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(54) Title: LATE BLIGHT RESISTANCE GENES AND METHODS

(57) Abstract: This invention provides novel gene sequences, compositions and methods for enhancing the resistance in crops, in particular but not limited to, potato, to late blight caused by the oomycete pathogen Phytophthora infestans.

LATE BLIGHT RESISTANCE GENES AND METHODS

FIELD OF THE INVENTION

Novel genes, compositions and methods for enhancing crop resistance to late blight.

BACKGROUND OF THE INVENTION

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

Two main types of resistance to late blight have been described in potato (Umaerus and Umaerus 1994). First, general resistance is often based on a major quantitative trait loci (QTL) and a few minor QTLs and results in partial resistance. Second, specific resistance is based on major dominant resistance (*R*) genes. In early breeding programs during the first half of last century, 11 *R* genes (*R1-R11*) derived from *S. demissum* were identified. Nine *R* genes, *R3* (now separated as *R3a* and *R3b*) and *R5-R11* were localized on chromosome 11 (Bradshaw et al. 2006; El-Kharbotly 1994, 1996; Huang et al. 2004; Huang 2005). Other *R* genes originating from *S. demissum* were mapped to different locations including *R1* on chromosome 5 (El-Kharbotly et al. 1994; Leonards-Schippers et al. 1992) and *R2* on chromosome 4 (Li et al., 1998). All *R* genes introgressed from *S. demissum* to cultivated potatoes have been overcome by the pathogen as new strains rapidly evolve that are virulent on the previously resistant hosts (Umaerus and Umaerus 1994). Consequently, partial resistance conferred by QTLs was thought to be more durable than resistance conferred by single *R* genes (Turkensteen 1993). However, partial resistance is strongly correlated with maturity type and makes resistance breeding more difficult (Wastie 1991). Also the genetic positions of QTLs often correspond to the region of *R* gene clusters (Gebhart and Valkonen 2001; Grube et al. 2000).

Hence, recent efforts to identify late blight resistance have focused on major *R* genes conferring broad-spectrum resistance derived from diverse wild *Solanum* species. Beside *S. demissum*, other wild *Solanum* species such as *S. acaule*, *S. chacoense*, *S. berthaultii*, *S. brevidens*, *S. bulbocastanum*, *S. microdontum*, *S. sparsipilum*, *S. spegazzinii*, *S., stoloniferum*, *S. sucrense*, *S. toralapanum*, *S. vernei* and *S. verrucosum* have been reported as new sources for resistance to late blight (reviewed by Jansky 2000; Hawkes 1990). To date, three *R* genes, *RB/Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* from *S. bulbocastanum* have been mapped on chromosome 8, 6 and 4, respectively (Nasess et al. 2000; Park et al. 2005a; van der Vossen et al. 2003, 2005). Another *R* gene, *Rpi-abpt*, probably from *S. bulbocastanum*, has been localized on chromosome 4 (Park et al. 2005b). *Rpi1* from *S. pinnatisectum* on chromosome 7 (Kuhl et al. 2001), *Rpi-mcq1* from *S. mochiquense* (Smilde et al. 2005) and *Rpi-phu1* from *S. phureja* on chromosome 9 (Śliwka et al. 2006) have also been reported.

It is evident from a review of the existing art in this area that a significant need remains for novel genes, compositions and methods for conferring late blight resistance. In this patent disclosure, we meet this need by screening wild *Solanum* species and by cloning, and introducing and expressing novel *Rpi* resistance genes into potato.

SUMMARY OF THE INVENTION

We have isolated, identified and characterised several different late blight *R* genes derived from the potato wild species *S. okadae* plus also from S. *mochiquense* and *S. neorossii*.

This invention provides novel gene sequences, compositions and methods for enhancing the resistance in crops, in particular but not limited to, potato, to late blight caused by the oomycete pathogen *Phytophthora infestans*.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 Pedigrees of the genotypes used to construct BAC libraries. (a): K39 and (b): K182

Fig. 2 Analysis of insert size of randomly selected BAC clones from two BAC libraries, K39 (a) and K182 (b) by pulsed-field gel electrophoresis. The BAC clones were digested with *Not*I. The bands at 7.5 Kb are derived from the cloning vector

pIndigoBAC-5. Molecular weight size markers given by the lambda ladder (Sigma Chemical) are indicated on the right of the pictures

Fig. 3 Map positions of the late blight resistance genes *Rpi-oka1, Rpi-oka2* and *Rpi-oka3* from *S. okadae* and *Rpi-nrs1* from *S. neorossii.*

Fig. 4 Pictures of SCAR markers used to screen two BAC libraries. TG551 digested with *Taq*I (a) is linked to *Rpi-oka1*, TG551 digested with *Mwo*I (b) and TG35 (c) are linked to *Rpi-oka1*, and U296361 (d) and TG591 (e) are linked to *Rpi-mcq1*. The 1 Kb size ladder and parental genotypes (A624, A613, A618 and A988) are indicated and the rests are BAC pools positive to certain markers identified by PCR followed by digestion with restriction enzyme. The resistant allele is indicated by '<<' on the right of each picture.

