen potato tissue was powdered in liquid nitrogen and sugars were extracted with 62.5% (v/v) methanol at 55 °C for 1 h. Sucrose, fructose, and glucose concentrations in the clarified extract were estimated against standards using a Sucrose, D-Fructose and D-Glucose assay kit (Megazyme, Bray, Ireland) according to the manufacturer's instructions.

Recombinant protein expression

Synthetic genes encoding INH2α*A1, INH2β*A1, and INH2β*B1, in each case harbouring an N-terminal tobacco etch virus (TEV) protease cleavage site and flanked by Gateway recombination sequences, were synthesized by GenScript (Piscataway, NJ, USA).

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For all three proteins, the encoded sequence began with the tyrosine residue at amino acid 27, since this appeared to be the mature protein based on structural studies (Hothorn *et al.*, 2004*a*). Genes were ligated into the pDEST566 vector (Addgene plasmid 11517, www.addgene.org) by Gateway recombination, to add an N-terminal His_6-maltose-binding protein (MBP) domain. Plasmids were transformed into *Escherichia coli* Rosetta Gami 2 cells (Novagen, San Diego, CA, USA) and grown at 37 °C in Terrific Broth until they reached a density of A_{600}=0.6, then induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and grown at 20 °C for 18 h. Harvested cells were resuspended in buffer containing 20 mM TRIS-HCl, pH 8.0, 300 mM NaCl, and antiprotease cocktail (Roche), and lysed using a C5 emulsifier (Avestin, Mannheim, Germany). The supernatant after centrifugation at 20 000 *g* for 30 min was loaded on a His-Trap HP column (GE Healthcare, Piscataway, NJ, USA), washed with 20 mM TRIS, pH 8.0, 300 mM NaCl, and fusion proteins were eluted with buffer containing 20 mM TRIS, pH 8.0, 150 mM NaCl, 300 mM imidazole. Fractions containing the fusion protein were pooled, and dialysed against 20 mM TRIS, pH 8.0, 50 mM NaCl at 4 °C for 18 h. The fusion proteins were then further purified by anion exchange on a HiTrap Q HP column (GE Healthcare), using a 50–500 mM NaCl linear elution gradient, and size exclusion chromatography on a Superdex 75pg HiLoad 16/60 column (GE Healthcare) using 20 mM TRIS, pH 8.0, 150 mM NaCl as eluant. In all cases protein was collected as soluble aggregates in the void volume of the column. The control was produced by TEV cleavage of a His-MBP-α/β hydrolase (from *Arabidopsis*) fusion protein and purification of the His-MBP domain as above. Protein concentrations were calculated from absorption at 280 nm, using extinction coefficients calculated from their respective amino acid sequences. For SDS–PAGE analysis, samples were denatured for 30 min at 37 °C with either SDS loading buffer or SDS loading buffer containing 6 M urea.

Invertase activity

Potato samples frozen in liquid nitrogen were powdered using a mortar and pestle in 3 vols of buffer containing 50 mM HEPES-KOH, pH 7.5, 15 mM $MgCl_2$, 2 mM EDTA, 2 mM dithiothreitol, 10% (v/v) glycerol, and 2% (w/v) polyvinylpolypyrrolidone. Crude extracts were centrifuged at 14 000 *g* and the supernatants desalted using Sephadex G-25 columns according to the manufacturer's instructions. The extracts were shaken for 90 min rapidly enough to generate foaming, to minimize the activity of endogenous invertase inhibitor proteins (Pressey, 1966). Enzyme assays contained 60 µl of protein extract, 60 mM phosphate-citrate buffer, pH 5.0, and 40 mM sucrose. Reactions were carried out at 30 °C for 30 min, and were stopped by heating to 94 °C for 1 min. Control reactions for each sample were performed by heating the reaction aliquots of protein extract to 94 °C for 1 min before addition of substrate and buffer. Glucose formed was determined against a standard curve using Sumner's reagent (Sumner, 1921). Protein was determined using a protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. Activity was expressed as nmol of glucose formed h^{-1} mg^{-1} protein.

In experiments where the activity of invertase inhibitors was examined, aliquots of crude protein extract from cold-stored tubers of cultivar Summer Delight prepared as above were incubated with dilutions of purified recombinant invertase inhibitor fusion proteins at 37 °C for 30 min to allow binding, prior to assay for invertase activity.

Results

Characterization of INH1 *and* INH2 *genes and mRNAs*

Library screening and PCR analysis identified several cDNAs with deduced proteins showing homology to known invertase inhibitors (Table 1). All deduced proteins possessed four conserved cysteine residues believed to be important in invertase inhibitor protein three-dimensional structure, but were of two distinct types. The first type had deduced proteins with a predicted signal peptide of 19 amino acids and a putative apoplastic localization (Fig. 1A). Two closely related cDNAs were isolated, probably representing allelic forms of the same gene. This gene was termed *INH1*, and the allelic forms *INH1*A* and *INH1*B*. INH1*A and INH1*B deduced proteins differed in only six amino acids, and both were highly homologous to invertase inhibitors from *Nicotiana tabacum* (NtINH1, GenBank accession no. Y12805) and *Solanum lycopersicum* (SlINH, AJ010943) (Fig. 1A). Variants of SlINH have been termed SolyCIF (Reca *et al.*, 2008) and SlINHINV1 (Jin *et al.*, 2009). Apoplastic invertase inhibitors do not appear to be glycosylated (Weil *et al.*, 1994; Reca *et al.*, 2008), and INH1*A and B lacked sites for *N*-glycosylation. Sequencing of two potato genomic clones found that both were *INH1*B*, and that the gene possessed an intron of 1242 bp located after nucleotide +297 (GenBank accession no. GU980592). The location of the intron was almost identical to that in the orthologous tomato gene *SolyCIF* (Reca *et al.*, 2008).

The second type of invertase inhibitor lacked a predicted signal peptide, and existed in several forms differing at the C-terminus of the deduced proteins and in the 3'-untranslated regions (UTRs). These were termed *INH2α* (encoding the full-length protein) and *INH2β* (encoding truncated and altered variants of the INH2 protein). Five alleles of *INH2α* were identified from sequencing of cDNA and genomic clones amplified from both potato cultivars (937/3 and 1021/1), although not all alleles were present in each cultivar. The alleles possessed four different deduced amino acid sequences, which were designated INH2α*A–D, all of which showed homology throughout their length to a putative

Table 1. INH forms isolated from potato genotypes 1021/1 and 937/3 using a combination of cDNA library screening and PCR amplification from genomic DNA and cDNA.
INH2 produced multiple transcripts from the same gene, with *INH2α* representing allelic versions of mRNAs encoding the full-length protein, and *INH2β*A* and *B* representing spliced, hybrid mRNAs encoding deduced proteins that are truncated and altered at the C-terminus.

Name	Introns	Deduced amino acids	Sites for *N*-glycosylation	Putative localization	Accession no.
INH1*A	1	171	0	Apoplastic	AY864819
INH1*B	1	171	0	Apoplastic	AY864820
INH2α*A1	0	181	1	Vacuolar	FJ810205
INH2α*A2	0	181	1	Vacuolar	FJ810206
INH2α*B	0	180	1	Vacuolar	FJ810207
INH2α*C	0	178	1	Vacuolar	FJ810208
INH2α*D	0	178	1	Vacuolar	FJ810209
INH2β*A1	Cryptic?	153	0	Vacuolar	AY864821
INH2β*A2	Cryptic?	153	0	Vacuolar	GU980593
INH2β*B1	Cryptic?	158	1	Vacuolar	GU980594
INH2β*B2	Cryptic?	158	1	Vacuolar	GU980595

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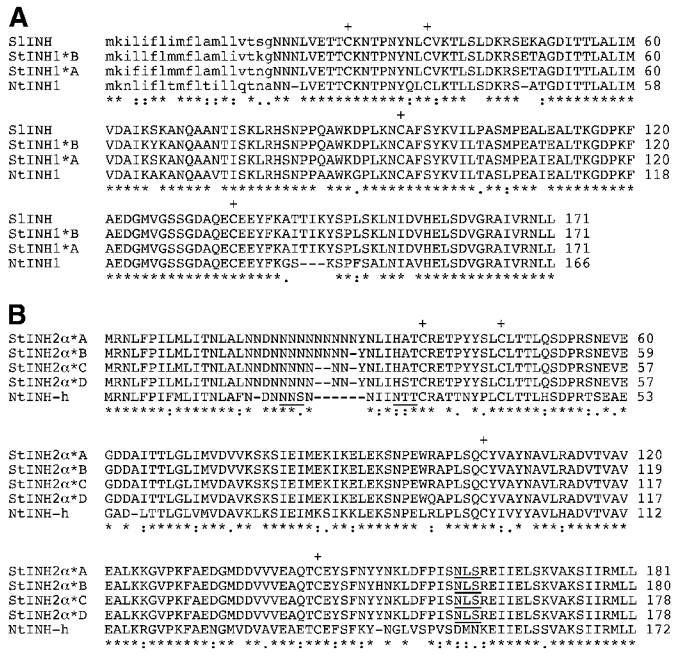


Fig. 1. Deduced amino acid sequences of StINH1 and StINH2α. (A) Two allelic forms of StINH1 aligned with NtINH1 from tobacco (GenBank accession no. Y12805) and SlINH from tomato (AJ010943). Predicted signal sequences are shown in lower case. (B) Four allelic forms of StINH2α aligned with NtINH-h from tobacco (Y12806). Potential sites for *N*-glycosylation are underlined. Four cysteine residues believed to be important in the formation of two disulphide bridges (conserved in both apoplastic and vacuolar invertase inhibitors) are marked with crosses. Amino acid identity is represented by asterisks, highly conservative changes by double dots, and conservative changes by dots.

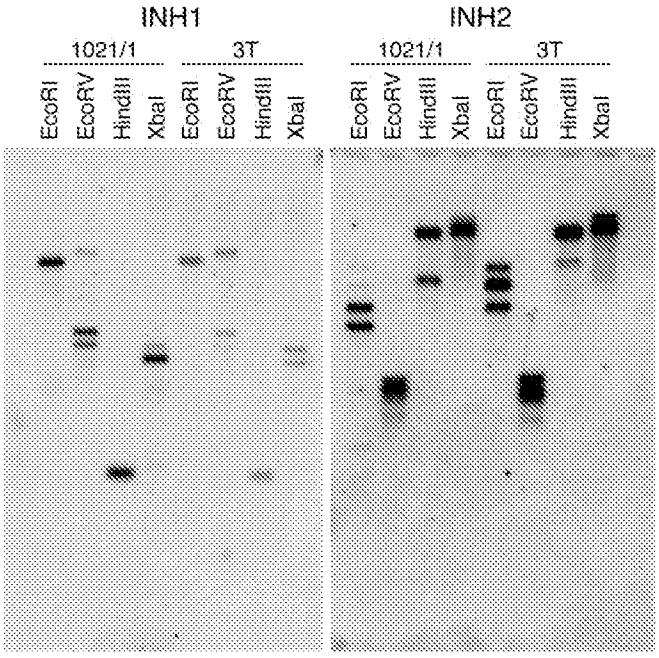


Fig. 2. DNA gel blot analysis. Genomic DNA of the potato tetraploid variety 1021/1 and the diploid variety 3T was digested with the indicated restriction enzyme and separated by gel electrophoresis. A blot of the gel was hybridized with labelled probes specific for *INH1* or *INH2* as shown, and washed at high stringency.

vacuolar invertase inhibitor from tobacco (Fig. 1B). INH2α*A–D differed from each other predominantly in the number of amino acids present in a run of consecutive asparagine residues near the N-terminus of the protein. INH2α*C and D also differed from each other by three amino acid residues elsewhere in the protein, as well as extensive differences in the 3′-UTR (data not shown). Two versions of *INH2α*A* were detected, differing only in the length of a stretch of adenine nucleotides upstream of the translation start codon. All of the potato INH2α forms had a single site for potential *N*-glycosylation, located near the C-terminus of the protein (Fig. 1B). No introns were detected in any *INH2α* genomic clones.

DNA gel blot analysis revealed that *INH1* and *INH2* are both likely to be encoded by very small gene families, probably two genes per haploid genome for *INH1* and with the possibility of a small number of closely related members for *INH2* (Fig. 2). Preliminary data released by the Potato Genome Sequencing Consortium (2009) have so far identified a single gene for each of these two families, both located on chromosome 12 with a tandem orientation (Fig. 3).

Subcellular localization of INH1 and INH2 proteins

Initial experiments using DNA constructs that fused the entire coding regions of *INH1* and *INH2* to GFP did not result in visible fluorescence upon transient transformation into onion bulb epidermis or flower petals. Consequently, constructs were produced that fused the N-terminal quarter of each INH protein, including any putative signal sequence, to GFP. As a control, GFP alone was transiently expressed in onion bulb epidermis cells by particle bombardment, and was detected as relatively weak GFP fluorescence present only in the cytoplasm (Fig. 4A–C). In contrast, the INH1Nterm–GFP fusion protein was detected strongly in the cytoplasm, but also in the cell wall of epidermal cells of both onion bulb (Fig. 4D–F) and lisianthus petal (Fig. 4J). Plasmolysis of lisianthus cells caused the cytoplasm to withdraw from the apoplast, revealing clear GFP fluorescence in the cell wall (Fig. 4K). INH2Nterm–GFP fusion protein was detected transiently in onion bulb epidermal cells, but degraded immediately upon exposure to light (data not shown). Expression of INH2Nterm–GFP fusion protein in lisianthus petals, where vacuolar proteins are more stable, showed that the majority of the fusion protein was in the cytoplasm, but that the protein also accumulated in the vacuole (Fig. 4G–I). These data show that although the bulk of INH1Nterm–GFP and INH2Nterm–GFP proteins was present in the cytoplasm, where proteins are synthesized, the signal sequences present on these fusion proteins caused a proportion to enter the cell wall or the vacuole, respectively.

Characterization of INH2β *mRNAs*

INH2β (subsequently termed *INH2β*A1*) was originally isolated from a cDNA library screen. An additional

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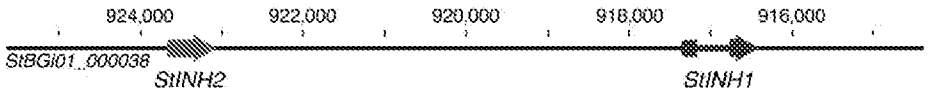


Fig. 3. Tandem location of *INH2* and *INH1* genes on potato chromosome 12. Numbers show nucleotide positions on the StBGI01_000038 Scaffold of version 1 of the potato (*S. tuberosum* RH) assembly (Potato Genome Sequencing Consortium, 2009).

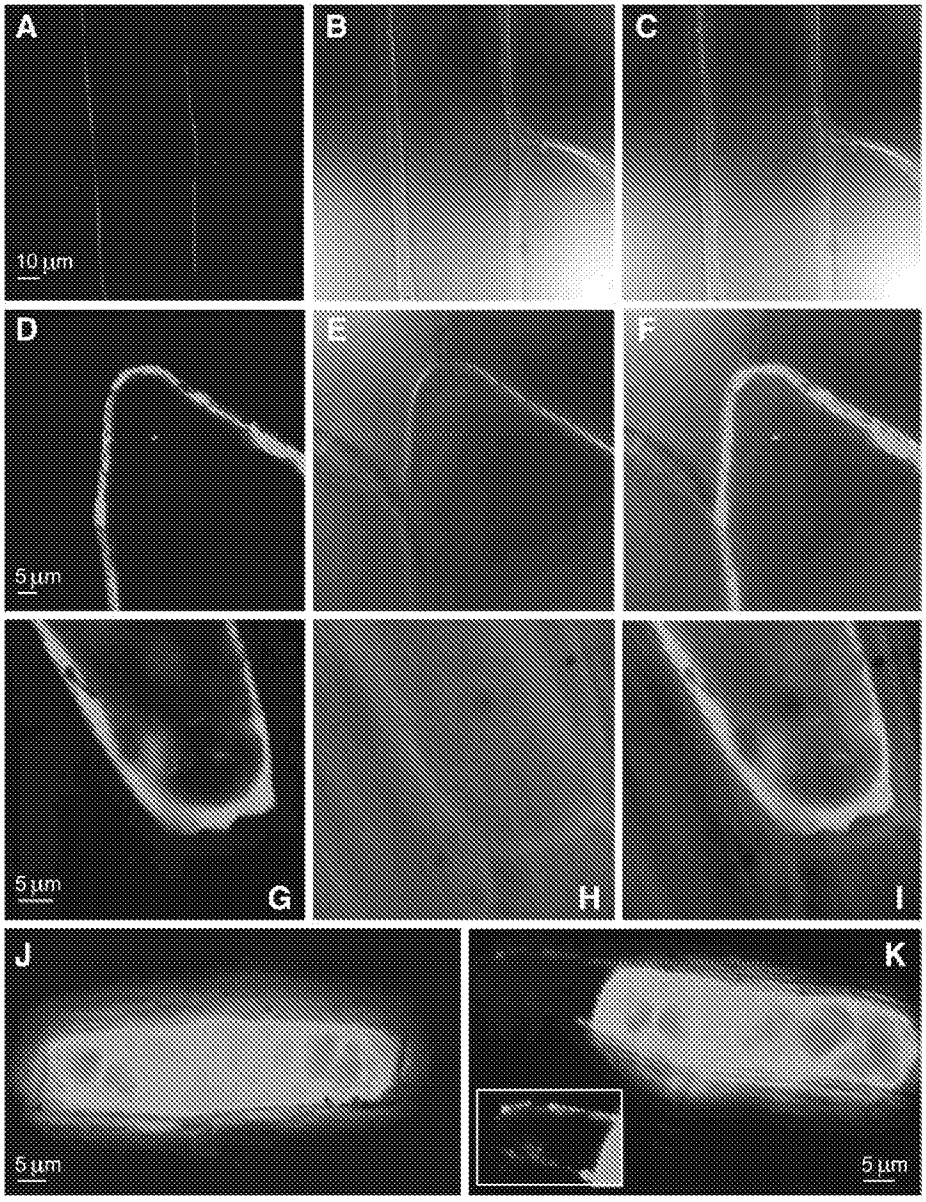


Fig. 4. Subcellular localization of INH1 and INH2 N-terminal peptides fused with GFP. GFP (control) and INH1Nterm–GFP and INH2Nterm–GFP fusion proteins were expressed transiently in adaxial epidermal cells of onion bulb or lisianthus flower petals and visualized using confocal laser scanning microscopy (A–I) or fluorescence microscopy (J and K) 36 h after particle bombardment with a gene gun. (A, D, G) Fluorescence image; (B, E, H) differential interference contrast image; (C, F, I) merged image. (A–C) Accumulation of GFP in the cytoplasm of an onion epidermal cell (0.2 µm optical section). (D–F) Accumulation of INH1Nterm–GFP fusion protein in the cytoplasm and cell wall of an onion epidermal cell (0.2 µm optical section). (G–I) Accumulation of INH2Nterm–GFP fusion protein in the cytoplasm and vacuole of a lisianthus petal cell (0.5 µm optical section). (J) Accumulation of INH1Nterm–GFP fusion protein in the cytoplasm and cell wall of a lisianthus petal cell. (K) A similar cell to that shown in J plasmolysed in 1 M sucrose for 10 min, showing fluorescence remaining in the wall after the protoplast has withdrawn. Inset boxed: the plasmolysed region of the cell with contrast enhanced to show GFP clearly in the cell wall.

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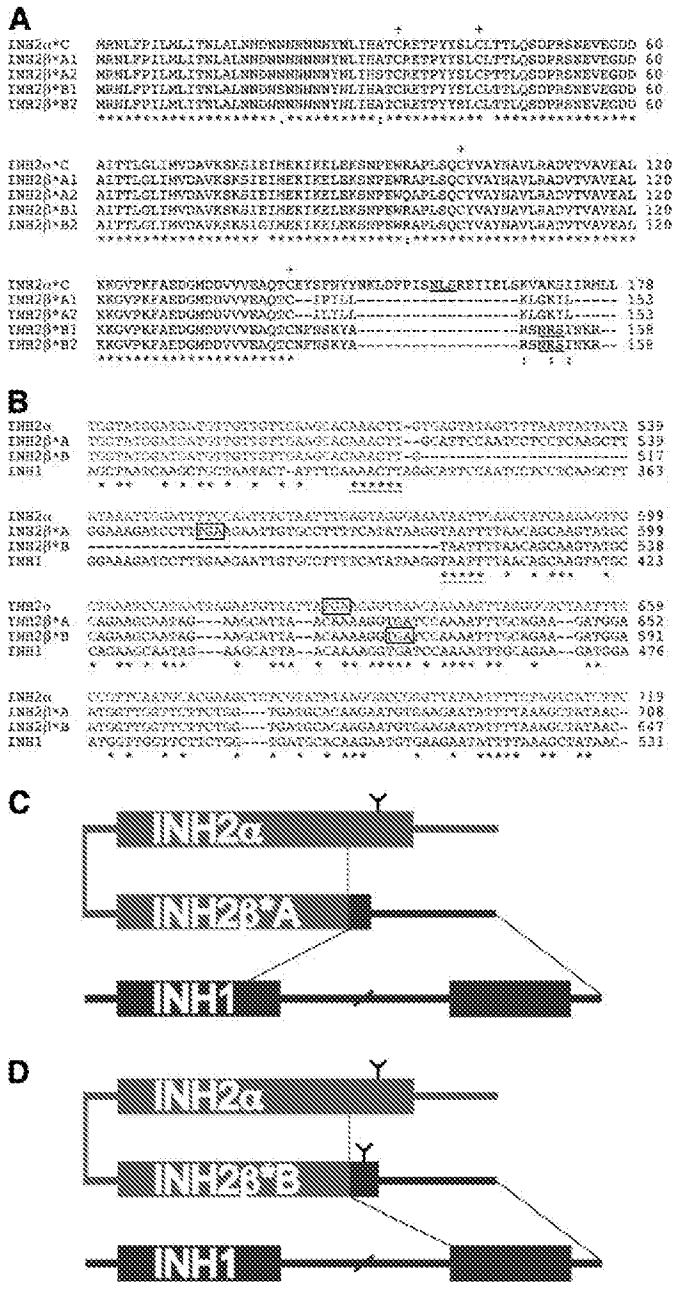


Fig. 5. *INH2β* is a hybrid RNA homologous to *INH2* upstream and to *INH1* downstream of the junction. (A) Alignment of the deduced amino acid sequence of four allelic forms of INH2β with that of INH2α*C. Potential sites for *N*-glycosylation are underlined, and four conserved cysteine residues are marked with crosses. Amino acid identity is represented by asterisks, highly conservative changes by double dots, and conservative changes by dots. (B) Part of the cDNA sequence of *INH2β*A* and *B* aligned with that of *INH2α* and *INH1*. The region shown is the junction where sequence conserved with that of *INH2α* (shown in green) is spliced to sequence conserved with that of *INH1* (shown in blue). Two different splice junctions are evident, *INH2β*A* possessing more upstream sequence derived from *INH1* than does *INH2β*B*. Putative short homologous sequences present in both *INH1* and *INH2α*, located at the apparent splice sites, are underlined in red. Translation stop codons are boxed. Nucleotide identity is indicated by asterisks, and gaps introduced into the alignment by dashes. (C) Schematic diagram showing the regions of *INH2α* (green) and *INH1* (blue) mRNA that make up the spliced *INH2β*A* transcript. Whether the intron derived from *INH1* appears in the pre-mRNA of *INH2β*A* and is then processed out, or splicing occurs after formation of the mRNA is unclear. Exons are shown as boxes, and introns and untranslated regions as lines. The exons derived from *INH1* are out-of-frame in *INH2β*, resulting in a shorter coding region. (D) Schematic diagram showing the regions of *INH2α* (green) and *INH1* (blue) mRNA that make up the spliced *INH2β*B* transcript. The exon derived from *INH1* is out-of-frame in *INH2β*B*, resulting in a shorter coding region. Potential sites for *N*-linked glycosylation in the corresponding deduced proteins are represented by Y-shaped symbols.

transcript termed *INH2β*A2* was isolated from cDNA of cultivar 1021/1 by PCR amplification, and differed from *INH2β*A1* by 10 nucleotides and four deduced amino acids. The deduced amino acid sequences of INH2β*A were highly similar to that of INH2α (almost identical to the *INH2α*C* allele) throughout most of the length, but diverged sharply near the C-terminus (Fig. 5A). At this point the DNA sequence differed from *INH2α* completely, and, surprisingly, the sequence downstream of this junction was identical to *INH1* (Fig. 5B) with a splice junction in the first exon (Fig. 5C). This apparent fusion of the mRNA sequences of *INH2* and *INH1* provided the hybrid *INH2β*A* mRNA with an alternative and longer 3′-UTR to that of *INH2α*, but had the effect of adding just 11 amino acid residues to the predicted protein before a translation stop occurred, so that INH2β*A was 25 amino acid residues shorter than INH2α*C (Fig. 5A, C).