Fig. 5 Contig of BAC clones identified from the K39 BAC library and covering the genomic region containing *Rpi-oka1* and *Rpi-oka2.*

Fig 6 High resolution and fine scale mapping of *Rpi* genes derived from *Solanum okadae* and *S. neorossii.*

Fig. 7 Alignment of the deduced protein sequences of *Rpi-oka1, Rpi-oka2* and *Tm-2*^{2}. The complete amino acid sequence of Rpi-oka1 is shown and dots indicate identical residues in the other two proteins. Where residues from Rpi-oka2 and Tm-2^2 differ from Rpi-oka1, the residues in these proteins are given. The two amino acid differences between Rpi-oka1 and Rpi-oka2 are indicated in bold type. Predicted coiled coil domains are underlined and the first and fourth hydrophobic residues of each heptad repeat are double-underlined. Conserved motifs within the NB-ARC domain are indicated in lower case italics. Putative leucine-rich repeats (LRRs) are indicated above the sequence line.

Fig. 8 Alignment of Rpi-oka1, Rpi-nrs1 and Tm-2^2 protein sequences. The CC, NB-ARC and LRR domains are highlighted in red, green and orange respectively. Conserved motifs within the NB-ARC domain are underlined in italic.

Fig. 9. Genetic linkage maps on chromosome IX of the *Rpi-oka1* (a) and *Rpi-nrs1* (b) loci mapped in the populations 7698 and 7663 respectively. Numbers on the left side

indicate genetic distances (cM). Relative positions of mapped loci are indicated by horizontal lines. The letter n represents the size of each population.

Fig. 10 Map position of the late blight resistance gene *Rpi-mcq1* from *S. mochiquense*.

Fig. 11 Contig of BAC clones identified from the K182 BAC library and covering the genomic region containing *Rpi-mcq1*.

DETAILED DISCLOSURE OF THE PREFERRED EMBODIMENTS OF THE INVENTION

The following sequences are annexed hereto as **Fig. 12** :

Seq ID	*Rpi* Sequence
1a	*oka1 nt*
1b	*oka2 nt*
1c	*oka1* transgene inc. promoter and terminator from pSLJ21152 nt
2a	*mcq1.1 nt*
2b	*mcq1.2 nt*
2c	*mcq1.1* transgene inc. promoter and terminator from pSLJ21153 nt
2d	*mcq1.2* transgene inc. promoter and terminator from pSLJ21148 nt
3	*nrs1 nt*
4a	*oka1 aa*
4b	*oka2 aa*
5a	*mcq1.1 aa*
5b	*mcq1.2 aa*
6	*nrs1 aa*

(nt = nucleotide sequence, aa = polypeptide sequence)

The above sequences represent extended sequences compared to those disclosed in GB0714241.7 from which the present application claims priority. Specifically, they have been extended as follows:

SEQ ID 1a – extended by 99 additional bases at beginning
SEQ ID 1b - extended by 141 additional bases at beginning
SEQ ID 3 - extended by the same 141 ditional bases at beginning

SEQ ID 4a - extended by 33 additional amino acids at beginning
SEQ ID 4b - extended by 47 additional amino acids at beginning
SEQ ID 6 - extended by the same 47 additional amino acids at beginning

Nevertheless that earlier subject matter is not abandoned. Thus where any aspect or embodiment of the present invention is disclosed in respect of the extended sequences defined above, it should be understood as applying *mutatis mutandis* the earlier shorter sequence. Thus each and everyone of such aspects or embodiments of the invention will apply *mutatis mutandis* also to:

SEQ ID 1a – nucleotides 100-2676
SEQ ID 1b - nucleotides 142-2718
SEQ ID 3 - nucleotides 142-2718
SEQ ID 4a - amino acids 34-891
SEQ ID 4b – amino acids 48-905
SEQ ID 6 – amino acids 48-905

The following sequences are expression cassettes including some of the above sequences:

In SEQ ID 1c (Rpi-oka1) - the Rpi-oka1 promoter is included within the bases 1-709, including a 5' untranslated region (UTR) from bases 627-709. The Rpi-oka1 open reading frame (ORF) is present at bases 710-3382 and the terminator from base 3383 onwards. This was cloned into pSLJ21152 and then used to transform *S. tuberosum* and *S. lycopersicum* to confer resistance against *P. infestans*.