Subsequent isolation of further *INH2β* cDNA clones by PCR amplification found that *INH2β*A1* was the predominant form, but that a variant, termed *INH2β*B*, also existed in both genotypes (Fig. 5A, B). In *INH2β*B*, the splice junction occurred further downstream, at the intron splice boundary at the beginning of exon 2 of *INH1* (Fig. 5D). A comparison of *INH2β*B* sequences isolated from genotypes 1021/1 and 937/3 found four single nucleotide differences, resulting in two amino acid residue changes, all of these differences being in the region of the DNA conserved with *INH2α*. These forms were termed *INH2β*B1* and *INH2β*B2*, respectively (Fig. 5A).

Although *INH2β*B* possessed the entire exon 2 from *INH1*, the exon was in a different reading frame so that translation caused the addition of only 16 amino acid residues before a translation stop codon occurred (Fig. 5A). This frameshift meant that there was no conservation in amino acid sequence between the C-terminal regions of INH2β*B and either INH2β*A or INH2α, although, like INH2α, the INH2β*B form had a site for potential *N*-glycosylation of the protein (Fig. 5A, D).

It was not possible to carry out genomic DNA gel blot analysis for *INH2β*, since this does not possess any unique DNA. Attempts to isolate genomic versions of *INH2β* using an array of nested primers (12 different primer pair combinations) spanning the apparent *INH2/INH1* junction

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were unsuccessful, although all primer pairs successfully amplified a product from tuber cDNA (data not shown). Data released by the Potato Genome Sequencing Consortium (2009) have also found no evidence for the existence of such hybrid sequences in the genome. These data suggest that *INH2β* may exist as a transcript but not as a gene.

Presence of alleles of INH2α *and* INH2β *in cultivars 1021/1 and 937/3*

Although several different allelic forms of the various genes are described above, it should be noted that not all alleles were found in each cultivar. This was particularly clear for *INH2α*, where cultivar 1021/1 had alleles *A1*, *A2*, and *D*, while cultivar 937/3 had alleles *A1*, *B*, and *C* (data not shown). Since the number of clones sequenced was limited and because only particular primer pairs were used, it is of course possible that other alleles of *INH2* (or related genes) are present in these genotypes. Both cultivars had the *INH2β*A1* mRNA, and both cultivars had an *INH2β*B* mRNA, although these were *INH2β*B1* and *INH2β*B2*, respectively.

Expression of invertase inhibitors in potato plants

Examination of various tissues of potato plants, including flower parts, vegetative tissues, and developing tubers, found that *INH2α* mRNA accumulated in all tissues, though to varying extents (Fig. 6A). *INH2α* mRNA was present at very low abundance in some flower parts (sepal, petal, and gynoecium) and leaf, and at higher abundance in stamen, stem, stolon, and developing tubers. In contrast, *INH2β* mRNA was undetectable in flower parts, leaf, stem, and stolon, and accumulated at low but increasing abundance in tubers as they developed (Fig. 6B). When present, the abundance of *INH2β* mRNA was several fold less than the accumulation of the *INH2α* transcript. These data suggest that *INH2β* transcript accumulation may be specific to tubers. *INH1* mRNA was present at very high abundance in flower parts, and at levels exceeding those of *INH2α* in all other tissues (Fig. 6C).

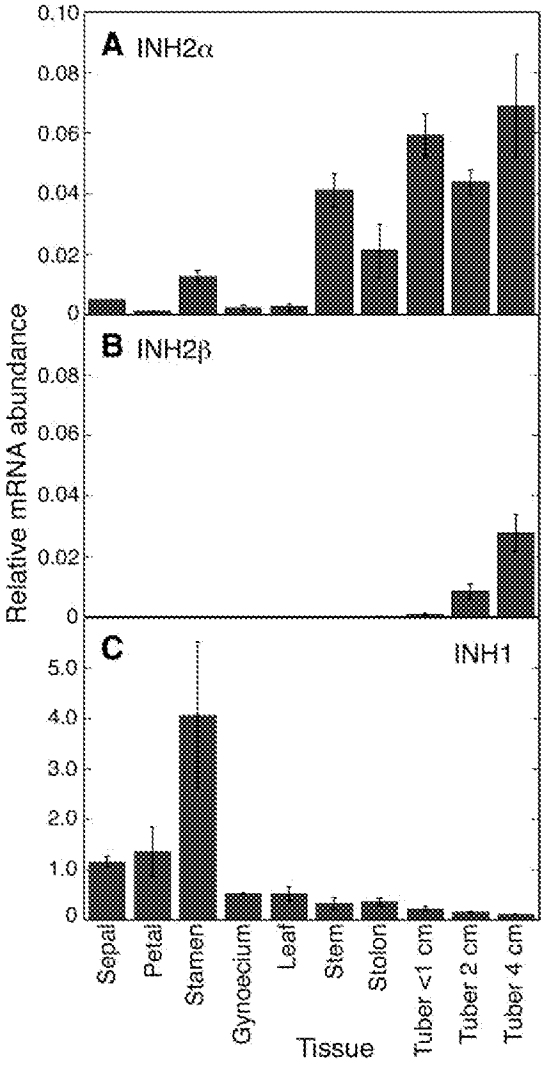


Fig. 6. mRNA accumulation of *INH1*, *INH2α*, and *INH2β* in various tissues of potato plants determined using quantitative real-time PCR. (A) Relative *INH2α* mRNA abundance. (B) Relative *INH2β* mRNA abundance. (C) Relative *INH1* mRNA abundance (note scale difference from A and B). Data shown are for cultivar 1021/1 (cultivar 937/3 was similar; data not shown). Bars denote SDs from a minimum of two replicate RNA preparations.

Abundance of invertase inhibitor mRNAs during potato tuber storage

In order to produce cold-induced sweetening, tubers of the susceptible cultivar 937/3 and the resistant cultivar 1021/1 were stored at 4 °C for 2 or 4 weeks and compared with tubers stored at 10 °C. In 937/3, soluble sugars accumulated drastically at 4 °C, increasing by 3-fold between 2 and 4 weeks (Fig. 7). The majority of this sugar was the reducing sugars glucose and fructose, with only a minor proportion being retained as sucrose. In contrast, in 1021/1, soluble sugars increased during cold storage to a lesser extent than in 937/3, and the majority of the soluble sugar was sucrose, with hexoses providing only one-fifth of the total sugar on a molar basis. In both genotypes, soluble sugar showed little change in amount during storage at 10 °C.

For examination of invertase inhibitor mRNA abundances (Fig. 8), RNA was prepared from the same samples of stored tubers as shown in Fig. 7. Since it was not possible to design a probe for RNA gel blot analysis that would be specific for *INH2β* and not show cross-hybridization with either *INH2α* or *INH1*, quantitative real-time PCR was used to discriminate between *INH2α* and *INH2β* mRNAs (Fig. 8B). *INH2α* and *INH2β* mRNAs accumulated at much higher levels in the resistant cultivar 1021/1 than they did in 937/3, especially after storage at 4 °C. In 1021/1, *INH2α* mRNA abundance increased several fold during treatment at 10 °C, and at 4 °C accumulated slightly later but to even higher levels. *INH2β* mRNA remained at very low abundance in 937/3 after all treatments, and in 1021/1 did not accumulate at 10 °C but increased during treatment at 4 °C.

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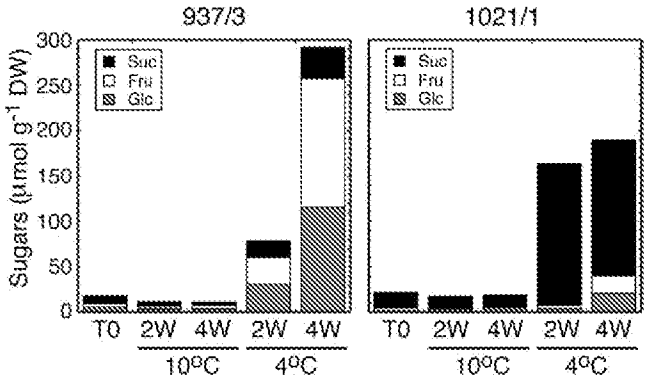


Fig. 7. Hexose sugars accumulate in cold-stored tubers of the cold-induced sweetening-sensitive cultivar 937/3, whereas sucrose accumulates in the resistant cultivar 1021/1. Tubers of two genotypes of potato with differing sensitivity to cold-induced sweetening were stored at 10 °C or 4 °C for 2 or 4 weeks (W). Contents of sucrose and the reducing sugars glucose and fructose were determined in extracts and are expressed on a molar basis.

The estimations of mRNA abundances by real-time PCR (Fig. 8B) were confirmed using RNA gel blot analysis (Fig. 8A). Hybridization of an RNA gel blot with a probe that would detect the sum of both *INH2α* and *INH2β* transcripts found that *INH2α+β* mRNA was at low abundance in cultivar 937/3 in all treatments, but accumulated substantially in cultivar 1021/1 (Fig. 8A). *INH2α+β* mRNA abundance increased during storage at 10 °C, and increased more slowly at 4 °C but to a greater extent by 4 weeks of treatment. The mRNA abundances determined by RNA gel blot analysis were in good agreement with those estimated by quantitative real-time PCR. These data show that accumulation of *INH2α* and *INH2β* mRNAs is independently developmentally regulated, and suggest that in cultivars resistant to cold-induced sweetening accumulation of the *INH2α* mRNA is promoted by both chilling (10 °C) and cold (4 °C), whereas *INH2β* mRNA accumulation is promoted by cold treatment. *INH1* mRNA was induced strongly after 2 weeks at 4 °C, and accumulated at much higher levels than *INH2α* or *INH2β* (Fig. 8C). However, the increase in mRNA accumulation was transient and occurred in both susceptible and resistant genotypes.

To investigate the wider applicability of these observations, lines with known cold-induced sweetening properties were selected from a mapping population, and fresh tubers were stored at 4 °C for 4 weeks. In the cold-induced sweetening-susceptible lines Karaka, Summer Delight, K056, S343, S190, and S130, cold storage resulted in a high accumulation of soluble sugars of which the majority was glucose and fructose (Fig. 9A). In the cold-induced sweetening-resistant lines K184, S028, S341, S217, S166, 1021/1, and K068, soluble sugars accumulated to low levels after storage and hexoses formed a minor proportion of the total. In general, the cold-induced sweetening-susceptible lines exhibited high acid invertase activity after storage, whereas the resistant lines had low activity (Fig. 9B). An exception was line S130, which had a high hexose sugar content but

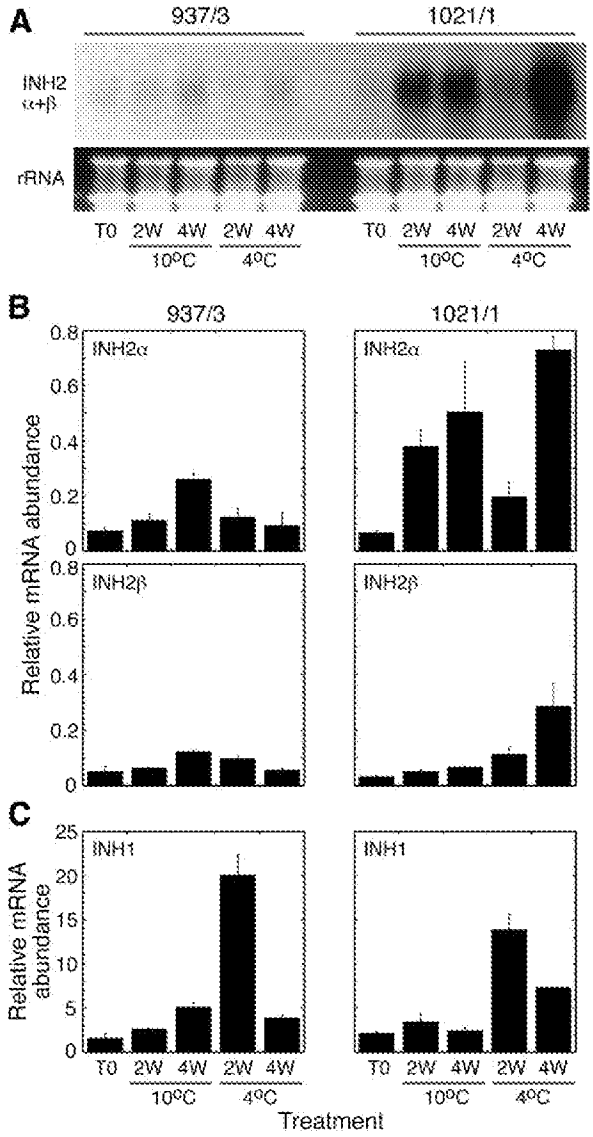


Fig. 8. mRNAs of *INH2α* and *β* accumulate during cold storage of tubers in the cold-induced sweetening-resistant cultivar 1021/1 but not in the sensitive cultivar 937/3. (A) RNA gel blot hybridized with a probe that will detect both *INH2α* and *β* mRNAs. (B) Relative abundance of mRNAs of *INH2α* and *β* determined using quantitative real-time PCR. (C) Relative abundance of mRNA of *INH1* determined using quantitative real-time PCR (note scale difference from B). Tubers were stored at 10 °C or 4 °C for 2 or 4 weeks (W). Bars denote SDs from three replicate RNA preparations.

low acid invertase activity. Examination of a time course of treatment would be necessary to determine if acid invertase activity had been higher at earlier points during storage in this line. In the majority of lines, low acid invertase activity was correlated with moderate or high accumulation of the sum of *INH2α* plus *INH2β* mRNAs (Fig. 9C), suggesting that invertase inhibitors may contribute to lower levels of acid invertase activity in certain cultivars. The correlation was not complete, however, with line S217 having low *INH2α+β* abundance and low acid invertase activity, and lines S130 and K184 having similar *INH2α+β* mRNA

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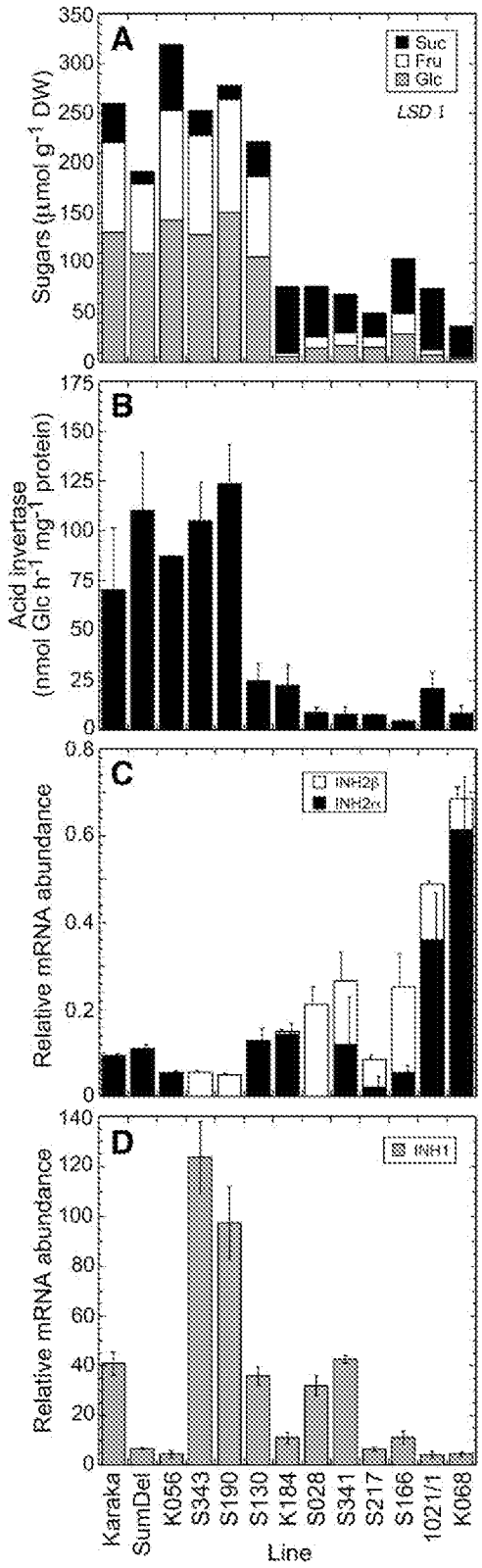


Fig. 9. Sugar content, acid invertase activity, and abundance of INH mRNAs in cold-stored tubers of lines selected from a mapping population. (A) Content and composition of soluble sugars. The least significant difference (indicated by a bar) at P=0.05 was 11.0 μmol g^{-1} DW. (B) Soluble acid invertase activity. Data are means of triplicate determinations from at least two independent protein extractions ±SE. (C) Relative abundance of mRNAs of *INH2α* and *INH2β* determined using quantitative real-time PCR. (D) Relative abundance of mRNA of *INH1* determined using quantitative real-time PCR. Tubers were stored at 4 °C for 4 weeks. In C and D, bars denote SDs of triplicate measurements from two independent cDNA preparations.

abundance and acid invertase activity but different sugar accumulation. It is interesting that most of the cold-induced sweetening-resistant lines had mRNAs of both *INH2α* and *INH2β*, whereas most of the susceptible lines had either *INH2α* or *INH2β* mRNA but not both (Fig. 9C). The significance of this, if any, remains to be established. The abundance of *INH1* mRNA did not correlate with acid invertase activity, sugar accumulation, or cold-induced sweetening status (Fig. 9D).

Activity of invertase inhibitor variants

Attempts to express INH proteins in *E. coli* or *Pichia pastoris* did not result in soluble or active protein and, although soluble protein could be obtained using a wheat germ cell-free system, no activity could be detected (data not shown). However, expression as fusion proteins with MBP in *E. coli* did result in soluble protein, although the fusion proteins were not susceptible to cleavage by TEV protease. Consequently, after purification, the fusion proteins were tested for inhibitory activity against acid invertase. All three of the recombinant His-MBP–INH fusion protein preparations were found to form high-ordered complexes, eluting in the void volume of the size-exclusion column. When examined by SDS–PAGE, they all showed the presence of a band of the expected size plus substantial amounts of higher molecular weight complexes (Fig. 10A). These complexes disappeared when samples were denatured in SDS buffer containing 6 M urea prior to SDS–PAGE analysis, showing that the preparations were reasonably pure with no major contaminants (Fig. 10A). The His-MBP control protein did not form high molecular mass complexes, suggesting that the presence of the INH domain was responsible for the aggregation of the proteins. The presence of mild detergents or 50 mM arginine and 50 mM glutamic acid reduced, but did not prevent, protein aggregation (data not shown), and recombinant fusion proteins were therefore tested for activity as high-ordered complexes.

The His-MBP–INH2α fusion protein was strongly inhibitory to acid invertase activity, with 94% inhibition being observed at the highest concentration of fusion protein tested (Fig. 10B). The His-MBP protein alone was not active (data not shown), and evidently the presence of the His-MBP domain in the fusion protein did not prevent interaction of the INH2α protein domain with acid invertase. However, the His-MBP–INH2β*A and His-MBP–INH2β*B fusion proteins were substantially less active. The His-MBP–INH2β*A protein inhibited invertase by only ~13% at the highest concentration tested, and so possessed only approximately one-seventh of the activity of

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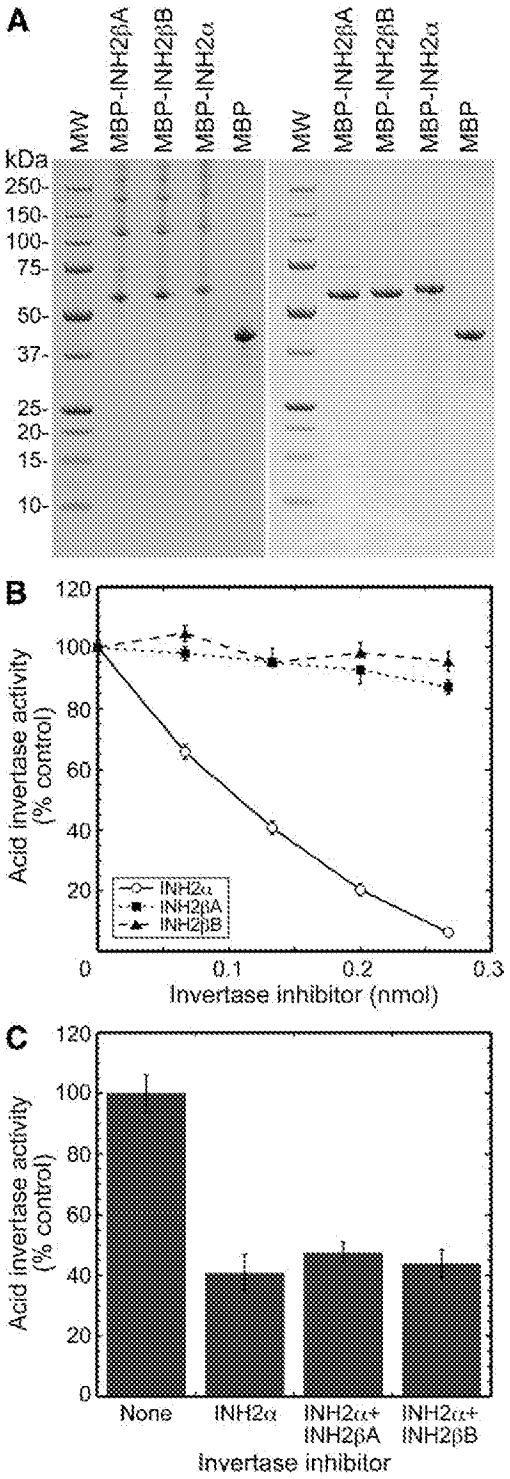


Fig. 10. Activity of recombinant His-MBP–INH fusion proteins. (A) SDS–PAGE of the three putative vacuolar His-MBP–INH fusion proteins and the His-MBP control. Samples were denatured in SDS loading buffer (left hand panel) or SDS–urea loading buffer (right hand panel) for 30 min at 37 °C prior to loading aliquots of 1.3 μg of protein per lane. Expected molecular masses were: His-MBP–INH2β*A, 59.4 kDa; His-MBP–INH2β*B, 60.2 kDa; His-MBP–INH2α, 62.5 kDa; His-MBP, 43.9 kDa. (B) Inhibition of acid invertase activity by recombinant His-MBP–INH fusion proteins. Dilutions of fusion protein were incubated at 37 °C for 30 min with crude protein extracts (~12.5 μg of protein) from potato tuber, prior to assay for acid invertase activity. Activity is shown as a percentage of that with no addition of recombinant protein. His-MBP was not inhibitory to acid invertase activity, and has been omitted for clarity. Means are calculated from three experiments carried out in triplicate ±SE. (C) Effect on acid invertase activity of the presence of 0.133 nmol of His-MBP–INH2α alone, or with an equivalent amount of either His-MBP–INH2β*A or His-MBP–INH2β*B. Means are calculated from one experiment carried out in triplicate ±SD.

His-MBP–INH2α. His-MBP–INH2β*B did not possess significant activity in the concentration range examined.

Since the presence of both *INH2α* and *INH2β* transcripts was detected in several of the lines most resistant to cold-induced sweetening, the effects of mixing His-MBP–INH2α with either His-MBP–INH2β*A or His-MBP–INH2β*B were examined (Fig. 10C). However, the presence of either His-MBP–INH2β*A or His-MBP–INH2β*B did not result in an inhibition of acid invertase activity greater than that due to His-MBP–INH2α alone.