SEQ ID 2b (Rpi-mcq1.1) - The Rpi-mcq1.1 promoter is included within the bases 1-2262, the Rpi-mcq1.1 open reading frame (ORF) is present at bases 2263-4848 and the terminator from base 4849 onwards. This was cloned into pSLJ21153 and then

used to transform *S. tuberosum* and *S. lycopersicum* to confer resistance against *P. infestans*.

SEQ ID 2d (Rpi-mcq1.2) - The Rpi-mcq1.2 promoter is included within the bases 1-1999, the Rpi-mcq1.2 open reading frame (ORF) is present at bases 2000-4567 and the terminator from base 4568 onwards. This was cloned into pSLJ21148 and then used to transform *S. tuberosum* and *S. lycopersicum* to confer resistance against *P. infestans*.

As shown in Figure 8, the *Rpi-oka1* and *Rpi-nrs1* sequences are extremely closely related.

As described below, it is believed that the sequences for *Rpi-oka2* are in fact identical to *Rpi-nrs1*, but these are included for completeness.

However the *Rpi-oka3* sequences referred to herein below are identical to *Rpi-oka2*, and are therefore not set out explicitly.

Finally, different candidate Rpi genes were identified from *S. mochiquense* and these are both set out in the sequences. These are both believed to be functional R genes with distinct recognition specificities.

Thus in a first aspect of the present invention there are disclosed isolated nucleic acid molecules encoding a functional Rpi gene, which may optionally be selected from *S. okadae*, *S. mochiquense* and *S. neorossii*.

In particular embodiments the invention provides an isolated Rpi resistance gene having a sequence provided herein as SEQ. ID. 1a, 1b, 2a, 2b or 3.

Nucleic acid molecules according to the present invention may be provided isolated and/or purified from their natural environment, in substantially pure or homogeneous form, or free or substantially free of other nucleic acids of the species of origin. Where used herein, the term "isolated" encompasses all of these possibilities.

The nucleic acid molecules may be wholly or partially synthetic. In particular they may be recombinant in that nucleic acid sequences which are not found together in nature (do not run contiguously) have been ligated or otherwise combined artificially.

Alternatively they may have been synthesised directly e.g. using an automated synthesiser.

Preferred nucleic acids consist essentially of the gene in question, optionally in an expression vector as described in more detail below.

Nucleic acid according to the present invention may include cDNA, RNA, genomic DNA and modified nucleic acids or nucleic acid analogs. Where a DNA sequence is specified, e.g. with reference to a figure, unless context requires otherwise the RNA equivalent, with U substituted for T where it occurs, is encompassed. Where a nucleic acid of the invention is referred to herein, the complement of that nucleic acid will also be embraced by the invention. The 'complement' of a given nucleic acid (sequence) is the same length as that nucleic acid (sequence), but is 100% complementary thereto.

Where genomic nucleic acid sequences of the invention are disclosed, nucleic acids comprising any one or more (e.g. 2) introns or exons from any of those sequences are also embraced.

A resistance gene in this context is one which controls resistance to the late blight caused by *P. infestans*. Such a gene may encode a polypeptide capable of recognising and activating a defence response in a plant in response to challenge with said pathogen or an elicitor or Avr gene product thereof.

Nucleic acids of the first aspect may be advantageously utilised, for example, in potatoes.

A nucleic acid of the present invention may encode one of the amino acid sequences described above (4a, 4b, 5a, 5b, 6) e.g. be degeneratively equivalent to the corresponding nucleotide sequences.

In a further aspect of the present invention there are disclosed nucleic acids which are variants of the sequences of the first aspect.

A variant nucleic acid molecule shares homology with, or is identical to, all or part of the coding sequence discussed above. Generally, variants may encode, or be used to isolate or amplify nucleic acids which encode, polypeptides which are capable of mediating a response against *P. infestans,* and/or which will specifically bind to an antibody raised against the polypeptides described above (4a, 4b, 5a, 5b, 6).