Discussion

The deduced proteins of invertase inhibitors isolated from potato were highly similar to deduced proteins from tobacco (Fig. 1), which is also a solanaceous species. Indeed, INH1 and INH2 were more similar to their orthologues from tobacco (86% and 71% for mature proteins, respectively) than they were to each other (43%). INH1 had a predicted signal peptide, which would target the mature proteins to the endoplasmic reticulum for eventual secretion to the apoplast, and the protein was shown to accumulate in the cell wall (Fig. 4). In tobacco, direct protein sequencing of NtINH1 determined that a signal peptide of 19 amino acids was removed to produce the mature protein (Greiner *et al.*, 1998). NtINH1 was localized in the apoplast and acted as an inhibitor of cell wall invertase (Weil *et al.*, 1994; Greiner *et al.*, 1998), as has also been shown for the tomato orthologue SolyCIF/SlINVINH1 (Reca *et al.*, 2008; Jin *et al.*, 2009).

The *INH2* gene showed developmentally regulated alternative splicing, with the unspliced *INH2α* mRNA encoding the full-length protein and the two hybrid *INH2β* mRNAs encoding altered INH2 proteins. Analysis of the deduced protein sequences of the *INH2α* and *β* transcripts with SignalP 3.0 did not unambiguously determine the intracellular target. The homologous protein NtINH-h from tobacco (Fig. 1B) has been assigned a putative vacuolar localization, since overexpression of NtINH-h in potato strongly inhibited vacuolar invertase but not cell wall invertase (Greiner *et al.*, 1999). A fusion of the N-terminus of potato INH2 to GFP was detected in the vacuole (Fig. 4), and the high homology between the INH2α and β forms suggests that all are probably localized to the vacuole.

INH1 mRNA accumulated to the highest levels in potato flower parts and was low in roots (Fig. 6C), similar to

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NtINH1 mRNA in tobacco (Greiner *et al.*, 1998) and *SolyCIF* in tomato (Reca *et al.*, 2008). *INH1* mRNA could, however, accumulate to high abundance in cold-stored tubers (Fig. 8C). The mRNA accumulation was substantial, but occurred transiently in both lines 937/3 and 1021/1 about 2 weeks into cold storage and had declined by 4 weeks (Fig. 8C). The reason for this transient increase is unclear, but may possibly be a part of the initial mechanism of coping with chilling stress. In two of the cold-induced sweetening-susceptible lines, S343 and S190, the abundance of *INH1* mRNA was very high after 4 weeks of cold storage (Fig. 9D), higher than in the other lines examined, although it is possible that the transient accumulation of *INH1* mRNA was occurring later in these two lines.

Resistance to cold-induced sweetening correlated, in general, with low acid invertase activity, several fold lower than in susceptible lines (Fig. 9B). The susceptible line 937/3 (Fig. 7) is also known to have high acid invertase activity, up to 7-fold greater than that of the resistant line 1021/1 (McKenzie *et al.*, 2005). These observations are consistent with analysis of other populations that also found a correlation between low acid invertase activity and either low sugar accumulation or a low hexose:sucrose ratio (Zrenner *et al.*, 1996; Matsuura-Endo *et al.*, 2004; McKenzie *et al.*, 2005), and indicate that the level of acid invertase activity is a major component of resistance to cold-induced sweetening. However, other factors may be involved since total invertase activity does not always correlate with hexose accumulation (line S130 of Fig. 9, and Richardson *et al.*, 1990), and quantitative trait loci (QTLs) for several genes other than acid invertase have been implicated (Menéndez *et al.*, 2002). The abundance of vacuolar invertase inhibitor proteins may be one such factor. *INH2α* mRNA was most abundant in potato tubers (Fig. 6A), particularly after cold stress (Fig. 8B). Lines resistant to cold-induced sweetening in general had greater accumulation of the sum of *INH2α* plus *β* mRNAs (Fig. 9C), presumably resulting in greater abundance of invertase inhibitor proteins. Attempts to destroy endogenous invertase inhibitors through foaming (Pressey, 1966) resulted in highly irreproducible data, although some increase in acid invertase activity was always seen (data not shown).

Cold stress can result in altered expression of genes, but also in altered processing of transcripts. In potato plants, exposure to cold stress caused exon skipping in an invertase mRNA, and presumably led to the formation of a version of the protein with altered properties (Bournay *et al.*, 1996). In stored tubers of some potato lines, cold stress caused the formation of the spliced *INH2β* mRNAs. There are two explanations for how a hybrid *INH2β* mRNA could arise. *INH2* and *INH1* are both located on chromosome 12, and have a tandem orientation, with *INH2* being upstream (Fig. 3). The genes are separated by ~5.5 kb with no obvious intervening genes. Although the two genes exhibit distinct developmental expression patterns, and the very different transcript abundances in different tissues indicate that they are probably independently transcribed (Figs 6, 8), one possibility is that a single transcript could be derived by read-through transcription from the *INH2* promoter into the downstream *INH1* gene. This large pre-mRNA transcript could then be processed *in cis* using cryptic splice sites into the *INH2β*A* and *INH2β*B* mRNAs observed. The tissue-specific accumulation of *INH2β* is related to that of *INH2α* (Fig. 6), which could be considered consistent with this possibility. Alternative splicing of exons *in cis* is a common feature of plant mRNAs (Wang and Brendel, 2006; Reddy, 2007). However, the *INH2* gene does not possess introns, and the splice junction found in the major *INH2* transcript (*INH2β*A*) did not correspond to an intron splice site in *INH1* (Fig. 5C). RNA folding into extensive secondary structures is exacerbated at low temperatures (Zhu *et al.*, 2007) which, combined with increased RNA splicing in the cold (Lee *et al.*, 2006), could result in multiple splicing products *in cis* using cryptic splice sites.

mRNAs can be also be spliced via *trans* mechanisms. *Trans* splicing occurs when two (or more) independently transcribed pre-mRNAs are *trans*-spliced to form a single functional mRNA. It has been best described in animals (Dorn *et al.*, 2001; Fischer *et al.*, 2008), but also occurs in organelles and seeds of plants (Chapdelaine and Bonen, 1991; Kawasaki *et al.*, 1999; Schmitz-Linneweber *et al.*, 2006). However, *trans*-splicing uses canonical splice sites to join together exons from different pre-mRNAs (Horiuchi and Aigaki, 2006), which is not the case for *INH2β* since *INH2* does not have an intron. The splicing of two separate mRNAs without the involvement of canonical splice sites produces what have been termed chimeric RNAs and, while rarely described in plants, 45 000 examples have been described in yeast, fruit fly, mouse, and human (Li *et al.*, 2009). In these organisms, about half of the chimeric RNAs had an non-conserved short homologous sequence of at least four nucleotides present at the junction site in both donor mRNAs. This was observed for both *INH2β*A* and *INH2β*B*, where a six nucleotide domain (AAACTT) and a five nucleotide domain (TAATT) were common to both *INH2* and *INH1* at the splice site, respectively (Fig. 5B). This short homologous sequence has been shown to be important for production of the spliced RNA (Li *et al.*, 2009).

It remains unclear whether the *INH2β* mRNA arises though splicing *in cis* or *trans*, since the presence at the splice junctions of weak cryptic splice sites (data not shown) and short homologous sequences (Fig. 5B) is consistent with either mechanism. The detection of *INH2β* mRNA only in tubers implies a tissue-specific regulation of this splicing event, which produces at least two variants. In *Arabidopsis*, alternative splicing of pre-mRNAs of serine/arginine-rich genes was also regulated in a tissue-specific manner (Palusa *et al.*, 2007), and was greatly increased by abiotic (particularly temperature) stress (Reddy, 2007). Indeed, increased RNA splicing under cold stress conditions appears to be part of the acclimation mechanism of plants (Lee *et al.*, 2006).

The deduced proteins of INH2α and both forms of INH2β possessed the four cysteine residues believed to be important in the formation of two disulphide bridges that

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stabilize the protein three-dimensional structure (Scognamiglio *et al.*, 2003; Hothorn *et al.*, 2004*a*). *INH2β* shared sequence identity with *INH2α* in the upstream region of the cDNA sequence, encoding most of the length of the deduced proteins. The sequence diverged within the codon for the fourth conserved cysteine, but in both *INH2β*A* and *B* the fusion with the mRNA of *INH1* provided the nucleotides necessary to complete the codon as a cysteine. However, when assayed as His-MBP–INH fusion proteins, only INH2α had strong inhibitory activity against potato acid invertase (Fig. 10B). INH2β*A exhibited weak inhibitory activity against acid invertase, while activity could not be detected for INH2β*B. Examination of the likely three-dimensional structure of invertase inhibitors (Hothorn *et al.*, 2004*a*) suggests that both INH2β*A and INH2β*B are likely either to be partially lacking or to possess a substantially altered fourth α-helix.

Low temperature stress causes large changes in the metabolome and transcriptome (Cook *et al.*, 2004; Lee *et al.*, 2005; Vogel *et al.*, 2005; Kaplan *et al.*, 2007), resulting in the accumulation of small molecules such as sucrose and other sugars that may be part of the mechanism for stabilizing proteins and membranes and maintaining cell osmotic pressure (Yamaguchi-Shinozaki and Shinozaki, 2006). Comparisons of different species of potato (*S. tuberosum* and *S. phureja*) have shown wide differences in their ability to acclimate to cold stress, which was correlated with differences in global gene expression including carbohydrate metabolism (Oufir *et al.*, 2008). In potato tuber many cold-responsive genes involved in carbohydrate metabolism showed a biphasic pattern of mRNA accumulation, with a rapid increase over 3–5 d being followed by a decrease and, in many cases, a subsequent sustained increase over the next 20 d (Bagnaresi *et al.*, 2008). Acid invertase mRNA showed this pattern (Bagnaresi *et al.*, 2008), and in a potato cultivar resistant to cold-induced sweetening (1021/1), *INH2α* mRNA also accumulated to high levels during the 28 d of cold storage (Fig. 8). Overall, the data are consistent with low acid invertase activity being the primary cause of resistance to cold-induced sweetening. Whether this is due to reduced expression of acid invertase or the expression of alleles with low activity will vary between genotypes, but may be assisted in some cultivars by a high mRNA accumulation of vacuolar invertase inhibitors. These proteins therefore play an important role in the resistance of certain potato cultivars to cold-induced sweetening by suppressing acid invertase activity.

Plants have several ways other than gene expression in which to modify the transcriptome. Three mechanisms used to produce more than one protein product from a single gene are alternative transcription initiation, alternative splicing of exons, and alternative translation initiation (Reddy, 2007; Wamboldt *et al.*, 2009; Tanaka *et al.*, 2010). Alternative splicing is regulated in a specific manner, since it can vary between tissues, during development, and in response to stresses (Reddy, 2007). Increased RNA splicing activity was detected in the tubers of many potato lines resistant to cold-induced sweetening, and the existence of such a mechanism under cold stress conditions may be a way for the plant to expand the variety of proteins beyond the one-gene one-protein model, and generate a range of proteins with the potential for additional and more diverse functional capacity to aid adaptability.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Primers used for PCR amplification and quantitative real-time PCR.

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Functional Genomics of *Phytophthora infestans* Effectors and *Solanum* Resistance Genes

Nicolas Champouret

Thesis committee

Thesis supervisors
Prof. dr. Richard G.F. Visser
Professor of Plant Breeding
Wageningen University

Prof. dr. Evert Jacobsen
Professor of Plant Breeding
Wageningen University

Thesis co-supervisor
Dr. Vivianne G.A.A. Vleeshouwers
Researcher
Wageningen University

Other members
Prof. Dr. Ir. Pierre J. G. M. de Wit, Wageningen University, The Netherlands
Prof. Dr. Martien Groenen, Wageningen University, The Netherlands
Prof. Dr. Ir. Corné Pieterse, Utrecht University, The Netherlands
Dr. Brande Wulff, The Sainsbury Laboratory, Norwich, UK

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Nicolas Champouret

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by the authority of the Rector Magnificus
Prof. dr. M.J. Kropff
in the presence of the
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ABSTRACT

Potato (*Solanum tuberosum* L.) is nowadays the most important non-cereal food crop in the world. It is prone to huge annual losses due to late blight, the disease caused by the oomycete pathogen *Phytophthora infestans*. Modern management of late blight necessitates the use of multiple resistance (*R*) genes, which requires efficient pipelines for identification, isolation and characterization of *R* genes. This thesis employs effectoromics, i.e. the use of effectors (pathogenic secreted protein) to probe corresponding *R* gene(s) in a host plant and sort out their functional redundancy and specificity. Using cytoplasmic RXLR effectors of *P. infestans* to probe resistant *Solanum* germplasm for late blight *R* genes, we were able to: (i) assess the biodiversity of *Avr-blb1*, characterize the genomic structure of virulent *P. infestans* isolates on *Rpi-blb1* plants and thus provide a technical solution for long-term disease management; (ii) identify the centre of origin of *R3a*, characterize *R3a* gene homologues and a functional *R* gene (*Rpi-sto2*), and (iii) uncover the potential co-evolution at both *R* and *Avr* side for the *R2/PiAvr2-PexRD11* interactions, providing more diversity and specificity of *R2* homologues, which may be valuable for potato breeding.

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CHAPTER 1

General Introduction

Part of this introduction will be published in:
Plants and oomycetes, an intimate relationship: Co-evolutionary principles and impact on agricultural practice (submitted).
Hendrik Rietman [1,3], Nicolas Champouret [1,3], Ingo Hein [2], Rients Niks [1] and Vivianne G.A.A. Vleeshouwers [1]

[1] Wageningen UR Plant Breeding, P.O. Box 386, 6700 AJ Wageningen, The Netherlands, [2] Scottish Crop Research Institute, Genetics Programme, Invergowrie – Dundee DD2 5DA, United Kingdom, [3] The Graduate School Experimental Plant Sciences, Wageningen University, Wageningen, The Netherlands

Potato

Potato (*Solanum tuberosum* L.) is currently, the most consumed crop worldwide by men after wheat and rice. In the early ages, potato was already an important food crop for people inhabiting the South American Andes region, and excavated food plant remains from archaeological sites date back as far as 2500 BC and 5000 BC (Hawkes 1990). The exact origin of European potato is still unclear, since Chile and Peru are both competing for this honour. However, potato was brought from South America to Europe in the late 16th century, in Spain (1570) and England (between 1588 and 1593) (Hawkes 1990). Since then European potato breeding came up and nowadays, the European Cultivated Potato Database (ECPD) records 4,100 cultivated varieties. In 2005, the estimated potato cultivation area was almost 20 million hectares with a total world production of over 300 million tons (Haverkort et al. 2009). Potato is used for human consumption (boiled, steamed, baked, "sauté" or fried, and processed into French fries, chips or noodles) or for industrial purposes mainly for starch production which can be processed in textile, papermaking, glue, coating, sizing, flocculating agents and building materials. More uses can be anticipated for the future such as biopharmaceuticals for encapsulation and controlled release of functional ingredients (Bradshaw et al. 2006a; Li et al. 2009).

Potato is part of the tuber-bearing *Solanum* species that belong to the section *Petota*. *Solanum* section *Petota* species occur in the Andes of South America and a secondary centre of diversity exists in the central Mexican highlands (Hawkes 1990; Spooner et al. 2004). Hawkes postulated that the ancestral potato species originated from Central America, subsequently migrated to South America, and a return migration back to Mexico, followed by hybridizations and allopolyploidisations within Central American taxa, then led to species with various ploidy levels (Hawkes 1990). *Solanum* species can easily hybridize with each other, making classification challenging (Hawkes 1990; Jacobs et al. 2008; Spooner and Hijmans 2001). Due to increased insights in potato taxonomy, the number of species is being reduced, in recent reviews. Hawkes (1990) recognized 227 tuber bearing species (7 cultivated species included) and 9 non-tuber-bearing species within section *Petota*. Spooner and Hijmans (Spooner and Hijmans 2001) recognized 203 tuber-bearing species including 7 cultivated species. Finally, Spooner and Salas (2006), reduced the number further to 189 species (including 1 cultivated species) in section *Petota*. However, one thing for sure, wild *Solanum* species are well spread from North to South America and highly diverse. Diploid, triploid, tetraploid, pentaploid and hexaploid species exist, and a polyploid species can contain genes with high homology to genes in one of its diploid progenitors, which increases the potential of diversification of genes in polyploid species to adapt to there environment. Consequently more diversity can occur for each gene homologue in the tuber-bearing *Solanum* species gene-pool, which can be used to improve various quality traits in breeding programs.

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Phytophthora infestans

Phytophthora infestans (Mont.) de Bary, the responsible pathogen for the Irish famine in the middle of the 1840's, was the first 'plant destroyer' described (Fry 2008; Large 1940; Schumann 1991). *P. infestans* is the causal agent of late blight, which is nowadays the most devastating disease on potato and tomato. It infects foliage, stems and can also infect fruits and tubers (Fry 2008). This notorious disease entails global yield losses of 16% of the potato crop, representing an annual financial loss of € 5.2 billion worldwide (Haverkort et al. 2009).

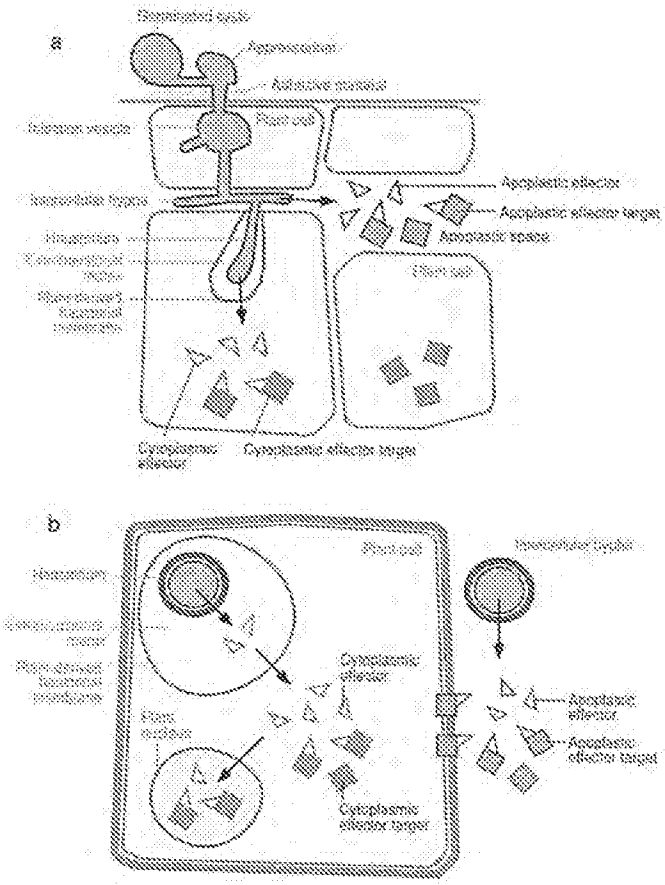


Figure 1. Plant pathogenic oomycetes secrete apoplastic effectors into the plant extracellular space and cytoplasmic effectors inside the plant cell.
A) Schematic view of the early stages of infection by *Phytophthora infestans* of a host plant illustrating the sites of action of the apoplastic and cytoplasmic effectors. B) Cross-section of the view in panel A illustrating the delivery of apoplastic and cytoplasmic effectors to their cellular target. One class of cytoplasmic effectors is known to target the plant nucleus. Apoplastic effectors interact with extracellular targets or surface receptors. Pathogen structures are shown in *dark grey* and plant structures in *clear grey*. Apoplastic effector and effector targets are outside of the plant cell, whereas cytoplasmic effectors and their targets are in the plant cell. Adapted from Kamoun (2006).

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P. infestans is a near-obligate hemi-biotrophic pathogen. The infection is based on a first biotrophic stage which is followed by a necrotrophic phase. Infection starts with the formation of an appressorium which breaks through the first physical layer of defence of the host (Figure 1). During the biotrophic stage, an intracellular infection vesicle is formed. From there, hyphae emerge, and hyphal growth expands in the extracellular space of the host. Then specialized infection structures called haustoria invaginate host cells and make near-direct contact with the host plasma membrane (Birch et al. 2006). From that point on, the infection switches to a necrotrophic phase and mycelium spreads rapidly through the host tissue and forms spores. During its life cycle *P. infestans* adopts two types of reproduction. The asexual cycle, as described above, enables dramatically rapid population growth in susceptible host tissue (Fry 2008). The sexual cycle, which requires both mating types (A1 and A2), leads to the formation of oospores that serve both as a survival structure and as a source of variation via sexual recombination (Fry 2008).

P. infestans belongs taxonomically to the oomycetes phylum. Traditionally *Phytophthora* was classified as a fungus due to its filamentous growth habit and common infection strategies (Latijnhouwers et al. 2003). However, genetic structure (Tyler et al. 2006), phylogeny (Baldauf 2003) and life cycle (Judelson and Blanco 2005) studies have demonstrated that oomycetes are closer to heterokont algae than to fungi (Figure 2). In the last decade various *Phytophthora* species such as *P. sojae*, *P. ramorum* and *P. infestans* have entered the genomics era (Haas et al. 2009; Lamour et al. 2007; Tyler et al. 2006). The major discovery has unquestionably been the variability and diversity of effectors these species contain.

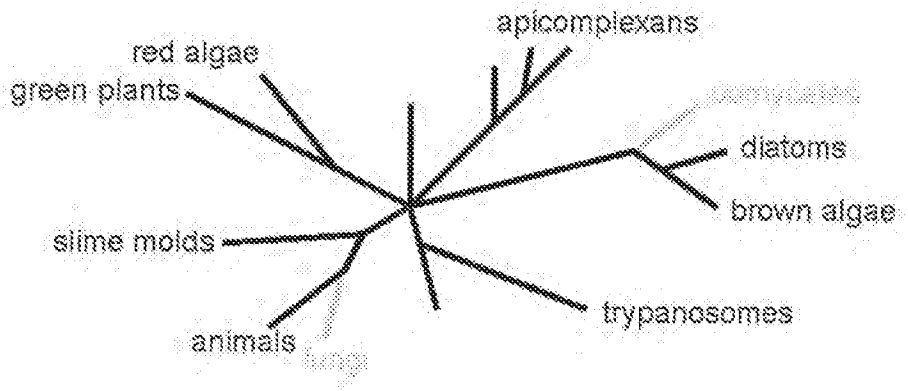


Figure 2. Schematic phylogenetic tree of the eukaryotes.
The tree is adapted from that of Baldauf et al. (Baldauf 2003) that is based on a concatenation of six highly conserved proteins. Oomycetes are more related to brown algae and diatoms (heterokonts) in the Stramenopiles, than fungi.

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Effectors

Effectors are pathogen molecules that manipulate host cell structure and function thereby facilitating infection and/or triggering defence responses. Unlike the terms "avirulence", "elicitor", "toxin", and "virulence", the term effector is neutral and does not imply a negative or positive impact on the outcome of the disease interaction. Two classes of effectors target distinct sites in the host plant: apoplastic effectors are secreted into the plant extracellular space, where they interact with extracellular targets and surface receptors; and cytoplasmic effectors are translocated inside the plant cell presumably through specialized structures like infection vesicles and haustoria that invaginate living host cells (Figure 1) (Kamoun 2006).