Variants of the present invention can be artificial nucleic acids (i.e. containing sequences which have not originated naturally) which can be prepared by the skilled person in the light of the present disclosure. Alternatively they may be novel, naturally occurring, nucleic acids, which have been or may be isolatable using the sequences of the present invention e.g. from *S. mochiquense, S. okadae* and *S. neorossii.*

Thus a variant may be a distinctive part or fragment (however produced) corresponding to a portion of the sequence provided. The fragments may encode particular functional parts of the polypeptide, e.g. LRR regions, or termini.

Equally the fragments may have utility in probing for, or amplifying, the sequence provided or closely related ones. Suitable lengths of fragment, and conditions, for such processes are discussed in more detail below.

Also included are nucleic acids which have been extended at the 3' or 5' terminus.

Sequence variants which occur naturally may include alleles or other homologues (which may include polymorphisms or mutations at one or more bases).

Artificial variants (derivatives) may be prepared by those skilled in the art, for instance by site directed or random mutagenesis, or by direct synthesis. Preferably the variant nucleic acid is generated either directly or indirectly (e.g. via one or amplification or replication steps) from an original nucleic acid having all or part of the sequences of the first aspect. Preferably it encodes a *P. infestans* resistance gene.

The term "variant" nucleic acid as used herein encompasses all of these possibilities. When used in the context of polypeptides or proteins it indicates the encoded expression product of the variant nucleic acid.

Some of the aspects of the present invention relating to variants will now be discussed in more detail.

Calculated nucleotide identities were as follows:

	Rpi-oka1	Rpi-oka2	Rpi-nrs1	Rpi-mcq1.1	Rpi-mcq1.2
Rpi-oka1					
Rpi-oka2	98%				
Rpi-nrs1	98%	100%			
Rpi-mcq1.1	84%	83%	83%		
Rpi-mcq1.2	83%	82%	82%	87%	
Tm2-2	80%	79%	79%	85%	84%

Calculated amino acid identities were as follows:

	Rpi-oka1	Rpi-oka2	Rpi-nrs1	Rpi-mcq1.1	Rpi-mcq1.2
Rpi-oka1					
Rpi-oka2	98%				
Rpi-nrs1	98%	100%			
Rpi-mcq1.1	76%	75%	75%		
Rpi-mcq1.2	76%	75%	75%	81%	
Tm2-2	72%	71%	71%	77%	75%

The above multiple comparisons were performed, using AlignX (Vector NTI Suite Invitrogen) with an engine based on the CLUSTAL matix.

More generally homology (i.e. similarity or identity) may be as defined using sequence comparisons are made using BestFit and GAP programs of GCG, Wisconsin Package 10.0 from the Genetics Computer Group, Madison, Wisconsin. CLUSTAL is also a matrix used by BestFit. Parameters are preferably set, using the default settings, as follows: Gap Creation pen: 9; Gapext pen: 2. Homology may be at the nucleotide sequence and/or encoded amino acid sequence level. Preferably, the nucleic acid and/or amino acid sequence shares at least about 50%, or 60%, or 70%, or 80% homology, most preferably at least about 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% homology with SEQ. ID. 1a, 1b, 2a, 2b or 3 or 4a, 4b, 5a, 5b, or 6 as appropriate.

In particular the invention provides an isolated Rpi resistance gene having a sequence which is at least about 80% homologous to the nucleic acid sequence provided herein as SEQ. ID. 1a, 1b, 2a, 2b or 3.

It further provides an isolated protein having an amino acid sequence which is at least 80% homologous to the amino acid sequence provided herein as SEQ. ID. 4a, 4b, 5a, 5b, or 6. Thus a variant polypeptide in accordance with the present invention may include within the sequences shown herien, a single amino acid or 2, 3, 4, 5, 6, 7, 8, or 9 changes, about 10, 15, 20, 30, 40 or 50 changes, or greater than about 50, 60, 70, 80, 90, 100, 200, 400 changes. In addition to one or more changes within the amino acid sequence shown, a variant polypeptide may include additional amino acids at the C-terminus and/or N-terminus.

Thus in a further aspect of the invention there is disclosed a method of producing a derivative nucleic acid comprising the step of modifying the coding sequence of a nucleic acid of the present invention e.g. SEQ. ID. 1a, 1b, 2a, 2b or 3.

Changes to a sequence, to produce a derivative, may be by one or more of addition, insertion, deletion or substitution of one or more nucleotides in the nucleic acid, leading to the addition, insertion, deletion or substitution of one or more amino acids in the encoded polypeptide. Changes may be desirable for a number of reasons, including introducing or removing the following features: restriction endonuclease sequences; codon usage; other sites which are required for post translation modification; cleavage sites in the encoded polypeptide; motifs in the encoded polypeptide (e.g. binding sites). Leader or other targeting sequences may be added or removed from the expressed protein to determine its location following expression. All of these may assist in efficiently cloning and expressing an active polypeptide in recombinant form (as described below).