Apoplastic effectors contain N-terminal signal peptides for secretion, followed by C-terminal effector module(s) (Figure 3), but are not known to carry additional host targeting signals (Damasceno et al. 2008; Tian et al. 2005; Tian et al. 2007; Tian et al. 2004). Some apoplastic effectors such as INF elicitins are down-regulated in early infection stages perhaps to avoid recognition. For example, INF elicitins are known to induce cell death when infiltrated on some *Solanum* species or *Nicotiana benthamiana* (Kamoun et al. 1998; Kamoun et al. 1997; van West et al. 1999; Vleeshouwers et al. 2006). Also others, like the NEP1-Like Family (necrosis- and ethylene-inducing peptides) are mainly expressed in late stages of infection (Kanneganti et al. 2006) when they might function in triggering tissue necrosis and enable necrotrophic colonization at later infection stages. Two other well-studied apoplastic effector classes of *P. infestans*, are the Serine and Cysteine protease

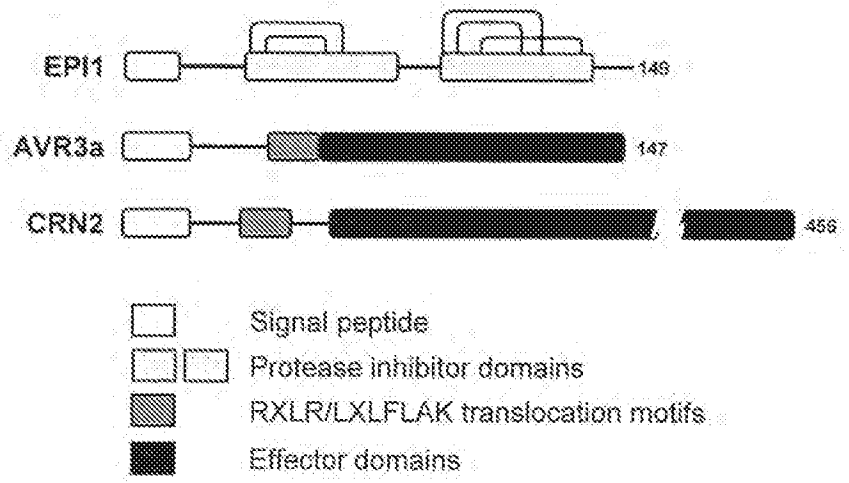


Figure 3. Oomycete effectors are modular.
All known effectors carry N-terminal signal peptides for secretion (white box). Cytoplasmic effectors, in addition, have conserved motifs in their N-termini (RXLR or LXLFLAK) that are required for host translocation. The C-terminal domain carries the module with biochemical effector activity. Adapted from Schornack et al. (2009b).

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inhibitors EPI1, EPI10 and EPIC1, EPIC2B respectively, which inhibit protease defence layers of the pathogen. Kazal-like extracellular serine protease inhibitors are e.g. EPI1 and EPI10, which bind and inhibit the pathogenesis-related P69B subtilisin-like serine protease from tomato (Song et al. 2009; Tian et al. 2005). Cystatin-like protease inhibitors, EPIC1 and EPIC2B, are subject to degradation by P69B. However, EPI1 protects the EPICs from degradation. EPIC2B binds and inhibits a salicylic acid–induced papain-like protease PIP1, and the related RCR3 in tomato, which are both under diversifying selection, but no virulent function for EPIC1 was found yet (Tian et al. 2007). These findings demonstrate a cascade of protein interactions between the pathogen and the host, to be able to establish infection (Song et al. 2009).

Oomycete cytoplasmic effectors (Figure 3) are characterized by an N-terminal signal peptide for type II secretion, a translocation motif (mainly RXLR and LXLFLAK) for trafficking inside host cells and a C-terminal domain for biochemical effector activity (Birch et al. 2008; Birch et al. 2006; Kamoun 2006; Morgan and Kamoun 2007; Tyler et al. 2006; Whisson et al. 2007). The RXLR effectors are defined by the amino acid (a.a.) sequence Arginine-X-Leucine-Arginine (where X is any residue) that designates a domain involved in translocation inside host cells (Dou et al. 2008; Whisson et al. 2007), this motif in an effector class is unique to oomycetes, revealing another clear distinction between oomycetes and true fungi. Elevated levels of polymorphism and signatures of positive (adaptive) selection have

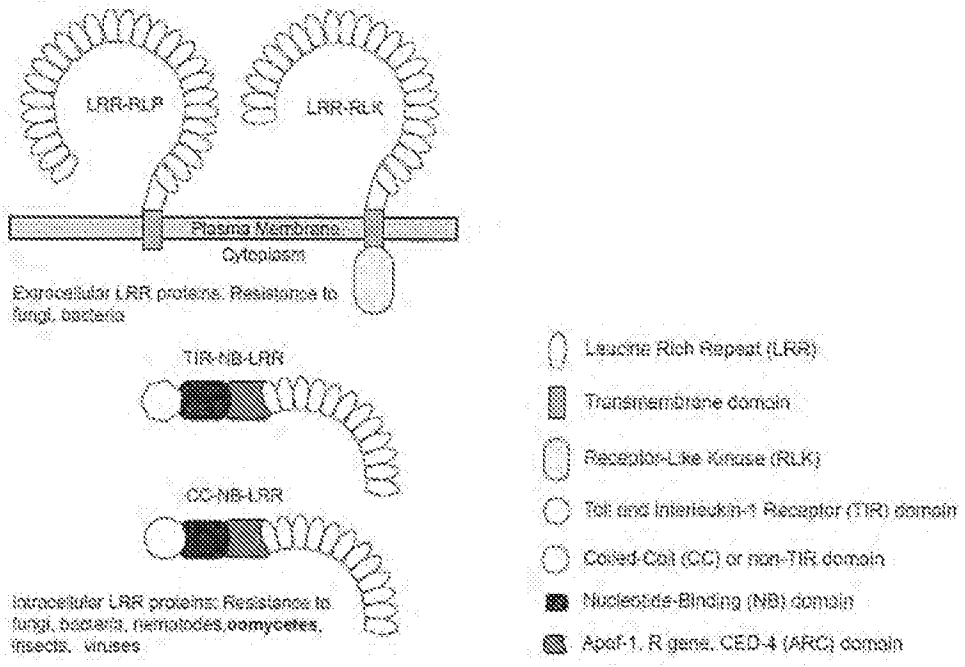


Figure 4. Schematic diagram of the proteins encoded by disease resistance genes along with a list of the types of pathogens recognized by these proteins.
These include proteins with extracellular moieties (top) or intra-cellular proteins (middle). The identity of individual protein domains are indicated at the bottom. Adapted from Moffett (2009).

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been observed in the C-terminal half of RXLR effectors, consistent with the view that this region executes the effector activity inside plant cells and coevolves with host factors (Allen et al. 2008; Oh et al. 2009; Rehmany et al. 2005; Win et al. 2007). The CRN effectors also contain N-terminal sequence domains, including the conserved LXLFLAK motif, which are implicated in host translocation (Haas et al. 2009; Win et al. 2007). Some of these cytoplasmic effectors are even known to target the nucleus of the host, like the NUK effectors of *P. infestans* that contain nuclear localization signals (NLSs) (Kanneganti et al. 2007). Interestingly, cytoplasmic RXLR effectors of *P. infestans* are the only effectors until now that were found to have an avirulence activity in potato, like AVR3a, PiAVR2, AVR-blb1, AVR4, AVR1 and more recently AVR-blb2 and AVR-vnt1 (Table 1) (Armstrong et al. 2005; Lokossou et al. 2009; Oh et al. 2009; Pel 2010; Tyler et al. 2006; Vleeshouwers et al. 2008). With the recent completion of the *P. infestans* genome (Haas et al. 2009), we can use effectors of this pathogen to functionally profile resistant *Solanum* plants for response to these effectors and mine their active *R* genes. This approach is known as effector genomics or effectoromics (Oh et al. 2009; Vleeshouwers et al. 2008). The characterisation of resistance genes by effectoromics is more accurate and provides more molecular insight in the potato/*P. infestans* pathosystem compared to determination of resistance spectra with *P. infestans* isolates.

Resistance genes

R proteins, often known as NB-LRR proteins, consist of three domains, the N-terminal, the NB-ARC (Nucleotide-Binding domain found in proteins such as human Apaf-1, plant R proteins and *Caenorhabditis elegans* Ced-4) and the LRR (Leucine Rich Repeat) domain. Two types of N-terminal domains are commonly observed (Figure 4). The Toll and Interleukin-1 Receptor (TIR), which is a protein-protein interaction domain, known to be associated with innate immune systems in multiple phyla (Staal and Dixelius 2007), and the coiled-coil (CC) N-terminal domain, which is in fact highly variable and not fully conserved between CC-NBS-LRR proteins (Cannon et al. 2002) forming a significant source of variation for fast evolution in this class of proteins. The NB-ARC domain, also known as NBS (nucleotide-binding site), is made of multiple conserved motifs (Albrecht and Takken 2006; McHale et al. 2006; Ooijen et al. 2007), which create a functional nucleotide-binding pocket capable of binding and hydrolyzing ATP (Tameling et al. 2002). This ATP/ADP binding site in the NB-ARC domain is thought to be the activation site for signal transduction of the R protein (Takken and Tameling 2009). The LRR domain is made up of individual repeats with the consensus sequence LxxLxxLxxLxLxx(N/C/T)x(x)LxxIPxx (Jones et al. 1997). The number of repeats and primary structure is highly variable between family members, and domain swapping studies have shown that recognition specificity maps to the LRR domain (Dodds et al. 2006; Ellis et al. 2007b; Kamoun 2006). Nevertheless recent

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studies let believe that not only the LRR but also the N termini of NB-LRR protein are involved in recognition of AVR protein and signal transduction, via a "bait" protein (Caplan et al. 2008; Sacco et al. 2009; Ueda et al. 2006). Collier and Moffett (2009) proposed a two-step recognition process to describe this mechanism, called "bait and switch" model, involving interactions of the AVR protein with a "bait" protein which could be the guardee or the decoy (see below) presented to the N terminal domain of the R protein. These inter-domain interactions bring the "bait" protein into close proximity with other domains of the NB-LRR protein which leads to a direct or indirect recognition of the AVR by the LRR domain and cause a flipping of the NB-LRR protein to a switch "on" mode starting the signal transduction for resistance. All these models try to explain the different mechanism observed in what is basically called R/AVR interaction.

During evolution, *R* genes under selective pressure can evolve at different rates, even within an individual cluster of similar sequences. Kuang et al. (2004) describe the fast and slow evolving extremes as type I and type II *R* genes, respectively. Type I *R* genes are characterized by frequent sequence exchanges between paralogs that obscure orthologous relationships, whereas type II resistance genes rarely undergo sequence exchanges with paralogs and maintain orthologous relationships (Friedman and Baker 2007). Within an *R* gene locus, the number of paralogs can vary considerably. For example, two *RPP8* homologous (type II) in *A. thaliana* (Kuang et al. 2008) and a few computationally recognized NBS-LRR singletons in sunflower (Radwan et al. 2008) represent rather small *R* gene families. In contrast, multi-copies of *R* genes can form highly extended *R* gene clusters, e.g. the maize *Rp1* (against *Puccinia sorghi*) cluster (Smith et al. 2004), the lettuce *Dm3* cluster (effective towards *Bremia lactucae*) and *RGC2* cluster (Kuang et al. 2004) and two potato Major *Late Blight* resistance (MLB) cluster at Linkage Group (LG) XI (Friedman and Baker 2007) harbouring up to 45 type I *R3a* homologous per haplotype, and at LG IV with the *R2* cluster of which up to 16 *R2* variants, or Resistance Gene Homologue (*RGH*), were observed in the Mastenbroek *R2* differential (Lokossou et al. 2009).

Defence mechanisms & Co-evolution potato – *P. infestans*

Pathogens exert significant constraints on host fitness. These pressures have consequently applied strong selection on the evolution of host genomes such that most hosts devote significant portions of their genomes to encoding defences against pathogens (Moffett 2009). In the last decade multiple theories have been put forward to describe this close co-evolution between the pathogen and its natural host. The most explicative mechanism is described by Zig-Zag model of Jones and Dangl (2006). In this model the first layer of defence is comprised of membrane-receptors that detect the widely conserved pathogen-associated molecular patterns (PAMPs), and activate responses that protect against colonization. This mechanism is called PAMP-Triggered Immunity (PTI). In some cases

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pathogens are able to escape PTI in their hosts by secreting effectors that interfere with PTI, and due to this effector-triggered suppression (ETS), infection can proceed. As a second defence layer, plants employ resistance (*R*) genes to activate ETI (Effector Triggered Immunity) which is often characterized by the hypersensitive response (HR), resulting in localized programmed cell dead. The effectors in that case act as avirulent factors (*Avr*) and their recognition by *R* genes is based in the gene-for-gene model (Flor 1971).

Direct interactions between an R protein and its matching AVR have relatively rarely been demonstrated (Deslandes et al. 2003; Dodds et al. 2006; Ellis et al. 2007a; Jia et al. 2000). Therefore new hypotheses of interaction were presented, for example the guard model, where R proteins monitor or guard host proteins (van der Biezen and Jones 1998). In that case, R proteins do not directly detect the presence of the pathogen, but perceive alterations of the protein they guard when pathogen effectors interact with it. This model is well illustrated with effectors proteins from *Pseudomonas syringae* AvrB, AvrRpm1, AvrRpt2, interacting with RIN4, which cause the activation of RPM1 and RPS2 resistance gene; and AvrPphB, interacting with PBS1 resulting in the activation of RPS5 of *Arabidopsis thaliana* (Axtell and Staskawicz 2003; Mackey et al. 2003; Mackey et al. 2002; Shao et al. 2003). Yet recognition specificity, or avirulence function, does not always correlate with virulence activity. In the case of AvrPto and AvrPtoB of *P. syringae*, the host target was thought to be Pto protein in tomato which is guarded by the R protein Prf (Pedley and Martin 2003). Recent studies have demonstrated that the virulence targets of these two effectors are in fact the receptor-like kinases CERK1, BAK1, EFR1 and FLS2, which mediate PTI responses (Gimenez-Ibanez et al. 2009; Göhre et al. 2008; Shan et al. 2008; Xiang et al. 2008). Therefore Pto is used as a decoy protein by the host to trap AvrPto and AvrPtoB and then activate Prf responses. These recent discoveries are supporting the decoy model established by van der Hoorn and Kamoun (2008). In this model of evolution, guardees will be subject to alternating selective pressures depending on the presence or absence of their guarding R protein. In the presence of the R protein, the guardee would be optimized for Avr interaction, and hence detection. In the absence of the R protein, the guardee would be under pressure to evade interaction with pathogen effectors in order to reduce virulence of the pathogen.

For *Solanum* section *Petota* species, two centres of diversity exist, one in the Andes of South America and another in the central Mexican highlands (Hawkes 1990; Spooner et al. 2004), as described above. Both areas have been suggested to be also the geographic origin of *P. infestans*, although this is still a subject of debate. The origin as Toluca Valley in Mexico is supported by the presence of sexual populations with both the A1 and A2 mating types in similar frequencies and the high degree of molecular genetic diversity in that region (Flier et al. 2003; Fry et al. 1992; Goodwin and Fry 1994; Grünwald and Flier 2005). The hypothetical South America origin is based on endemic coexistence of distinct lineages as well as nucleotide and haplotype diversity in the Andes (Gomez-Alpizar et al. 2007). These studies suggest that the *P. infestans* isolates may have adapted to different host range species

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in both centres. As a consequence, *R* loci that co-evolved with *P. infestans* in South American *Solanum* species are not necessarily expected to display functional similarity to those from the Central American species, and vice versa. Therefore studying *R* gene diversity in these two centres of origin will provide insight into the co-evolution of this pathosystem and hopefully new tools to manage the disease.

Late blight potato breeding

Early potato breeding efforts focussed on the characterisation of the resistance spectrum and the introgression of 11 *R* genes from *Solanum demissum*, a wild relative of potato. The exploitation of new cultivars containing these *R* genes proved to be successful (Black et al. 1953; Malcolmson and Black 1966). However, rapidly changing populations of *P. infestans* overcame the *R1-R11* genes, even in pyramiding systems (Hein et al. 2009). In the second half of the twentieth century, potato farmers resorted largely to fungicides to control the disease. Nevertheless *P. infestans* continued to damage potato crops, and at the beginning of the 1980s even gained in resistance to metalaxyl, a phenylamide fungicide, in Europe (Cooke 1981; Davidse et al. 1981) and in Middle East (Cohen and Reuveni 1983), in the 1990s in North America (Deahl et al. 1993; Miller et al. 1997), and beginning of the 21st century in Asia (Deahl et al. 2002). At the end of the 20th century, a new interest for the protection of the environment and for a more economical management system by farmers, made such pesticide inputs undesirable. Contemporary potato breeding for *P. infestans* resistance is exploring the wealth of *R* gene diversity in a broad range of tuber-bearing *Solanum* section *Petota* species to build up a collection of diverse *Rpi* (*Resistance* to *P. infestans*) genes. A number of *R* genes and new *Rpi* genes have recently been cloned from Mexican *Solanum* species, i.e. *R1*, *R2* and *R3a* from *S. demissum* (Ballvora et al. 2002; Huang et al. 2005; Lokossou et al. 2009), *Rpi-blb1/RB* (Song et al. 2003; van der Vossen et al. 2003), *Rpi-blb2* (van der Vossen et al. 2005), and *Rpi-blb3* from *S. bulbocastanum* (Lokossou et al. 2009), *Rpi-sto1* and *Rpi-pta1* from *S. stoloniferum* (Vleeshouwers et al. 2008), and *Rpi-abpt / R2-like* from unknown species used in a pre-breeding program (Lokossou et al. 2009). Also, the first *Rpi*-genes of South American origin are being cloned, such as *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* from *S. venturii* (Foster et al. 2009; Pel et al. 2009) (Table 1). Many more *Rpi* genes are expected to be cloned in the coming years so that the set of cloned *R* genes against late blight is rapidly growing (Hein et al. 2009; Hein et al. 2007; Jacobs et al. 2010; Kuhl et al. 2001; Rauscher et al. 2006; Sandbrink et al. 2000; Smilde et al. 2005; Tan et al. 2008; Villamon et al. 2005) (Table 1). Interestingly all cloned *R* genes from wild *Solanum* species against late blight are intracellular LRR proteins from the CC-NBS-LRR category (Figure 4).

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Table 1. Late blight *R* genes, their origin, cloning status and their cognate *Avr*

Gene	Species	Comments	Matching *Avr*	References
R genes from Central America				
R1	*S. demissum*	Cloned	*Avr1*	Ballvora et al. 2002; Tyler 2009
R2	*S. demissum*	Cloned	*PiAvr2, PexRD11, PITG_21949, PITG_21645*	Lokossou et al. 2009; This thesis chapter 3
R2-like	*Unknown*	Cloned		Lokossou et al. 2009; This thesis chapter 3
Rpi-abpt	*Unknown*	Cloned		Lokossou et al. 2009; This thesis chapter 3
Rpi-blb3	*S. bulbocastanum*	Cloned		Lokossou et al. 2009; This thesis chapter 3
Rpi-edn1.1	*S. edinense*	Cloned	*PiAvr2, PexRD11, PITG_21949, PITG_21645, PITG_13940*	This thesis chapter 3 and 4
Rpi-snk1.1	*S. schenckii*	Cloned		This thesis chapter 3 and 4
Rpi-snk1.2	*S. schenckii*	Cloned		This thesis chapter 3 and 4
Rpi-hjt1.1	*S. hjertingii*	Cloned		This thesis chapter 3 and 4
Rpi-hjt1.2	*S. hjertingii*	Cloned		This thesis chapter 3 and 4
Rpi-hjt1.3	*S. hjertingii*	Cloned		This thesis chapter 3 and 4
Rpi-dms1	*S. demissum*	Mapped		Hein et al. 2007
R3a	*S. demissum*	Cloned	*Avr3a*	Armstrong et al. 2005; Huang et al. 2005
Rpi-sto2	*S. stoloniferum*	Cloned	*Avr3a*	This thesis chapter 5
R3b, R5-R11	*S. demissum*	Mapped		Bradshaw et al. 2006b; El-Kharbotly et al. 1996; Huang et al. 2005; Huang et al. 2004
Ma-R4	*S. demissum*		*Avr4*	van Poppel et al. 2008
Rpi-blb1/RB	*S. bulbocastanum*	Cloned	*Avr-blb1 (ipiO)*	Song et al. 2003; van der Vossen et al. 2003; Vleeshouwers et al. 2008
Rpi-sto1	*S. stoloniferum*	Cloned	*Avr-blb1 (ipiO)*	Vleeshouwers et al. 2008; Wang et al. 2008
Rpi-pta1	*S. papita*	Cloned	*Avr-blb1 (ipiO)*	Vleeshouwers et al. 2008; Wang et al. 2008
Rpi-plt1	*S. polytrichon*	Mapped		Wang et al. 2008
Rpi-blb2	*S. bulbocastanum*	Cloned	*Avr-blb2*	van der Vossen et al. 2005
Rpi-bst1	*S. brachistotricum*	Mapped		Hein et al. 2009
Rpi1	*S. pinnatisectum*	Mapped		Kuhl et al. 2001
R genes from South America				
Rpi-mcd1	*S. microdontum*	Mapped		Sandbrink et al. 2000; Tan et al. 2008
Rpi-mcq1	*S. mochiquense*	Mapped		Smilde et al. 2005
Rpi-vnt1.1	*S. venturii*	Cloned	*Avr-vnt1*	Foster et al. 2009; Pel 2010; Pel et al. 2009
Rpi-vnt1.2	*S. venturii*	Cloned	*Avr-vnt1*	Foster et al. 2009; Pel 2010; Pel et al. 2009
Rpi-vnt1.3	*S. venturii*	Cloned	*Avr-vnt1*	Foster et al. 2009; Pel 2010; Pel et al. 2009
Rpi-cap1	*S. capsicibaccatum*	Mapped		Jacobs et al. 2010
R_{ber}	*S. berthaultii*	Mapped		Rauscher et al. 2006
Rpi-pcs	*S. paucissectum*	Mapped		Villamon et al. 2005

P. infestans populations exhibit a high risk of evolution owing to high mutation rates, large effective populations, a high gene/genotype flow, a mixed reproduction system and an efficient directional selection (McDonald and Linde 2002). Moreover, the revelation of *P. infestans* genome plasticity and its abundance of putative effectors, 563 genes predicted for the RXLR family (Haas et al. 2009), show that this oomycete remains a formidable foe. This explains why single-dominant host *R* genes, that were deployed separately, have been rapidly overcome (Wastie 1991). Also pyramiding approaches with the first R1 to R11 *R* genes

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rendered only little success (Hein et al. 2009). Nevertheless, in natural populations, *Solanum* host species are not extinct in areas where *P. infestans* is dominantly present. This co-evolution situation is most-likely the one we have to be inspired by for future breeding, and we postulate that understanding the molecular insight of R/AVR interactions in this pathosystem can provide rational solutions for application of a durable resistance strategy to manage late blight in agriculture.

First of all, increasing information about the molecular basis for *R* and *Avr* genes and their interactions is being uncovered (Oh et al. 2009; Vleeshouwers et al. 2008). New *R* genes with broad or less broad spectrum are being cloned, and *RGH* identification and cloning is also providing new tools to breeders. For example, *Rpi-sto1* and *Rpi-pta1* from the crossable *S. stoloniferum*, which are homologues of *Rpi-blb1* from *S. bulbocastanum*, can be used for breeding programs because *S. bulbocastanum* is less easy crossable with *S. tuberosum* (Vleeshouwers et al. 2008). To contour classical breeding problems, like time and non desired genome linkage dragging, a new Cisgenesis technology is being developed (Jacobsen and Schouten 2007; Vetten et al. 2003). In this technique plants are genetically modified by cisgene only. A cisgene is a natural gene, coding for an (agricultural) trait, from the crop plant itself or from a sexually compatible donor plant that can be used in traditional plant breeding. In potato breeding against late blight, *R* genes from crossable or crossable by bridging *Solanum* species is inserted in susceptible or new cultivars by genetic modification without any bacterial markers. This technique also provides new opportunities for *R* gene stacking (Jacobsen and Schouten 2007). Another example is the quest for complementary *R* genes, which are targeted at the full spectrum of allelic variants of an Avr gene, for stacking approaches to provide broad-spectrum resistance. In the case of R3a/AVR3a, with only *Avr3a*^{KI} and *Avr3a*^{EM} present in *P. infestans* population (Armstrong et al. 2005), combining an R gene responding to AVR3a^{EM} with R3a, is likely to cover a full spectrum of resistance against *P. infestans*. For the deployment of *R* genes in agriculture, the availability of effectors that match particular *R* genes are useful for example via effectoromics studies (Oh et al. 2009; Vleeshouwers et al. 2008). The diversity of effector variants across pathogen populations, coupled with functional assays, can be used to monitor pathogen populations for the potential occurrence or emergence of races that overcome the *R* genes (Schornack et al. 2009b; Vleeshouwers et al. 2008). Also the identification of an *R* gene responding to an effector with a virulent function crucial for *P. infestans* infection process could be perceived as a "Holy Grail" for potato breeders. All these strategies and ideologies to manage late blight require the identification and characterization of more *R* genes and *RGH*, as well as the diversity of the corresponding *Avr* genes in the *P. infestans* natural population to be able to setup a durable strategy against late blight.

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Scope of this thesis

The aim of this thesis was to provide new insights in diversity and co-evolution of *Solanum* section *Petota* resistance genes and corresponding *Phytophthora infestans* effectors by functional genomics to provide new genetic tools for management of late blight and potato resistance breeding. We focussed our research on three diverse R-AVR interactions, namely *Rpi-blb1 – Avr-blb1*, *R2 – Avr2/PexRD11*, and *R3a-Avr3a* and dedicated effectoromics in combination with disease tests and population studies to uncover new diversities on *R* and *Avr* gene families by targeted allele mining. Our finding with genotypes originating from natural environments brought new genetic material, information regarding gene stacking, copy numbers of resistance gene homologues and tools to potato breeders for predicting and deploying a durable, broad-spectrum resistance in contemporary agricultural practice.

In Chapter 2, we challenge the classification of *Rpi-blb1* from *S. bulbocastanum* as a broad spectrum *R* gene using information on the matching effector *Avr-blb1* (*ipiO*). We examined the diversity of *ipiO* in a wide set of *P. infestans* isolates and tested them for virulence on potatoes expressing *Rpi-blb1*. We found the first *P. infestans* isolates that defeated *Rpi-blb1* plants, and showed that profiling the *ipiO* variation is instrumental for predicting the effectiveness of *Rpi-blb1* in potato.

Chapter 3 presents an effectoromics approach to identify R-Avr interactions and rapidly characterize functional RGH from other wild *Solanum* species. By functional assays we detect that two effectors of *P. infestans*, PiAVR2 and PexRD11, interact with R2 homologues. We amplified a number of *R2GH* from various wild *Solanum* species, and uncovered a Mexican origin of this *R2* gene family. Among the members, we identified six new functional *R2* homologues, i.e. *Rpi-edn1.1*, *Rpi-snk1.1*, *Rpi-snk1.2*, *Rpi-hjt1.1*, *Rpi-hjt1.2*, and *Rpi-hjt1.3* that confer resistance to *P. infestans* Positive selection modelling on the R2GH revealed amino acids under diversifying selection in the N-terminus, the NB and the LRR domain, and we postulated a guard model function for *R2* with a bait and switch mechanism for recognition and signal transduction. In addition a discussion on copy number of functional *R* homologues in wild *Solanum* species is presented.

In Chapter 4 we further investigate the R2-PiAvr2/PexRD11 interaction, and use the *P. infestans* genome sequence to retrieve the *PiAvr2/PexRD11* gene family. We analyzed the diversity and functionality of *PiAvr2/PexRD11* and *R2* gene families for avirulence activity, and discussed functional evidence for an arm-race or co-evolution between *P. infestans* and Mexican *Solanum* species.

Chapter 5 presents effectoromics using different transient assays in combination with disease tests to characterize *Solanum* species containing functional homologues of *R3a*. One functional R3a homologue, Rpi-sto2 that interacts with Avr3a, was cloned from *S. stoloniferum*. The origin of *R3a*, its diversity and the implications of for breeding purposes are discussed.

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CHAPTER 2

Phytophthora infestans isolates lacking class I *ipiO* variants are virulent on *Rpi-blb1* potato

Nicolas Champouret, Klaas Bouwmeester, Hendrik Rietman, Theo van der Lee, Chris Maliepaard, Anika Heupink, Peter J.I. van de Vondervoort, Evert Jacobsen, Richard G.F. Visser, Edwin A.G. van der Vossen, Francine Govers, and Vivianne G.A.A. Vleeshouwers

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Phytophthora infestans isolates lacking class I *ipiO* variants are virulent on *Rpi-blb1* potato

Nicolas Champouret[1*], Klaas Bouwmeester[2*], Hendrik Rietman[1*], Theo van der Lee[3], Chris Maliepaard[1], Anika Heupink[1], Peter J.I. van de Vondervoort[2], Evert Jacobsen[1], Richard G.F. Visser[1], Edwin A.G. van der Vossen[1], Francine Govers[2*], and Vivianne G.A.A. Vleeshouwers[1*]

[1] Wageningen UR Plant Breeding, P.O. Box 386, 6700 AJ, Wageningen, The Netherlands, [2] Laboratory of Phytopathology, Wageningen University, Droevendaalsesteeg 1, 6708 PB, The Netherlands, [3] Plant Research International, P.O. Box 16, 6700 AA Wageningen, The Netherlands, * The first three and last two authors share first and last authorship, respectively.

Abstract

A strategy to control the devastating late blight disease is providing potato cultivars with *R genes* that are effective to a broad spectrum of *Phytophthora infestans* isolates. Thus far, most late blight *R* genes that were introgressed in potato were quickly defeated. In contrast, the *Rpi-blb1* gene originating from *Solanum bulbocastanum* has performed as an exclusive broad-spectrum *R* gene for many years. Recently, the RXLR effector family *ipiO* was identified to contain *Avr-blb1*. Monitoring the genetic diversity of the *ipiO* family in a large set of isolates of *P. infestans* and related species resulted in 16 *ipiO* variants in three distinct classes. The class I and class II, but not class III, *ipiO* variants induce cell death when co-infiltrated with *Rpi-blb1* in *Nicotiana benthamiana*. Class I is highly diverse and represented in all analyzed *P. infestans* isolates except in two Mexican *P. infestans* isolates, and these were found virulent on *Rpi-blb1* plants. In its C-terminal domain, IPI-O contains a W-motif that is essential for triggering Rpi-blb1-mediated cell death and is under positive selection. This study shows that profiling the variation of *Avr-blb1* within a *P. infestans* population is instrumental for predicting the effectiveness of *Rpi-blb1*-mediated resistance in potato.

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Introduction

Late blight caused by the oomycete *Phytophthora infestans* is one of the most severe threats to potato production worldwide (Fry 2008). Despite many efforts, effective methods to control late blight epidemics are still not available. In recent years, however, intensified research on both the pathogen and the host plant has deepened our insight into the molecular basis of virulence and avirulence determinants in *P. infestans*, and of host defense responses. This knowledge is instrumental for obtaining genetic resistance in potato (Bryan and Hein 2008; Govers and Gijzen 2006; Park et al. 2009).

In order to invade plant cells without hindrance, pathogens secrete effector proteins that can manipulate host defense responses thus resulting in effector-triggered susceptibility (ETS) (Jones and Dangl 2006; Kamoun 2006; Kamoun 2007). When an effector is recognized by a resistance (R) protein, effector triggered immunity (ETI) is activated often resulting in a hypersensitive response (HR). Effectors then act as avirulence (Avr) factors and the encoding *Avr* genes interact with *R* genes according to the gene-for-gene model (Flor 1971). The molecular arms race between the pathogen and its host drives co-evolution of *R-Avr* gene pairs, as has been clearly demonstrated with the *Arabidopsis R* gene *RPP13* and the corresponding *Avr* factor *ATR13* of the oomycete *Hyaloperonospora arabidopsidis* (formerly *H. parasitica*) (Allen et al. 2008). Both *RPP13* and *ATR13* are highly variable and by examining natural variants of ATR13 key amino acids were identified that are functionally essential for interaction with RPP13 (Allen et al. 2008). In *P. infestans* only a few *Avr* genes have been studied at the molecular level, so far. *Avr3a*, the counterpart of the *Solanum demissum* gene *R3a*, has two alleles – a virulent and avirulent one – that differ only two amino acids (Armstrong et al. 2005). The latter, $Avr3a^{KI}$, not only triggers a *R3a*-dependent HR; it is also capable to suppress a cell death response induced by the elicitin INF1. These two activities, however, are conditioned by distinct amino acids (Bos et al. 2009). On the plant side, *R3a* unleashed its evolutionary potential with numerous *R3a*-like genes, which resulted in a major late blight locus on chromosome 11 of *Solanum demissum* (Friedman and Baker 2007; Huang 2005; Huang et al. 2005). *Avr4*, which interacts with *S. demissum R4*, has one predominant avirulent allele in nature but unlike *Avr3a*, the virulent allele has frameshift mutations and can no longer produce an effector protein (van Poppel et al. 2008). Recently, we described the identification of another potential *P. infestans Avr* gene, i.e. *ipiO*, the *in planta induced* gene that was postulated as being involved in pathogenicity based on its expression profile (van West et al. 1998). The identification of *ipiO* as *Avr-blb1* resulted from an effector genomics approach that is based on high throughput functional profiling of effector genes in plants carrying *R* genes (Vleeshouwers et al. 2008). In the effector screening, two variants of *ipiO*, i.e. *ipiO1* and *ipiO2*, triggered a cell death response in *S. bulbocastanum* plants carrying the late blight *R* gene *Rpi-blb1* (alternatively named RB) (Song et al. 2003; van der Vossen et al. 2003; Vleeshouwers et al. 2008). Cell death

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responses to *ipiO1* and *ipiO2* were also noted in *S. stoloniferum*, which is the source of the *Rpi-blb1* homologues *Rpi-sto1* and *Rpi-pta1*. Accordingly, agro-coinfiltration of *Rpi-blb1*, *Rpi-sto1* and *Rpi-pta1* with *ipiO1* and *ipiO2* in *Nicotiana benthamiana* resulted in specific cell death, and provided functional evidence for the *R-Avr* interaction. However, *ipiO4* – a genetically more distant variant – did not elicit cell death when agro-coinfiltrated with *Rpi-blb1*, nor with its homologues. This suggested that alleles or variants of the *ipiO* gene family vary with respect to recognition by *Rpi-blb1* and hence, in avirulence to *Rpi-blb1*.

Like Avr3a and Avr4, IPI-O contains at its N-terminus a signal peptide for type II secretion and a RXLR-dEER motif for host cell internalization whereas the C-terminal domain is required for effector functions (Govers and Bouwmeester 2008; Rehmany et al. 2005; Whisson et al. 2007). The RXLR domain of IPI-O partly overlaps with a RGD cell adhesion motif, which has been shown to bind to a lectin receptor kinase in *Arabidopsis* that may function as an effector target (Gouget et al. 2006). This lectin receptor kinase participates in protein-protein interactions to mediate cell wall-plasmamembrane adhesions and it has been observed that IPI-O can disrupt these adhesions (Senchou et al. 2004). In many of the RXLR-dEER effectors the C-terminal domain consists of a variable number of motifs that occur in a repeated fashion (Jiang et al. 2008). This allows rapid evolution and diversification within this effector family, which is consistent with a role for *Avr* genes in gene-for-gene interactions with their hosts.

A wealth of resistance (*R*) genes is present in botanical *Solanum* species, but in spite of that, resistance breeding has thus far been unsuccessful. Introgressed *R* genes from e.g. *S. demissum* and *S. berthaultii* were quickly defeated and virulent *P. infestans* isolates were detected in the field sometimes even before introduction into cultivated potato (Flier et al. 2003; Grünwald et al. 2001; Rauscher et al. 2006; Wastie 1991). Recently, renewed hope for resistance breeding emerged with the identification of the so-called broad-spectrum *R* genes *Rpi-blb1* and *Rpi-blb2* from *S. bulbocastanum* (Song et al. 2003; van der Vossen et al. 2003; van der Vossen et al. 2005). Since the introduction in potato, *Rpi-blb1* appears to have remained effective in various geographical areas and over several growing seasons (Colton et al. 2006; Helgeson et al. 1998; Naess et al. 2000). The identification of *ipiO* as the candidate for *Avr-blb1* enables us to address the question how widespread avirulent alleles or variants occur in *P. infestans* populations, and related to this, how likely it is that *Rpi-blb1*-mediated resistance will last in the field. In this study, we monitored the genetic variation of *ipiO* in a highly diverse set of *P. infestans* isolates and related *Phytophthora* species, and identified 16 naturally occurring *ipiO* variants that could be grouped in three different classes. The *P. infestans* isolates lacking one specific class of *ipiO* variants appeared to be virulent on plants carrying *Rpi-blb1*, thus confirming the gene-for-gene interaction between *ipiO* and *Rpi-blb1*. We also show that IPI-O contains a W-motif in the C-terminal domain that is subject to positive selection and that this domain is sufficient to trigger *Rpi-blb1*-dependent cell death.

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Results

The *ipiO* gene is highly diverse

For assessing natural genetic variation of *ipiO*, we compiled a set of 29 *P. infestans* isolates that were collected from various geographic regions, including the European potato growing areas and the Central Highlands of Mexico that are both known to harbour genetically highly diverse *P. infestans* populations (Flier 2001; Flier et al. 2003; Rivera-Pena 1990a) (Table 1). Furthermore, the *P. infestans* isolates originated from diverse host plants, including potato, tomato and botanical *Solanum* species, and they vary for year of collection, mating type, and virulence pattern on the potato *R1-R11* differential set. To examine the genetic diversity we fingerprinted 21 *P. infestans* isolates with SSR markers and showed that nearly all isolates had a unique genotype. Only two isolates, i.e. IPO-0 and UK7824, had identical genotypes, and PRC505705 and PRC506303 have a highly similar genotype. No clear supported branching was observed upon Neighbor Joining in the cluster analysis, illustrating that the assembled set of *P. infestans* isolates is genetically highly diverse (Figure 1).

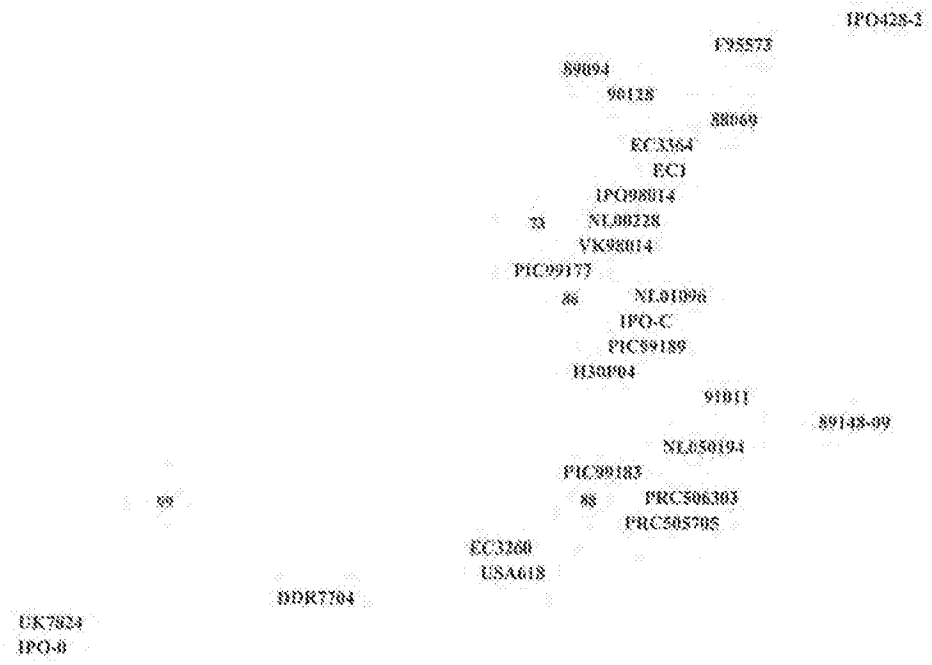


Figure 1. Genetic diversity of the Phytophthora infestans isolates in this study. Bootstrap values in percentage (>60) are shown at the nodes.

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Table 1. *Phytophthora* isolates used in this study

Phytophthora species	Isolate	Origin			MT	Race	Reference
		Country	Year	Source			
P. infestans	F95573	The Netherlands	1995	Potato cull pile	A1	1.3.4.7.10.11	Flier et al. (2003)
	89148-09	The Netherlands	1989	Potato crop	A1	0	
	PIC99177	Mexico	1999	*S. stoloniferum*	A2	1.2.3.4.7.9[a].11	Flier et al. (2002)
	88069	The Netherlands	1988	Tomato crop	A1	1.3.4.7	van West et al. (1998)
	PIC99189	Mexico	1999	*S. stoloniferum*	A2	1.2.5.7.10.11	Flier et al. (2002)
	90128	The Netherlands	1990	Potato crop	A2	1.3.4.7.8.10.11	Vleeshouwers et al. (1999)
	EC1	Ecuador	n.d.[b]	unknown	n.d.	1.3.4.7.10.11	Armstrong et al. (2005)
	H30P04	The Netherlands	1985	-[c]	A1	3a.7.10.11	Drenth et al. (1995)
	USA618	Mexico	n.d.	Potato crop	A2	1.2.3.6.7.10.11	Fabritius et al. (2002)
	IPO-0	unknown	n.d.	unknown	n.d.	3b.4.7.10.11	
	IPO-C	Belgium	1982	Potato crop	A2	1.2.3.4.5.6.7.10.11	
	PIC99183	Mexico	1999	*S. stoloniferum*	A2	1.2.3.4.5.7.8.10.11	Flier et al. (2002)
	NL01096	The Netherlands	2001	Potato crop	A2	1.3.4.7.8.10.11	
	VK98014	The Netherlands	1998	Potato crop	A1	1.2.4.11	
	IPO428-2	The Netherlands	1992	Potato crop	A2	1.3.4.7.8.10.11	Flier et al. (2003)
	NL00228	The Netherlands	2000	Potato crop	A2	1.2.4.7	
	DDR7704	Germany (fGDR[d])	1977	Potato crop	A1	1.2.4	
	UK7824	United Kingdom	1978	Potato crop	A1	1.2.3.6.7	
	89094	The Netherlands	1989	Potato cull pile	A2	1.2.3.6.7.10.11	Kamoun et al. (1998)
	91011	The Netherlands	1991	Experimental field	A2	3.4.5.10	
	PIC97757	Mexico	1997	*S. demissum*	A1	n.d.	Flier et al. (2001)
	IPO98014	The Netherlands	1998	Potato crop	A1	1.3.3.4.7.11	Flier et al. (2003)
	NL050194	The Netherlands	2005	Potato crop	A2	n.d.	
	NL05105	The Netherlands	2005	Potato crop	A2	n.d.	
	PRC505705	China	2005	Potato crop	A2	1.2.3.4.5.6.7.9.10.11	
	PRC506303	China	2005	Potato crop	A2	1.2.3.4.5.6.7.9.10.11	
P. infestans s.l.[e]	EC3280	Ecuador	2001	*S. betaceum*	A1	non-host	Adler et al. (2004)
	EC3304	Ecuador	2001	*S. betaceum*	A1	non-host	Adler et al. (2004)
	EC3364	Ecuador	2001	*S. betaceum*	A1	non-host	Adler et al. (2004)
P. andina	EC3414	Ecuador	2001	*Anarrhichomenum* complex	A1	non-host	Gomez-Alpizar et al. (2008)
P. ipomoeae	PIC99193	Mexico	1999	*Ipomoea longipedunculata*	s.f.[f]	non-host	Flier et al. (2002)
P. phaseoli	CBS556.68	unknown	1968	unknown	s.f.	non-host	Flier et al. (2002)
P. mirabilis	PIC99111	Mexico	1999	*Mirabilis jalapa*	A2	non-host	Flier et al. (2002)
	CBS150.88	Mexico	1988	*Mirabilis jalapa*	A2	non-host	Kamoun et al. (1998)

[a] not conclusive, [b] n.d., not determined, [c] F1 progeny from a cross between *P. infestans* isolates 80029 and 88133 (Drenth et al. 1995), [d] fGDR, the former German Democratic Republic, [e] s.l., sensu lato, [f] s.f., self fertile

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To determine the genetic variation at the *ipiO* loci, we PCR amplified *ipiO* on genomic DNA derived from the 29 *P. infestans* isolates and from five isolates of other clade 1c species, i.e. *P. andina*, *P. ipomoeae*, *P. phaseoli*, and *P. mirabilis* (Blair et al. 2008). Sequence analyses revealed 16 variants of *ipiO* with a minimum of one and a maximum of four *ipiO* variants per isolate (Table 2, Figure 2 & 3). So far, no *ipiO* homologues have been detected in *Phytophthora* species outside clade 1c (data not shown). As described previously, IPI-O1 and IPI-O2 are highly similar with only four different amino acids (Pieterse et al. 1994). Most of the newly identified IPI-O variants have amino acid changes due to point mutations, i.e. IPI-O3 to IPI-O9, IPI-O11, IPI-Om1 and IPI-Om2. IPI-O10 is identical to IPI-O2, although this variant contains two nonsynonymous nucleotide polymorphisms. Another variant, IPI-O13 has a C-terminal extension of 10 amino acids compared to IPI-O3. *P. infestans* variant IPI-O12 and *P. phaseoli* IPI-Op1 have frameshift mutations that result in truncated proteins.

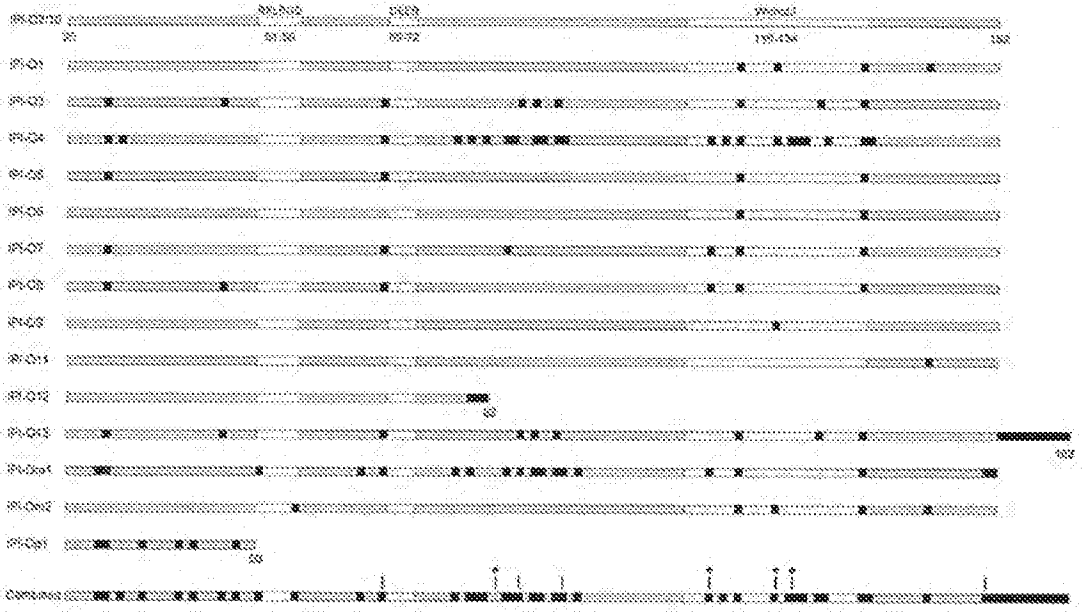


Figure 2. Thirteen variants of the mature IPI-O protein. The motifs RXLR, RGD and DEER and a predicted W-motif are indicated. Numbers refer to the amino acid positions. Amino acid polymorphisms in IPI-O variants, as compared to IPI-O2, are depicted in black. In the lower bar (combined) all the amino acids that show polymorphism are indicated. The vertical lines refer to positively selected sites (see table 2). *= P<99%.

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Table 2. Occurrence of *ipiO* variants and classes in isolates of *Phytophthora infestans* and related species

		I											II		III		
Phytophthora spp	Isolate	*ipiO1*	*ipiO2*	*ipiO5*	*ipiO6*	*ipiO7*	*ipiO8*	*ipiO9*	*ipiO10*	*ipiO11*	*ipiO12*	*ipiOm2*	*ipiO3*	*ipiO13*	*ipiO4*	*ipiOm1*	*ipiOp1*
P. infestans	P95573	x	x														
	89148-09	x															
	PIC99177												x				
	88069	x	x										x				
	PIC99189												x		x		
	90128	x	x							x							
	EC1	x	x							x			x				
	H30P04	x								x			x				
	USA618	x	x														
	IPO-0	x													x		
	IPO-C	x											x				
	PIC99183									x	x						
	NL01096	x	x										x				
	VK98014	x	x											x			
	IPO428-2	x	x							x							
	NL00228	x	x											x			
	DDR7704		x														
	UK7824		x												x		
	89094			x			x	x					x				
	91011	x			x	x			x								
	PIC97757	x			x	x			x								
	IPO98014			x			x	x					x				
	NL050105		x										x				
	NL05194		x										x				
	FRC305705		x														
	FRC306303		x														
P. infestans s.l. [a]	EC3260														x		
	EC3394														x		
	EC3364	x	x										x				
P. andina	EC3414														x		
P. ipomoeae	PIC99193											x					
P. phaseoli	CBS556.88																x
P. mirabilis	PIC99111											x					
	CBS150.88															x	

[a] s.l., sensu lato

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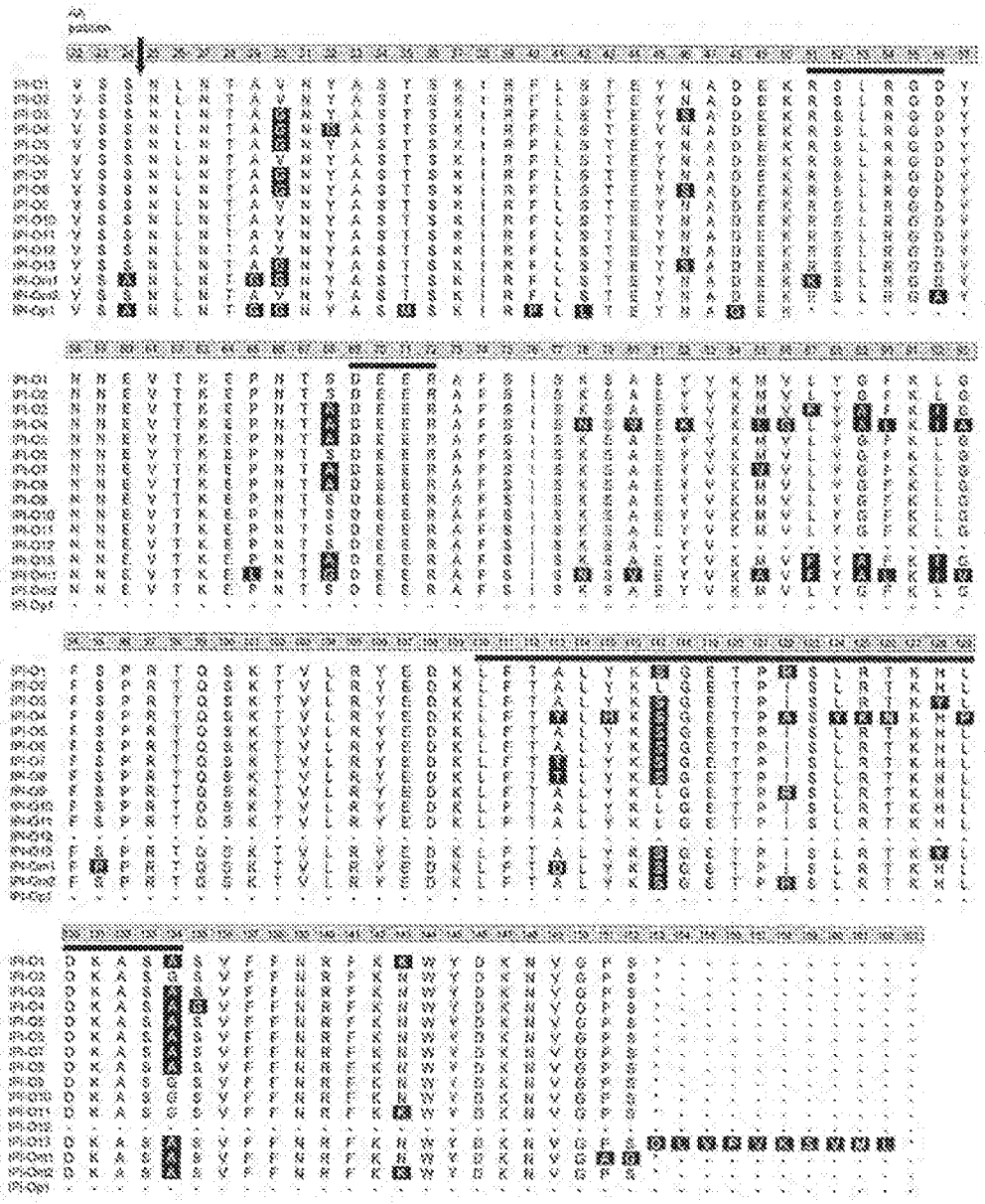


Figure 3. Amino acid alignment of *ipiO* variants. The motifs RXLR, RGD, DEER and a predicted W-motif are indicated. Numbers refer to the amino acid positions. The arrow depicts the predicted signal peptide cleavage site. Amino acid polymorphisms in IPI-O variants, as compared to IPI-O2, are depicted in black background.

Phylogenetic analyses on the protein alignment of the 16 IPI-O variants showed a grouping into three classes, which were designated as class I, II and III (Figure 4). Phylogenetic trees based on Neighbor-joining, minimal evolution or maximum parsimony algorithms resulted in similar clustering (not shown) and also analyses based on nucleotide alignments yielded comparable results. Most IPI-O variants group in class I, together with IPI-O1 and IPI-O2. Class II is significantly different from class I and includes IPI-O3 and IPI-O13. Class III comprises IPI-O4, which has 16 amino acid differences compared to the

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class I and II IPI-O variants and is the most divergent IPI-O variant in *P. infestans*.

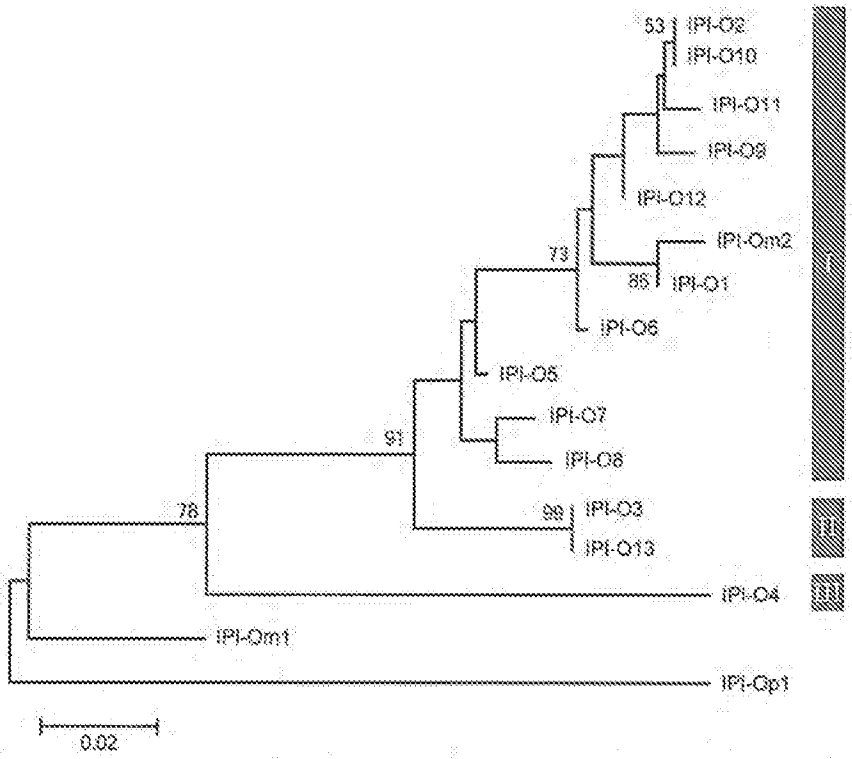


Figure 4. Phylogenetic relationship and clustering of the IPI-O variants. The minimum evolution (ME) tree was rooted with IPI-Op1. Rooting with IPI-Om1, IPI-O4, or *P. sojae* Avr1b resulted in similar clustering. Bootstrap values of 1000 replicates are indicated at the nodes; values less than 50% are omitted (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Class I, II and III IPI-O variants are indicated.

IPI-O has one W-motif with several positively selected sites

The majority of the RXLR-dEER effectors contain positively selected amino acid residues in the C-terminus (Jiang et al. 2008; Win et al. 2007). To investigate whether *ipiO* is also under diversifying selection, we assessed positive selection per residue on two sets of *ipiO* variants. The first set, called Pi, is composed of the 11 full length *ipiO* variants as found in *P. infestans*. The other set, Pi+Pm, includes the variants detected in the sibling species *P. mirabilis*, in addition to the Pi set. Of the different evolutionary models (Yang et al. 2005), model M2a for positive selection fits well on both data sets with ℓ values of -822.55 and -960.76 for Pi and Pi+Pm, respectively. With this model, several positively selected amino acid residues were identified, four of which overlap in the two data sets. Also the selection model M8 gave high log likelihood values for both sets. For the Pi set, M2a and M8 identified the same seven positively selected sites. For the Pi+Pm set, M8 identified the same set of four overlapping positively selected sites and four additional ones, one of which is also selected by the M2a model for the Pi+Pm set (Table 3).

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Table 3. Evidence for positively selected sites in *ipiO*

	Model code	Parameters estimates	ℓ [a]	Positively diversified codons [b]
Pi [c]	M0: one ratio	ω=1.089	-839.78	Not allowed
	M1a: nearly neutral	ω0=0, ω1=1, *p*0=0.580, *p*1=0.420	-835.50	Not allowed
	M2a: positive selection	ω0=0, ω1=1, ω2=28.638, *p*0=0.353, *p*1=0.637, *p*2=0.010	**-822.55**	46N, 82Y, 85M, 87L, 113A*, 122R*, 124L*
	M7: beta	*p*=0.012, *q*=0.005	-837.03	Not allowed
	M8: beta & ω	*p*0=0.990, *p*1=0.010, *p*=0.008, *q*=0.005, ω=27.108	**-822.57**	46N, 82Y, 85M, 87L, 113A*, 122R*, 124L*
Pi + Pm [d]	M0: one ratio	ω=0.970	-980.05	Not allowed
	M1a: nearly neutral	ω0=0, ω1=1, *p*0=0.599, *p*1=0.401	-972.94	Not allowed
	M2a: positive selection	ω0=0.476, ω1=1, ω2=12.487, *p*0=0.971, *p*1=0, *p*2=0.028	**-960.76**	85M*, 93G, 113A*, 122R*, 124L
	M7: beta	*p*=0.005, *q*=0.007	-972.94	Not allowed
	M8: beta & ω	*p*0=0.983, *p*1=0.017, *p*=0.005, *q*=0.005, ω=14.665	**-960.34**	68S, 85M*, 87L, 93G, 113A*, 122R*, 124L*, 151P

[a] Log likelihood value
[b] Bayes Empirical Bayes (BEB) analysis (Yang et al. 2005). Positively selected sites (P>95%, *=P>99%)
[c] based on full length *P. infestans ipiO* variants, n=11
[d] based on full length *P. infestans* and *P. mirabilis ipiO* variants, n=13

In a recent study that used Hidden Markov Model (HHM) searches to find motifs in RXLR-dEER effectors, it was shown that many of the RXLR-dEER effectors contain conserved C-terminal motifs that may occur in repeated fashion (Jiang et al. 2008). These motifs were named W, Y and L after the amino acid at a fixed position in each motif. IPI-O contains a single W-motif with moderate to strong HMM scores ranging from 6.5 to 12.3 among the IPI-O variants (Figure 2 & 5). Interestingly, three of the four positively selected sites that overlap in the M2a and M8 model of the two sets have a high posterior probability (P>99%) and are located within the conserved W-motif (Figure 2).

The region comprising the W-motif is sufficient to trigger *Rpi-blb1* mediated cell death

To investigate whether or not the W-motif is involved in triggering *Rpi-blb1*-mediated cell death we analysed several deletion mutants of *ipiO2* (Figure 6A). In co-agroinfiltration assays in *N. benthamiana* IPI-O2 – with or without its signal peptide – triggers cell death in the presence of Rpi-blb1. Deleting the domain comprising the RXLR, RGD and dREER motifs did not abolish recognition and even an additional deletion of the first 26 amino acids of the C-terminal domain did not change the cell death response. Agroinfection and rub-inoculation assays on *S. stoloniferum* accession sto17605-4, which harbors the *Rpi-blb1* homologue *Rpi-sto1*, resulted in similar responses and showed that recognition of IPI-O by *Rpi-sto1* follows

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the same pattern. The results show that the region spanning the last 54 amino acids of IPI-O and comprising the W-motif is sufficient for recognition by Rpi-blb1 and Rpi-sto1. Since the only mutations that are consistent between, on the one hand, the class I and II variants and, on the other hand, the class III variant are located within the W-motif it is conceivable that this motif plays a role in recognition of IPI-O by Rpi-blb1.

IPI-O variants of class I and II, but not class III, trigger *Rpi-blb1*-mediated cell death

In a previous study, we showed that the class I *ipiO* variants, *ipiO1* and *ipiO2*, trigger *Rpi-blb1*-mediated cell death. To assess whether the newly identified *ipiO* variants are also recognized by *Rpi-blb1* we used agroinfiltration in *Nicotiana benthamiana* to reconstruct the interaction between the *ipiO* variants and *Rpi-blb1*. Coinfiltration of *N. benthamiana* leaves with an *A. tumefaciens* strain carrying a construct expressing *Rpi-blb1* as well as a strain carrying a construct expressing either class I *ipiO* genes (*ipiO1*, *ipiO2*, *ipiO5*, *ipiO7*, and *ipiO8*), or class II *ipiO* genes (*ipiO3*) resulted in a confluent cell death response (Figure 6B). Also class I *ipiO* variant *ipiOm2* of *P. mirabilis* co-infiltrated with *Rpi-blb1* resulted in cell death (data not shown). In contrast, co-expression of *Rpi-blb1* with the class III *ipiO4* gene did not elicit a *Rpi-blb1*-mediated response, as no visible cell death was observed in the infiltrated leaves.

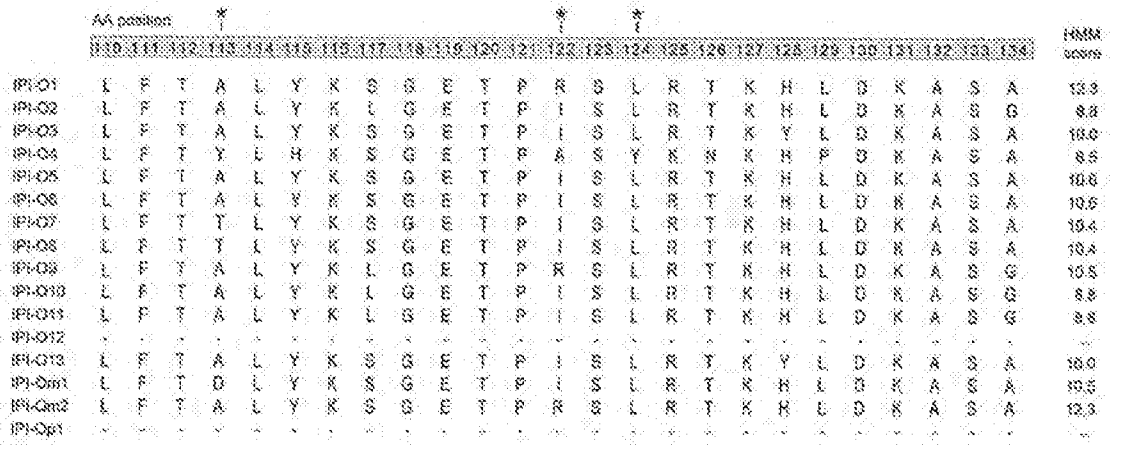


Figure 5. Hidden Markov model (HMM) scores of the conserved W-motif in the IPI-O variants. HMM scores >10 are considered as strong. The vertical lines refer to positively selected sites (Table 2 and Fig. 1). * = $P < 99\%$.

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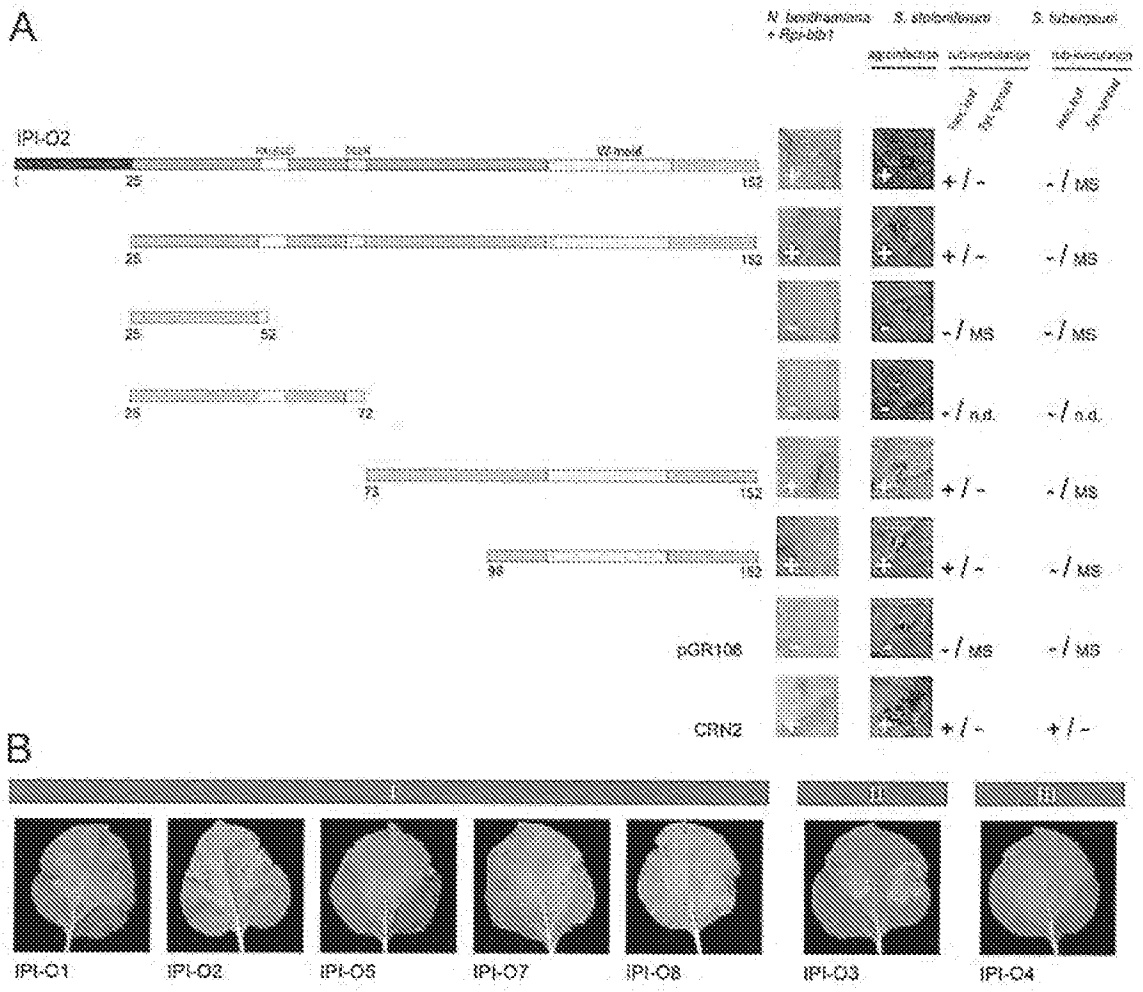


Figure 6. (A) The C-terminus of IPI-O comprising the W-motif is sufficient for recognition by Rpi-blb1. Deletion mutants of *ipiO2* were co-agroinfiltrated with *Rpi-blb1* in *N. benthamiana* or were agroinfected or rub-inoculated with PVX particles carrying the deletion mutants on *S. stoloniferum* accession 17605-4. Potato cultivar Bintje was used as control. Mosaic symptoms (MS) indicate virus spread. Pictures were taken at 5 days after infiltration or agroinfection. (B) Class I and II IPI-O variants are recognized by Rpi-blb1. *N. benthamiana* leaves were agroinfiltrated – at both sides of the leaf midrib – with *Rpi-blb1* in combination with *ipiO* variants of class I, II or III. Pictures were taken at 5 days after infiltration.

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IpiO variants are expressed *in planta*

To enable expression analyses of *ipiO* variants belonging to the three classes, class specific primers were designed that were tested for specificity on genomic DNA (Figure 7). Subsequently, RNA isolated from potato leaves infected with *P. infestans* isolates PIC99183, PIC99189 and PIC99177 was analyzed by semi-quantitative RT-PCR. As shown in Figure 7, mRNA derived from class I, class II and class III *ipiO* genes is present demonstrating that all three classes comprise functional genes that are expressed during *in planta* growth.

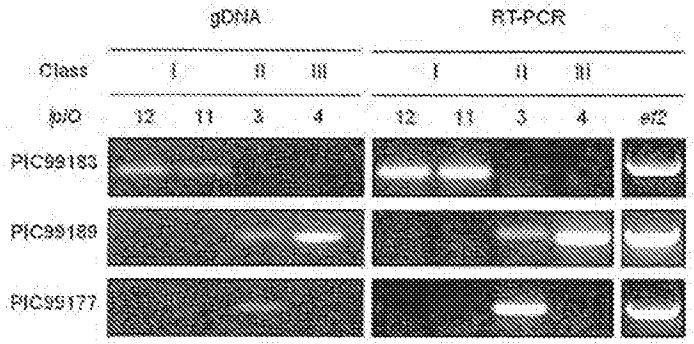


Figure 7. Expression of *ipiO* in infected potato leaves. *IpiO* class specific primers were used for semi-quantitative RT-PCR on RNA isolated from infected leaf tissue of potato cultivar Bintje at 6 dpi. To demonstrate that the primers are specific for each of the three *ipiO* classes, genomic DNA of *P. infestans* isolates PIC99183, PIC99189 and PIC99177 was used as template and detection of specific *ipiO* variants was confirmed. The *P. infestans* elongation factor 2 gene (*ef2*) was used as a control to determine the integrity of the RNA.

Isolates lacking class I *ipiO* variants are virulent on *Rpi-blb1* plants

To test whether the identified *ipiO* variants determine *Rpi-blb1*-mediated cell death, we performed infection assays using *P. infestans* isolates that are genetically diverse and carry different classes of *ipiO* variants (Figure 2, Table 2). To allow a correct interpretation of the virulence phenotypes of the isolates on *Rpi-blb1* plants we first tested 16 selected isolates for their infection capabilities on potato. Detached leaves of universal susceptible potato cultivar Désirée were inoculated and at 6 days post inoculation (dpi) lesion diameters were measured. Based on the lesion size (LS) the isolates were grouped in three classes of aggressiveness (Table 4). To investigate the specificity spectrum of *Rpi-blb1*, we inoculated the 16 isolates on the *S. bulbocastanum* accession blb8005-8, which is the genotype from which *Rpi-blb1* was isolated, and included Désirée and *S. bulbocastanum* blb2002 – containing *Rpi-blb2* – as susceptible and resistant controls, respectively (Table 5). Lesion diameters were measured at 4, 5, and 6 dpi and LS, lesion growth rates (LGR) and infection efficiency (IE) were calculated. Large lesions exceeding 25 mm^2 always coincided with massive sporulation and were scored as compatible interactions. In contrast, smaller lesions typically did not sporulate or showed a 'black-spot' phenotype, indicating a HR. As expected for the so-called 'broad-spectrum' *R* gene *Rpi-blb1*, blb8005-8 was incompatible with nearly

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all isolates. Two Mexican isolates PIC99177 and PIC99189 however, were clearly compatible with blb8005-8 and both developed sporulating lesions on blb8005-8 leaves.

Table 4. Aggressiveness of *P. infestans* isolates on potato cultivar Désirée

P. infestans isolate	LS [a]	Scale of aggressiveness [b]
PIC99177	123.0	
F95573	146.2	
89148-09	151.3	moderately aggressive
88069	248.7	
PIC99189	332.6	
90128	370.7	
EC1	391.7	
IPO-0	399.3	
PIC99183	425.7	aggressive
H30P04	431.6	
IPO-C	488.9	
USA618	518.6	
NL01096	572.4	
VK98014	603.8	
IPO428-2	655.2	highly aggressive
NL00228	721.4	

[a] The lesion size (LS) at 6 dpi determined in two independent experiments, the mean LS are presented.
[b] Grouping is based on significant differences between LS (ANOVA, $P < 0.05$).

To investigate the correlation between compatibility or incompatibility, and *ipiO* variants, we compared the virulence phenotypes of the isolates on *Rpi-blb1*-containing host plants with the occurrence or the absence of specific *ipiO* variants. All avirulent isolates contained at least one class I *ipiO* variant (Table 2 & 5). In contrast, no class I *ipiO* variants were found in the two virulent isolates PIC99189 and PIC99177; only class II and/or III *ipiO* variants were detected. These results suggest that class I *ipiO* variants determine avirulence of *P. infestans* isolates on *Rpi-blb1* plants.

To verify these findings, we tested the *P. infestans* isolates on transgenic lines of cultivar Impala and cultivar Désirée containing *Rpi-blb1* as transgene. In general, *Rpi-blb1*-mediated resistance levels in the potato transgenic lines were lower than in its wild *Solanum* background, blb8005-8 (Table 5). In accordance with the previous experiment, isolate PIC99189 was able to establish sporulating lesions on transgenic Impala RGC-2A9 expressing *Rpi-blb1*, whereas isolates IPO-C and 90128 displayed a HR (Figure 8). On five Désirée *Rpi-blb1* transformants including A01-20, we quantitatively assessed the resistance

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Table 5. *P. infestans* isolates virulent on *Solanum* plants containing *Rpi-blb1* lack class 1 *ipiO* variants

P. infestans isolate [a]	Plant material												[c]	*ipiO* class		
	blb8005-8		sto17605-4		pta17831-8		A01-20		Désirée		blb2002					
	IE [c]	LGR [b]	IE	LGR	IE	LGR	IE	LGR	IE	LGR	IE	LGR		I	II	III
E95573	0	0	0	0	0	0	3	1.4	69	4.4	0	0	A	x		
88148-09	0	0	0	0	0	0	16	1.1	69	2.7	0	0	A	x		
88069	0	0	0	0	0	0	22	0.6	100	3.2	0	0	A	x	x	
90128	0	0	0	0	0	0	50	3.7	78	3.4	0	0	A	x		
EC1	0	0	0	0	0	0	31	2.5	84	3.7	0	0	A	x	x	
IPO-0	0	0	0	0	0	0	78	3.4	91	3.8	0	0	A	x		x
PIC99183	0	0	50	0.5	63	1.2	88	3.6	91	3.6	0	0	A	x		
H30P04	0	0	0	0	0	0	9	2.4	100	4.4	0	0	A	x	x	
IPO-C	0	0	0	0	0	0	50	2.9	100	3.9	0	0	A	x	x	
USA618	0	0	0	0	0	0	19	3.5	100	3.4	0	0	A	x		
NL01096	0	0	0	0	0	0	66	3.4	100	3.8	0	0	A	x	x	
VK98014	0	0	0	0	0	0	66	1.8	100	4.7	0	0	A	x	x	
IPO428-2	0	0	0	0	0	0	38	1.9	100	4.2	0	0	A	x		
NL00228	0	0	0	0	0	0	47	4.6	100	4.8	0	0	A	x	x	
PIC99177	72	1.1	75	2.6	81	2.9	50	2.8	88	3.0	0	0	V		x	
PIC99189	34	1.1	75	0.9	100	4.3	91	4.5	91	3.6	0	0	V		x	x

[a] Additional information about the aggressiveness of P. infestans isolates on potato cultivar Désirée can be found in Table 4.
[b] Isolates were inoculated on different *Solanum* plants containing *Rpi-blb1* or its homologues (Table 7) and mean infection efficiency (IE) and lesion growth rate (LGR) were determined.
[c] *Phenotype on *Rpi-blb1* plants. A = avirulent; V = virulent.

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levels. The virulent isolates PIC99177 and PIC99189 infected A01-20 (Table 5) and the other four transgenic lines (data not shown) equally well as the Désirée control plants (ANOVA, $P<0.05$). To the other 14 isolates, the Rpi-blb1 transgene conferred enhanced resistance at various levels, and generally the level of resistance negatively correlated with the aggressiveness of the isolates. The moderately aggressive isolates F95573, 89148-09 and 88069 reached only low levels of IE and LGR on A01-20, and *Rpi-blb1* clearly conferred a high level of resistance. Highly aggressive isolates, however, achieved slightly reduced or similar IE and LGR on A01-20 as compared to Désirée control plants, and were often able to establish high percentages of fast growing lesions on A01-20, despite the fact that these isolates contain a class I *ipiO* variant. Obviously the aggressiveness of the isolates overrules the recognition by *Rpi-blb1* in the transgenic potato background. The observation that the level of resistance conferred by *R* genes is influenced by the genetic background in which they reside, is in line with previous studies. For example, it was shown that expression of *Rpi-blb1* in *S. bulbocastanum* is dramatically higher than in potato lines carrying *Rpi-blb1* as a transgene (Bradeen et al. 2009; Kramer et al. 2009).

Recognition specificity of *ipiO* variants by *Rpi-blb1* is conserved in *Rpi-sto1* and *Rpi-pta1*

Recently, we described the identification and cloning of functional homologues of *Rpi-blb1* in the distantly related *Solanum* species *S. stoloniferum* (Vleeshouwers et al. 2008). The homologues *Rpi-sto1* and *Rpi-pta1* are nearly identical to *Rpi-blb1*; they only differ in 3 and 5 non-synonymous nucleotide substitutions, respectively. To test these *R* genes for *ipiO* specificity, we co-infiltrated *N. benthamiana* leaves with *A. tumefaciens* strains carrying constructs expressing either *Rpi-sto1* or *Rpi-pta1* combined with *A. tumefaciens* strains expressing *ipiO* variants of class I, II or III. Leaves co-infiltrated with the *Rpi-blb1* homologues and class I or class II *ipiO* variants showed a confluent cell death response, but leaf panels co-infiltrated with class III *ipiO* did not show cell death (Figure 9). These results demonstrate that similar to *Rpi-blb1*, *Rpi-sto1* or *Rpi-pta1* display differential specificity towards the different classes of *ipiO* variants.

We also investigated how *S. stoloniferum* sto17605-4 and pta17831-8 responded to infection with the 16 *P. infestans* isolates that posses different variants of *ipiO*. In detached leaf assays, infection of sto17605-4 and pta17831-8 with the isolates PIC99189 and PIC99177 resulted in sporulating lesions, in accordance with the results obtained with blb8005-8. Of the other 14 isolates only PIC99183 formed a few lesions on sto17605-4 and pta17831-8. There was, however, no biotrophic growth nor sporulation and the lesions expanded very slowly. Since the LGR and IE parameters were much lower than expected for such highly aggressive isolate we suspect that the necrotic spots are due to a trailing HR following invasion attempts, but not to specific virulence of PIC99183 on *S. stoloniferum* sto17605-4 and pta17831-8.

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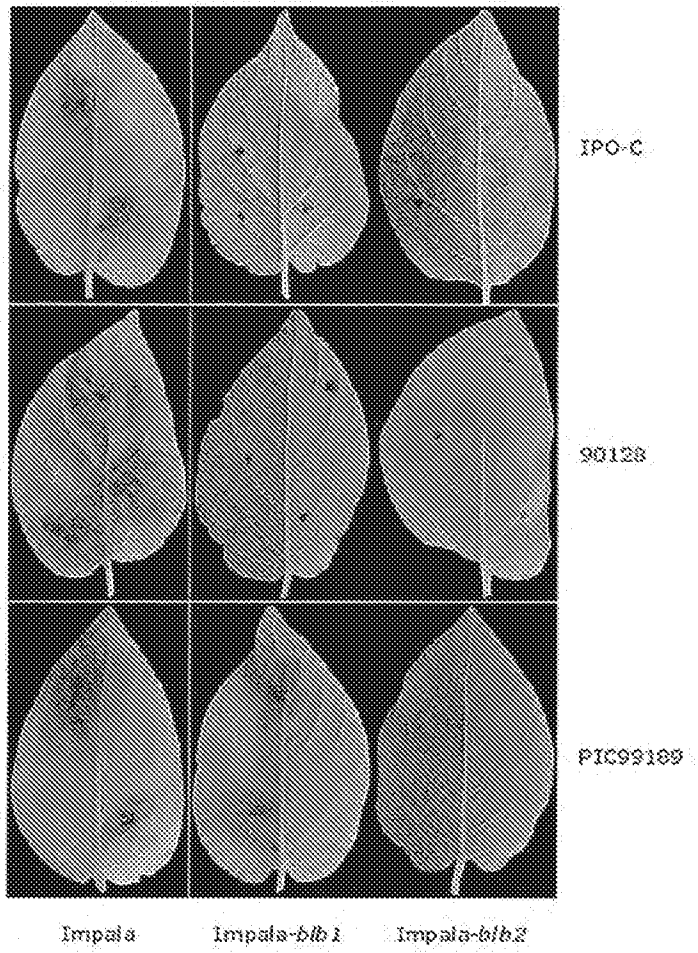


Figure 8. *Rpi-blb1* transgenic potato lines are resistant to *P. infestans* isolates carrying *ipiO* class I and/or II variants. Pictures were taken at 6 dpi.

Discussion

Rpi-blb1 is classified as a so-called broad-spectrum *R* gene that confers resistance to a broad range of *P. infestans* isolates (Song et al. 2003; van der Vossen et al. 2003). In a recent study we identified the RXLR-dEER effector IPI-O as the candidate for the cognate Avr factor of Rpi-blb1 (Vleeshouwers et al. 2008). In this study, we detected isolates that are virulent on Rpi-blb1 plants, and showed that *Rpi-blb1* – *ipiO* is an *R-Avr* pair that basically interacts according to the gene-for-gene model (Flor 1971). IPI-O is the effector that triggers ETI in plants carrying *Rpi-blb1* whereas changes in the IPI-O effector repertoire result in loss of ETI.

The *ipiO* gene family is highly diverse but restricted to *Phytophthora* species that belong to clade 1c. Profiling 34 isolates of *P. infestans* and its close relatives revealed 16 *ipiO* variants, 14 of which were grouped in three distinct classes. In *P. infestans* sensu latu, *P. andina*,

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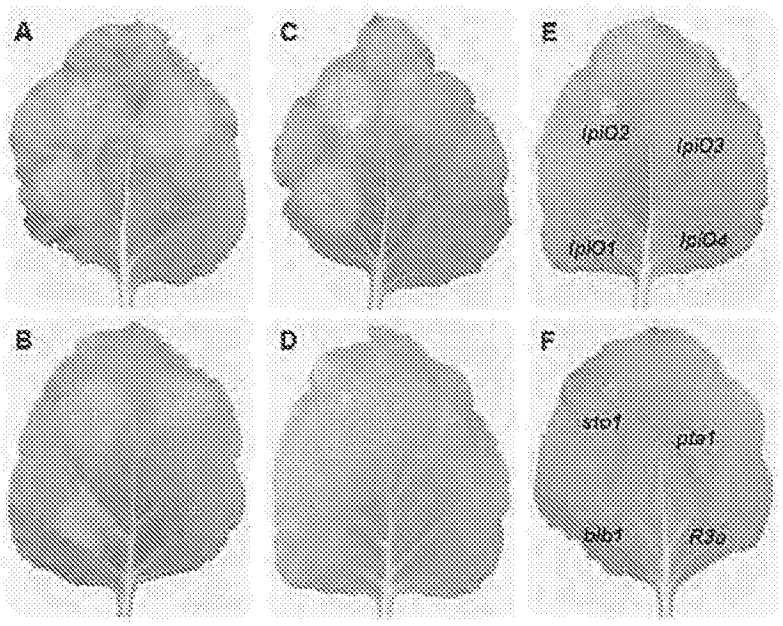


Figure 9. The homologues *Rpi-blb1* (A), *Rpi-sto1* (B) and *Rpi-pta1* (C) induce cell death when co-agoinfiltrated with *ipiO* variants of class I and II. In negative control *R3a* (D), and single infiltration of effectors (E) or *R* genes (F) no cell death is observed. Pictures were taken at 8 days after infiltration. Infiltrated leaf panels in A-D are as indicated in E.

P. ipomoeae, *P. phaseoli*, and *P. mirabilis*, which all evolved on host species distant from *Solanum* section *Petota*, a more or less equal distribution of class I, II, and III variants was found and, in addition, two more distant *ipiO* variants were detected. In *P. infestans* however, class I was significantly expanded, with one to four class I variants in most isolates, of which the majority also has one class II and/or one class III variant. Class I includes *ipiO1* and *ipiO2*, the variants that were already isolated in the early 1990s (Pieterse et al. 1994), and more recently identified as *Avr-blb1* (Vleeshouwers et al. 2008). Disease testing on *Solanum* species showed that all isolates with class I *ipiO* variants were avirulent on *Rpi-blb1* plants, which is in line with the previously reported broad-spectrum character of this *R* gene (Song et al. 2003; van der Vossen et al. 2003). In contrast, two Mexican *P. infestans* isolates, PIC99189 and PIC99177, that both lack class I *ipiO* variants appeared to be virulent on *Rpi-blb1* plants. These data suggest that absence of class I *ipiO* genes is correlated with virulence on *Rpi-blb1* plants, and this is supported by the observation that co-infiltration of class I *ipiO* variants and *Rpi-blb1* in *N. benthamiana* leaves results in cell death. Apparently, class I IPI-O variants elicit HR in *Rpi-blb1* plants and this arrests pathogen invasion. Class III *ipiO* appeared unable to induce cell death when co-infiltrated with *Rpi-blb1*, thus strongly suggesting that the presence of class III *ipiO* in *P. infestans* strains is indeed unlikely to confer avirulence. It should be noted, though, that we have not used epitope tagged-constructs in our *in planta* expression assays to monitor the stability of the various IPI-O variants and, hence, can not exclude the possibility that class III IPI-O is less stable than class I or II IPI-O.

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A more puzzling issue is the finding that class II variants elicit cell death when co-infiltrated with *Rpi-blb1* in *N. benthamiana* leaves. The class II variant *ipiO3* was found to be expressed *in planta* in the virulent strain and one would expect that the presence of a class II variant together with the Rpi-blb1 resistance protein in one cell would lead to HR. This is not the case and raises the question how the numerous RXLR effectors that are predicted to be targeted to the host cell interact with each other. In reconstruction experiments, for example, some RXLR effectors suppress cell death induced by other effectors such as elicitins or BAX (Bos et al. 2009; Bouwmeester et al. 2008; Dou et al. 2008; Whisson et al. 2007). *In vivo*, there could well be a kind of synergism between class I and class II IPI-O variants or even other RXLR effectors. For example, when class I is lacking the class II variant might not be potent enough to act as Avr factor by itself. Alternatively, the presence of a class I variant could suppress the virulence function of a class II variant by competition for the same virulence target. One can also not rule out the possibility that the class II variants are less stable *in planta* than the class I variants. The finding of a class III variant that is not recognized by *Rpi-blb1* but is expressed *in planta* makes the situation even more complex.

Nevertheless, the clear distinction between the three classes helped us to define which parts of the protein are involved in recognition by Rpi-blb1. Deletion analysis showed that the C-terminal part of the IPI-O effector protein is required for recognition, which is in line with other studies (Bos et al. 2006; Dou et al. 2008). The smallest fragment that we tested is 54 amino acids in length and comprises the single W-motif that is present in IPI-O. For *Phytophthora sojae* Avr1b, specific amino acid residues in the W- and Y-motifs in Avr1b are responsible for recognition by the RPS1 protein as well as suppressor activity of cell death. In IPI-O, the W-motif is the region that is the most divergent in the class III variants and the three amino acids that show positive selection are all located within the W-motif. More detailed analysis of the role of each individual amino acid residue in the W-motif will reveal the exact determinants of recognition by Rpi-blb1.

In recent years, insights into *R* gene-based resistance in potato and the role of cognate *Avr* genes from *P. infestans* have increased. For example, many studies were performed for the *R3a-Avr3a* model system. The *R3a*-harboring species *S. demissum* coexists with *P. infestans* in the cool and humid mountain forests in Toluca Valley, a perfect condition for a tight co-evolution between *R3a* and *Avr3a*. *R3a* is a typical fast evolving type I *R* gene (Huang et al. 2005; Kuang et al. 2005) resulting in numerous *R3a*-like genes (Friedman and Baker 2007; Huang 2005). For Avr3a, only two alleles have been detected; $Avr3a^{EM}$ is present in most *P. infestans* isolates world-wide (Armstrong et al. 2005; Rivera-Pena 1990b), whereas $Avr3a^{KI}$ is much less abundant. $Avr3a^{KI}$ but not $Avr3a^{EM}$ is recognized by R3a thus resulting in defeat of *R3a* by most *P. infestans* isolates. *R1*, *R2*, and *R4* also originate from *S. demissum* and similar to *R3a*, these *R* genes were quickly defeated in the field (Fry 2008). Similar to *Avr3a*, *Avr4* alleles in field isolates show very little variation and in all virulent strains, the *Avr4* gene is out of frame due to a one base pair deletion (van Poppel et al. 2008).

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Preliminary analysis of *Avr1* and *Avr2* indicate that also these *Avr* genes are represented by only a few alleles (Francine Govers, Paul Birch and Eleanor Gilroy, personal communication). A completely different scenario exists for the *Rpi-blb* and *ipiO* interaction. *Rpi-blb1* originates from *S. bulbocastanum*, which occurs in more arid climates and most likely has less intensive encounters with *P. infestans*. Presence of *Rpi-blb1* homologues in other Mexican species such as *S. stoloniferum* (Wang et al. 2008) that partly grow in *P. infestans* conducive climates, creates the opportunity for virulent strains to evolve on these species. Indeed, the virulent strains PIC99189 and PIC99177 described in this study were collected from *S. stoloniferum* host plants in Mexico (Flier et al. 2002). Both *Rpi-sto1* and *Rpi-pta1* are almost identical to *Rpi-blb1*, and *Rpi-blb1* fulfills the criteria of a type II *R* gene with only little diversifying selection that is typically slow evolving (Kuang et al. 2005; Liu and Halterman 2006). Thus, for the *Rpi-blb1-ipiO* interaction, not the *R* gene, but the *Avr* gene is represented by a highly diverse and extensive gene family and the notable expansion of class I *ipiO* in *P. infestans*, but not in related *Phytophthora* species that host on other plant species, might be due to a certain degree of co-evolution between *Avr-blb1* and *Rpi-blb1* homologues in *Solanum* host plants. Also *Rpi-blb2*, another broad-spectrum *S. bulbocastanum R* gene that is not (yet) defeated, is interacting with a highly diverse *Avr* gene family, and perhaps such *Avr* genes may be less easy to overcome (Kamoun, personal communication).

In the wild *Solanum* species that contain *Rpi-blb1* or a functional homologue, full resistance was conferred to all tested avirulent *P. infestans* isolates, with the exception of the two Mexican strains. The transfer of *Rpi-blb1* into potato cultivars resulted in improved protection to *P. Infestans* isolates. The enhanced resistance however, could only arrest the growth of mild isolates. Aggressive isolates were not blocked and could still cause high percentages of lesions expanding at high rates. The influence of the genetic background on the performance of *Rpi-blb1* was also reported in other studies, in which the basal *Rpi-blb1* expression levels were found dramatically higher in its wild *S. bulbocastanum* origin compared to transgenic potato (Bradeen et al. 2009; Kramer et al. 2009). Also the increase in expression after *P. Infestans* inoculation was higher. These studies suggest that expression of *Rpi-blb1* as transgene in potato is not high enough to provide satisfactory resistance in the field, and this implies that stacking with other R genes, or engineering enhanced transgene expression in cultivars, is recommended.

The future of late blight resistance breeding is controlled application of well-studied *R* genes in high quality potato cultivars, and devising inherent durability predictions based on the interacting *Avr* gene. Potato cultivars engineered with *Rpi-blb1* and *Rpi-blb2* are expected to be the first GM potatoes to be cultivated for consumption purposes in Europe (Application GM field trial 2005). *Rpi-blb1* is still effective to a broad range of isolates; virulent isolates similar to PIC99189 and PIC99177 have not (yet) been detected in the Netherlands and neighboring countries and class I *ipiO* is well-represented in the *P. infestans*

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isolates analyzed thus far. Future large-scale monitoring aimed at diagnosing the ipiO classes in the *P. infestans* population can help answering the question whether virulence to *Rpi-blb1* is evolving in commercial potato growing areas, or whether accidental introduction of potentially virulent *P. infestans* has occurred. When the first virulent isolates are detected, selection pressure towards losing class I *ipiO* might be avoided by omitting cultivars with *Rpi-blb1* for a certain period of time and apply other *R* genes in stead.

Materials and Methods

Phytophthora isolates, culture conditions and inoculum preparation

The *Phytophthora* isolates used in this study are listed were retrieved from our in-house collection or kindly provided by colleagues. *Phytophthora* isolates were routinely grown in the dark at 15°C in liquid Plich medium (Lee et al. 1997) prior to DNA extraction (Lees et al. 2006), or on solid rye sucrose medium (Caten and Jinks 1968) prior to disease tests. To isolate zoospores for plant inoculations, sporulating mycelium was flooded with cold water and incubated at 4°C for 1-3 h.

Cloning of *ipiO* variants

Primers (Table 6) and *Pfu* DNA polymerase (Promega) were used to amplify *ipiO* on genomic DNA. After 30 cycles Super*Taq* polymerase (HT Biotechnology) and its buffer were added followed by 15 minutes at 72°C. The obtained amplicons were cloned into pGEM®-T Easy vector (Promega) and transformed in DH5α competent cells (Invitrogen). Sequencing was performed using universal M13 primers and DNA sequences were analysed using DNAstar v6, Chromas 2.3 (Technelysium) and Vector NTI software.

SSR genotyping

SSR genotyping was performed using two multiplex set of four SSR markers each (SSR1, SSR3, SSR7, SSR11 and SSR2, SSR4, SSR6, SSR8). Experimental details can be found at the Eucablight site (http://www.eucablight.org/EucaBlight.asp).

Phylogenetic data analyses

The SSR data were analyzed by the phylogenetic software package TREECON® for Windows Version 1.3b (Van de Peer and De Wachter 1994). The evolutionary distance estimation was performed according to Nei and Li (1979) and clustering was performed using the Neighbor Joining algorithm. The tree was rooted using isolate IPO-0. Bootstrap values in percentage (>60) from 1000 replicate trees are shown at the nodes. The scale bar shows genotype divergence in percentage. Phylogenetic analyses of *ipiO* sequences were conducted

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using the Minimum Evolution (ME) method (Rzhetsky and Nei 1992) in MEGA version 4 (Tamura et al. 2007).

Positive selection analysis

To test for amino acids under purifying or diversifying selection we used codon-based analysis (Codeml) implemented in PAML v.4 package (Yang 2007). Maximum-likelihood codon substitution models M0, M1a, M2a, M7, and M8 were used for analysis. Models M2a and M8 are capable to detect sites under positive selection. Bayes Empirical Bayes statistics was used to calculate positively selected sites with high posterior probability (Yang et al. 2005).

***IpiO* expression analysis**

The zones surrounding the water-soaked lesions, where *ipiO1* is known to be highly expressed (van West et al. 1998), were cut from infected leaves of cultivar Bintje at 6 dpi. RNA was isolated using the RNeasy Mini Kit (QIAGEN), incubated with DNase and purified with the RNA clean-up protocol. The purity of the RNA was confirmed on gel. Semi-quantitative RT-PCR was performed with the OneStep RT-PCR kit (QIAGEN) using the specific primers RT-ipiO-I-F and RT-ipiO-I12-R for *ipiO12* (class I), RT-ipiO-I-F and RT-ipiO-I11-R for *ipiO11* (class I), RT-ipiO-II-F and RT-ipiO-II-R for *ipiO3* (class II), and RT-ipiO-III-F and RT-ipiO-III-R for *ipiO4* (class III). *P. infestans* elongation factor 2 gene (*ef2*) was used as a control (Torto et al. 2002).

Table 7. *Solanum* plant material used in this study

Solanum species	Abb.[a]	Genotype	*R* gene	Background	Reference
S. bulbocastanum	blb	8008-5	*Rpi-blb1*		(van der Vossen et al. 2003)
S. bulbocastanum	blb	2002	*Rpi-blb2*		(van der Vossen et al. 2005)
S. stoloniferum	sto	17605-4	*Rpi-sto1*		(Vleeshouwers et al. 2008)
S. stoloniferum[b]	pta	17831-8	*Rpi-pta1*		(Vleeshouwers et al. 2008)
Potato cultivars					
S. tuberosum	tbr	Désirée	-		(van Berloo and Hutten 2005)
S. tuberosum	tbr	Impala	-		(van Berloo and Hutten 2005)
S. tuberosum	tbr	Bintje	-		(van Berloo and Hutten 2005)
Potato transformants					
S. tuberosum	tbr	A01-20	*Rpi-blb1*	Désirée	this study
S. tuberosum	tbr	RGC-2A9	*Rpi-blb1*	Impala	(van der Vossen et al. 2003)
S. tuberosum	tbr	T5/7	*Rpi-blb2*	Impala	(van der Vossen et al. 2005)

[a] Abbreviation of the *Solanum* species
[b] Previously classified as *S. papita*

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Table 6. Primers used in this study

Target	Primer[a]	Primer sequence (5'-3')
SSR fingerprinting	SSR1-F	GGCGCCCTACCCACCGTC
	SSR1-R	GTTTGCGCCTCTTCGCGGACGC
	SSR2-F	CGACTTCTACATCAACCGGC
	SSR2-R	GTTTGCTTGGACTGCGTCTTTAGC
	SSR3-F	ACTTGCAGAACTACCGCCC
	SSR3-R	GTTTGACCACTTTCCTCGGTTC
	SSR4-F	TCTTGTTCGAGTATGCGACG
	SSR4-R	GTTTCACTTCGGGAGAAAGGCTTC
	SSR6-R	TCGCCACAAGATTTATTCCG
	SSR6-F	GTTTCATCATGGAGCGTAGGATGG
	SSR7-F	GTCCTCGGCGTTCTATGAC
	SSR7-R	GTTTCCGAGTACCGAATGAGGC
	SSR8-F	AATCTGATCGCAACTGAGGG
	SSR8-R	GTTTACAAGATACACACGTCGCTCC
	SSR11-F	TTAAGCCACGACATGAGCTG
	SSR11-R	GTTTAGACAATTGTTTTGTGGTCGC
ipiO variants	ipiO-F	ATGGTTTCATCCAATCTCAACACC
	ipiO-R	CTATACGATGTCATAGCATGACAC
	F-dT-IpiOwoL	CACCATGGTTTCATCCAATCTCAACACC
	R-dT-IpiOwoL+	CTAGCTAGGGCCAACGTTTTTATC
	PIET10	AAGGCTACGACATGTCC
	ipiO-R-long	GAATTAGAAAAAGACACGTGG
	Cla1-FR1a-ipiOF	CATCGATATGGGATTTGTTCTCTTTTCAC
	Cla1-IPIOF	CATCGATGGTTTCATCCAATCTCAACACCG
	Cla1-IPIO1/3F	CATCGATGGCGTTTTCTATCTCAAA
	Cla1-IPIO2/3F	CATCGATGCGCACTCAGTCCAAGACG
	StopoIPIO1/3R	CACCCTATGAGATAGAAAACGCCCGC
	StopoIPIO2/3R	CACCCTACTTGGACTGAGTGCG
	Not1IPIOR	CGCGGCCGCCTAGCTAGGGCCAACGTTT
Rpi-blb1	AL57-Rpi-blb1	TCGGCTATGACTGGGCACAACAGACA
	AL58- Rpi-blb1	AAGAAGGCGATAGAAGGCGATGCG
RT-PCR	RT-ipiO-I-F	GATGGTACTTTATGCATTCAAAC
	RT-ipiO-I11-R	CTTCTCGGCGTCTCTCCTA
	RT-ipiO-I12-R	CTTCTCGGCGTCTCTCCGG
	RT-ipiO-II-F	GATGGTATTTTATGCATTCAAAA
	RT-ipiO-II-R	GCGGAAGCCTTATCGAGATA
	RT-ipiO-III-F	GTTGGGATTGTATGCATTAAAGA
	RT-ipiO-III-R	ATCGGGATGCTTGTTCTTGTA

[a] F is forward primer, R is reverse primer

Plant material and generation of *Rpi-blb1* transgenic potato plants

Solanum plant material used is this study is listed in Table 7. Potato cultivars and wild *Solanum* accessions were obtained from our-in-house collection and the Center of Genetic Resources CGN, Wageningen, The Netherlands, respectively (http://www.cgn.wur.nl/uk). *Solanum* plants were maintained *in vitro* on Murashige and Skoog medium (Duchefa) supplemented with 20% sucrose (MS20) in climate chambers at 18°C with a 16h

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photoperiod. Top shoots were transferred to fresh medium and 1-2 weeks later rooted plantlets were transferred to the soil and grown under greenhouse conditions. *N. benthamiana* plants used for agroinfiltration were grown in climate chambers at 22-25°C and high light intensity. The generation of the *Rpi-blb1* transformant in cultivar Impala has been described previously (van der Vossen et al. 2003), and the transformant A01-20 of cultivar Désirée was generated using identical procedures. Briefly, the binary vector pBINPLUS containing *Rpi-blb1* under control of its native promoter and terminator (van der Vossen et al. 2003) was transformed to *A. tumefaciens* strain COR308 and introduced into cultivar Désirée according to standard protocols (Visser et al. 1991). Regenerative shoots were transferred on solid selective medium Zcvk (MS20 with zeatine 1 mg l^{-1}, claforan 200 mg l^{-1}, vancomycine 200 mg l^{-1} and kanamycine 100 mg l^{-1}) and transformants to solid MS30 with 100 mg l^{-1} of kanamycine.

Disease tests

Leaves from 6-8 week old plants were detached and placed in water-saturated oasis in trays, according to (Vleeshouwers et al. 1999). Leaves were spot-inoculated at the abaxial leaf side with 10 µl droplets containing $5*10^4$ zoospores ml^{-1} and incubated in a climate chamber at 15°C with a 16h photoperiod. Lesion diameters were measured at 4, 5, and 6 dpi. The area of the lesions (LS), the infection efficiency (IE) representing the percentage of successful infections were calculated, and the lesion growth rate (LGR) was estimated using linear regression in GenStat 10.

***In planta* expression assays**

IpiO variants without signal peptide were introduced in pK7WG2 and pGR106 (Karimi et al. 2002). *R3a*, *Rpi-blb1*, *Rpi-sto1* and *Rpi-pta1* with their native expression elements were introduced into the pBINPLUS binary vector (Engelen et al. 1995). *Agrobacterium tumefaciens* strain GV3101, and AGL1 (Lazo et al. 1991) in combination with the ternary plasmid pBBR1MCS-5.*virGN54D* (Fits et al. 2000), were used for transformation. For agroinfiltration, *A. tumefaciens* strains were grown as described previously (van der Hoorn et al. 2000) to a final OD600 of 0.4. Leaves of 4-5 week old *N. benthamiana* plants were infiltrated with the *A. tumefaciens* suspensions (at a 1:1 ratio for co-infiltration) in MMA induction buffer (1 l MMA: 5 g MS salts, 1.95 g MES, 20 g sucrose, 200 µM acetosyringone, pH 5.6), and responses were scored from 3-8 days post-infiltration. To obtain PVX particles for PVX agroinfection, *Agrobacterium* strains containing the various recombinant pGR106-*IpiO* plasmids were agroinfected on *N. clevelandii* plants. After appearance of mosaic symptoms leaves were grinded in 50 mM potassium phosphate buffer (pH 7.0). Potato plants were rub-inoculated with the obtained homogenate after light dusting with carborundum powder.

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Author recommended Internet Resources

http://www.eucablight.org/EucaBlight.asp

http://www.cgn.wur.nl/UK/CGN+Plant+Genetic+Resources/Collections/Potato/-+Species/

Data deposition

DNA sequences were deposited in NCBI Genbank under the accession numbers GQ371190 - GQ371203.

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CHAPTER 3

Evolutionary and Functional Analyses Reveal a Diverse Family of *R2* Late Blight Resistance Genes in Mexican *Solanum* Species

Nicolas Champouret, Estelle Verzaux, Anoma A. Lokossou, Hendrik Rietman, Joe Win, Liliana M. Cano, Dennis A. Meijer, Dirk Budding, Koen Pelgrom, Mingxia Yang, Edwin A. G. van der Vossen, Evert Jacobsen, Richard G. F. Visser, Paul R J Birch, Sophien Kamoun, Vivianne G. A. A. Vleeshouwers

Submitted for publication

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Evolutionary and Functional Analyses Reveal a Diverse Family of *R2* Late Blight Resistance Genes in Mexican *Solanum* Species

Nicolas Champouret[1,4], Estelle Verzaux[1,4], Anoma A. Lokossou[1,4], Hendrik Rietman[1,4], Joe Win[2], Liliana M. Cano[2], Dennis A. Meijer[1], Dirk Budding[1], Koen Pelgrom[1], Mingxia Yang[1], Edwin A. G. van der Vossen[1], Evert Jacobsen[1], Richard G. F. Visser[1], Paul R J Birch[3], Sophien Kamoun[2], Vivianne G. A. A. Vleeshouwers[1]

[1] Wageningen UR Plant Breeding, Wageningen University, Wageningen, The Netherlands, [2] The Sainsbury Laboratory, Norwich, United Kingdom, [3] Division of Plant Sciences, University of Dundee (at SCRI), Invergowrie, Dundee DD2 5DA, United Kingdom, [4] The Graduate School Experimental Plant Sciences, Wageningen University, Wageningen, The Netherlands

Abstract

Potato, the third most important mankind-feeding crop, is prone to huge annual losses due to late blight, the disease caused by the oomycete pathogen *Phytophthora infestans*. Modern management of late blight necessitates the use of multiple resistance (*R*) genes, which requires efficient pipelines for identification and characterization of *R* genes. In this study, we used 55 RXLR effectors of *P. infestans* to screen and probe 24 resistant *Solanum* genotypes for late blight *R* genes. One effector, PEXRD11, was recognized by two resistant genotypes. We examined PEXRD11 recognition on a further 100 genotypes representing the broad *Petota* genepool and detected specific cell death responses in the phylogenetically diverse species *S. edinense*, *S. schenckii* and *S. hjertingii*, which all originate from Mexico. The *R* genes responding to PEXRD11 were localized in the *R2* cluster. Allele-mining for *R2* yielded 17 new variants. We identified positively selected amino acid sites predicted to be functionally important in *R2*, principally in the LRR domain but also in the leucine-zipper and the NB domain. Association between phylogenetic relatedness and functional activity was evident. Six new *R2* homologues, i.e. *Rpi-edn1.1*, *Rpi-snk1.1*, *Rpi-snk1.2*, *Rpi-hjt1.1*, *Rpi-hjt1.2* and *Rpi-hjt1.3*, recognised *PexRD11* and *PiAvr2*, a previously described related RXLR effector gene, and conferred resistance to *P. infestans*. We conclude that *R2* family has evolved in Mexico, the centre of origin of the pathogen, and provide an overview of the evolution and functional diversification of this *R* gene family in its natural environment. In addition, we demonstrate that effectoromics is a fast approach to identify functional *R* gene homologs in phylogenetically diverse *Solanum* and to sort out their functional redundancy. These results highlight the high degree of natural variation in functional late blight resistance genes, and offer to potato breeders, a number of potential important and R genes that can be translated to agriculture.

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Introduction

Potato (*Solanum tuberosum*) is currently, one of the three most consumed crops worldwide in addition to wheat and rice (Haverkort et al. 2009). Potato is susceptible to many pathogens, and the most devastating disease hampering potato production is late blight, caused by the oomycete *Phytophthora infestans*, which destroys leaves, stems, and tubers. Late blight causes global yield losses of 16% of the potato crop, representing an annual loss of € 5.2 billion (Haverkort et al. 2009). To manage the disease, early potato breeding efforts focussed on characterisation of resistance spectrum and introgression of 11 resistance (*R*) genes obtained from *Solanum demissum*, a wild relative of potato. The exploitation of new cultivars containing these *R* genes proved to be successful (Black et al. 1953; Malcolmson and Black 1966). Rapidly changing populations of *P. infestans*, however, overcame the deployment of *R1-R11* genes, even after pyramiding (Hein et al. 2009). Contemporary potato breeding for *P. infestans* resistance is exploring the wealth of *R* gene diversity in a broad range of botanical *Solanum* section *Petota* species, to build up a collection of diverse *Rpi* (*R*esistance to *P. infestans*) genes available for stacking in existing or new cultivars (Jacobsen and Schouten 2007). To this end, we initiated an extensive survey of about 1,000 wild *Solanum* accessions for resistance to *P. infestans*, and established a useful resource for breeding and cloning *Rpi* genes (*SolR*gene database). A number of new *Rpi* genes have been cloned recently from Mexican *Solanum* species, e.g. *R1*, *R2*, and *R3a* from *S. demissum* (Ballvora et al. 2002; Huang et al. 2005; Lokossou et al. 2009); *Rpi-blb1/RB* (Song et al. 2003; van der Vossen et al. 2003), *Rpi-blb2* (van der Vossen et al. 2005), and *Rpi-blb3* from *S. bulbocastanum* (Lokossou et al. 2009); *Rpi-sto1* and *Rpi-pta1* from *S. stoloniferum* (Vleeshouwers et al. 2008); and *Rpi-abpt / R2-like* from unknown species used in a pre-breeding program (Lokossou et al. 2009). The first *Rpi*-genes of South American origin also are being cloned, such as *Rpi-vnt1.1*, *Rpi-vnt1.2*, and *Rpi-vnt1.3* from *S. venturii* (Foster et al. 2009; Pel et al. 2009). Many more *Rpi* genes are expected to be cloned in the future, so that the set of cloned *R* genes against late blight is rapidly growing (*SolR*gene database ; Kuhl et al. 2001; Rauscher et al. 2006; Sandbrink et al. 2000; Smilde et al. 2005; Villamon et al. 2005).

The co-evolution of *R-Avr* gene pairs is driven by a molecular arms race between the pathogen and its host (Flier et al. 2003; Flor 1971; Jones and Dangl 2006; Thompson and Burdon 1992). The central highlands of Mexico and, particularly, the Toluca valley are considered to be a center of genetic diversity for the potato late blight pathogen (Flier et al. 2003; Fry et al. 1993; Goodwin et al. 1992; Grünwald et al. 2001) and for tuber-bearing *Solanum* spp. (Hawkes 1990; Hijmans and Spooner 2001; Spooner et al. 2004). *R* genes for resistance to potato late blight were first discovered in endemic *Solanum* species, such as *S. demissum* (Black and Gallegly 1957; Black et al. 1953; Malcolmson and Black 1966). The revelation of *P. infestans* genome plasticity, and its abundance of putative effectors (Haas et

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al. 2009), shows that this oomycete remains a formidable foe. Not only is the discovery of new *R* genes important, but the identification of functional *R* gene homologs (*RGH*) from a family could also provide a first hint about co-evolution within this pathosystem.

R genes typically occur in clusters in various linkage groups (LG). A particularly interesting family of *P. infestans R* genes is located in a Major Late Blight (MLB) locus in LG IV (Park et al. 2005b). Of this MLB locus, *R2*, *R2-like*, *Rpi-blb3*, and *Rpi-abpt* were cloned recently. Up to 16 *R2* variants, or *R2GH*, furthermore, were observed in the Mastenbroek *R2* differential (Lokossou et al. 2009). These so-called *R2*-family genes have the closest homology to *RPP13* of *Arabidopsis thaliana*, i.e. 39.4% identity at the amino acid level (Bittner-Eddy et al. 2000; Lokossou et al. 2009), but no other homologs in *Solanaceae* have been identified. Structural analyses of the *R2*-family members revealed clear blocks of sequence exchange between paralogs (Lokossou et al. 2009). This family has evolved, therefore, as a type I *R* gene (Friedman and Baker 2007; Kuang et al. 2005). Another MLB cluster is located in LG XI, which is highly extended and harbours up to 45 type I *R3a* homologs per haplotype (Huang et al. 2005; Kuang et al. 2005). Various *R* genes acting against *P. infestans* have been shown to occur in this MLB, namely *R3a*, *R3b*, and *R5 - R11* (Bradshaw et al. 2006b; El-Kharbotly et al. 1996; Huang et al. 2005; Huang et al. 2004). Homologues of *Rpi-blb1* reside in LG VIII (Vleeshouwers et al. 2008; Wang et al. 2008); these type II *R* genes are thought to rarely experience sequence exchanges between paralogs and maintain orthologous relationships (Friedman and Baker 2007; Liu and Halterman 2006). Finally in LG V, a diversity of *RGH* have been characterized. *R1* confers resistance to *P. infestans*, *Rx2* to potato virus X and *Gpa2* to potato cyst-nematodes (Kreike et al. 1994; Ritter et al. 1991).

Similar to other plant pathogens *P. infestans* translocates effector proteins inside host cells (Birch et al. 2006; Dou et al. 2008; Rehmany et al. 2005; Whisson et al. 2007). The primary activity of these effectors is to promote virulence, for instance by suppressing plant immunity (Schornack et al. 2009b). A number of these effectors, namely members of the RXLR family, however, trigger hypersensitive cell death and resistance responses by activating particular NBS-LRR immune receptors, generically known as CC-NBS-LRR (Dangl and Jones 2001). Six *Avr* genes were characterized recently for the potato – *P. infestans* pathosystem, i.e. *Avr1*, *PiAvr2*, *Avr3a*, *Avr4*, *Avr-blb1 (IpiO)*, and *Avr-blb2* (Armstrong et al. 2005; Champouret et al. 2009; Lokossou et al. 2009; Oh et al. 2009; Tyler 2009; van Poppel et al. 2008; van Poppel et al. 2009; Vleeshouwers et al. 2008). Except for *Avr4*, the corresponding *R* genes to the other five *Avr* genes have been cloned (Ballvora et al. 2002; Huang et al. 2005; Lokossou et al. 2009; Song et al. 2003; van der Vossen et al. 2003; van der Vossen et al. 2005). Two of these *Avr* genes, occur as one or two copies in the genome and show limited allelic diversity: *Avr3a* which is known to have two major alleles $Avr3a^{KI}$ and $Avr3a^{EM}$ in *P. infestans* populations (Armstrong et al. 2005), and *Avr4* of which the avirulent form are the full length protein with few a.a. changes and the virulent form has a

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disrupted ORF (van Poppel et al. 2008). Others of these *Avr* genes occur in multiple copies in the genome and are highly diverse: *Avr-blb1*, which is represented by up to four paralogous copies per genotype, with 13 allelic variants identified in *P. infestans* isolates (Champouret et al. 2009), and *Avr-blb2*, which is diverse and occurs as seven paralogues in the *P. infestans* genome (Oh et al. 2009). Identification of allelic diversity of an *Avr* in *P. infestans* populations, as well as detection of paralogous copies in a genome, therefore, are two factors to consider to categorize *R* genes that could stand in agriculture, if matching *R* variants can be found and combined to recognise natural variation in the pathogen population. It was discovered recently, that effectors can be useful to facilitate breeding, cloning, and deployment of *R* genes by screening plant germplasm for response to candidate effectors (Schornack et al. 2009b). This approach has been used to identify novel R proteins and to classify their activities into discrete recognition specificities (Ellis et al. 2009; Oh et al. 2009; Vleeshouwers et al. 2008). Effectoromics can accelerate the cloning of functional *R* genes. The screen and probe of multiple *Solanum* germplasm for late blight *R* genes can provide a pattern of responses to a specific effector from a group of *Solanum* species, and therefore, can help to avoid redundant cloning efforts. For these reasons, we have based our approach to identify the diversity of one *R* gene in the wild *Solanum* population not on the resistance spectrum to *P. infestans* isolates but rather on the responses to a specific effector.

Allele-mining strategy is emerging as an efficient approach to identify genetic variation of *R* genes, especially now that sequence and positional information is expanding in the genome era (Leister et al. 1996; Lokossou et al. 2009; Pel et al. 2009; Wang et al. 2008). After genetic mapping of *Rpi-vnt1* in *S. venturii* at the locus of *Tm-2*^{2}, a Tomato mosaic virus resistance gene (Foster et al. 2009; Pel et al. 2009), allele-mining methods quickly led to full-length *Tms-GHs* that confer resistance to *P. infestans* isolates (Pel et al. 2009). In some cases, however, numerous *Rpi*-candidate genes are amplified and the challenge is to identify efficiently the functional *RGH* among these. We argue that functional allele-mining, by using the effector, is a new way to delimit the number of *RGH* to analyse for functional *R* genes.

In this study, we functionally profiled *Solanum* species with specific RXLR effectors of *P. infestans*. Based on observed responses, we focussed on the recognition of one effector, PEXRD11, an RXLR effector related to the previously described PiAVR2 (Lokossou et al. 2009). Exploring the stretch of specific recognition to PEXRD11 by functional allele-mining on a large set of *Solanum* section *Petota* species yielded three species from phylogenetically diverse series (Jacobs et al. 2008) and originating from Mexico. Genetic approaches located the *Rpi* genes responsive to PEXRD11 in the *R2* cluster. PCR allele-mining on the three genotypes led to the identification of 17 *R2* homologues (*R2GH*), divided in six clades. Diversifying selection in the R2-GHs was confirmed by positive selection analyses, and could be the result of a molecular arms race between wild populations of *P. infestans* and the three *Solanum* species in Mexico. Combination of transient assays for specific effector recognition and transient complementation in *Nicotiana benthamiana* resulted in the

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identification of six new *R2* homologues that confer resistance to *P. infestans* and recognized PEXRD11 and PiAVR2 with varying degrees of intensity.

Results

Effectoromics screens reveal specific recognition of PEXRD11 in *Solanum* spp.

A broad diversity of the *Solanum* section *Petota* gene pool was subjected to disease assays with *P. infestans* isolate 90128, and 24 resistant *Solanum* genotypes that belong to 18 wild species within eight taxonomic series were selected (*Sol*Rgene database ; Jacobs et al. 2008). Three susceptible cultivars and one transgenic plant, Impala-*Rpi-blb1*, were added as controls. To functionally assess these 28 genotypes for their recognition spectra towards *P. infestans* effectors, we assayed them for response to 55 putative RXLR effectors, previously described by Oh et al. (2009) and Vleeshouwers et al. (2008), using an expression system based on potato virus X (PVX) and *Agrobacterium tumefaciens* and known as PVX agroinfection (Oh et al. 2009; Vleeshouwers et al. 2008; Vleeshouwers et al. 2006). Based on observed responses, the effectors were classified in two groups: specific and non-specific responses (Table 1). The non-specific group contained effectors that activated responses in resistant and susceptible genotypes. Our study priority is the identification of new specific interactions; therefore, we did not pursue further with this group. The specific group, represented by 41 effectors, contained 86 specific genotype-effector combinations, where effectors triggered cell death in resistant but not in susceptible plants (Table 1), which suggests that these effectors might exhibit avirulence activity and might activate *R* genes that are absent in susceptible *Solanum* plants (Vleeshouwers et al. 2008). Eight specific combinations correspond to the known R-AVR interaction of *Rpi-blb1* homologues with *Avr-blb1* (*IpiO*): namely *S. bulbocastanum* (8005-8) carrying *Rpi-blb1*, *S. stoloniferum* (CGN 17605-4) carrying *Rpi-sto1*, *S. papita* (CGN 17831-8-1) carrying *Rpi-pta1* and Impala-*Rpi-blb1* transformants carrying *Rpi-blb1*. These responses showed a localized cell death to *IpiO1* and *piO2* (see [1] Table 1), as described in previous studies (Champouret et al. 2009; Vleeshouwers et al. 2008), which indicates that the screen was relevant.

To determine the extent to which the remaining 78 specific combinations correspond to bona fide *R* gene-mediated recognition, we used a genetic approach. We crossed resistant *S. polyadenium* (pld17749-4), *S. stoloniferum* (sto17605-4), and two *S. microdontum* (mcd23050-1 and mcd18200-6) with susceptible *Solanum* genotypes, and assessed the populations for segregation of cell-death response to candidate effectors and for resistance to *P. infestans* isolates. Segregation of response to candidate effectors at different ratios was observed in 11 combinations: with the genotype pld17749-4, mcd23050-1, and sto17605-4 (see [3] Table 1). Because there was no co-segregation between effector response and resistance to *P. infestans*, we did not pursue this further.

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