Other desirable mutation may be random or site directed mutagenesis in order to alter the activity (e.g. specificity) or stability of the encoded polypeptide. Changes may be by way of conservative variation, i.e. substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine. As is well known to those skilled in the art, altering the primary structure of a polypeptide by a conservative substitution may not significantly alter the activity of that peptide because the side-chain of the amino acid which is inserted into the sequence may be able to form similar bonds and contacts as the side chain of the amino acid which has been substituted out. This is so even when the substitution is in a region which is critical in determining the peptides conformation.

Also included are variants having non-conservative substitutions. As is well known to those skilled in the art, substitutions to regions of a peptide which are not critical in determining its conformation may not greatly affect its activity because they do not greatly alter the peptide's three dimensional structure.

In regions which are critical in determining the peptides conformation or activity such changes may confer advantageous properties on the polypeptide. Indeed, changes such as those described above may confer slightly advantageous properties on the peptide e.g. altered stability or specificity. For instance, the manipulation of LRR regions of the polypeptides encoded by the nucleic acids of the present invention may allow the production of novel resistance specificities e.g. with respect to *P. infestans* isolates.

LRR regions may also be grafted on to other NBS regions (e.g. from other resistance genes). Thus methods for generating novel specificities may include mixing or incorporating sequences from related resistance genes into the *Rpi* sequences disclosed herein. An alternative strategy for modifying *Rpi* sequences would employ PCR as described below (Ho et al., 1989, Gene *77*, 51-59) or DNA shuffling (Crameri et al., 1998, Nature *391*).

A detailed analysis of some of the ORFs of the present invention is provided in the Examples below, including the existence of variants having substitutions, and identification of regions of interest.

In a further aspect of the present invention there is provided a method of identifying and/or cloning a nucleic acid variant from a plant which method employs a distinctive *Rpi* nucleotide sequence (e.g. as present in SEQ. ID. 1A, 1B, 2A, 2B or 3 or the complement thereof, or degenerate primers based thereon).

An oligonucleotide for use in probing or amplification reactions comprise or consist of about 30 or fewer nucleotides in length (e.g. 18, 21 or 24). Generally specific primers are upwards of 14 nucleotides in length. For optimum specificity and cost effectiveness, primers of 16-24 nucleotides in length may be preferred. Those skilled in the art are well versed in the design of primers for use processes such as PCR. If required, probing can be done with entire restriction fragments of the gene disclosed herein which may be 100's or even 1000's of nucleotides in length.

Preferably the probe/primer is distinctive in the sense that it is present in all or some of the *Rpi* sequences disclosed herein, but not in resistance gene sequences of the prior art.

For instance, the functional allele data presented herein (see e.g. Fig 10 or Fig 11) permits the identification of functional *Rpi* alleles as follows.

In a further embodiment, a variant in accordance with the present invention is also obtainable by means of a method which includes:

(a) providing a preparation of nucleic acid, e.g. from plant cells,

(b) providing a nucleic acid molecule which is a probe as described above,

(c) contacting nucleic acid in said preparation with said nucleic acid molecule under conditions for hybridisation of said nucleic acid molecule to any said gene or homologue in said preparation, and identifying said gene or homologue if present by its hybridisation with said nucleic acid molecule.

Probing may employ the standard Southern blotting technique. For instance DNA may be extracted from cells and digested with different restriction enzymes. Restriction fragments may then be separated by electrophoresis on an agarose gel, before denaturation and transfer to a nitrocellulose filter or nylon. Labelled probe may be hybridised to the DNA fragments on the filter and binding determined. DNA for probing may be prepared from RNA preparations from cells.

Test nucleic acid may be provided from a cell as genomic DNA, cDNA or RNA, or a mixture of any of these, preferably as a library in a suitable vector. If genomic DNA is used the probe may be used to identify untranscribed regions of the gene (e.g. promoters etc.), such as is described hereinafter. Probing may optionally be done by means of so-called "nucleic acid chips" (see Marshall & Hodgson (1998) Nature Biotechnology 16: 27-31, for a review).

Preliminary experiments may be performed by hybridising under low stringency conditions. For probing, preferred conditions are those which are stringent enough for there to be a simple pattern with a small number of hybridisations identified as positive which can be investigated further.

For instance, screening may initially be carried out under conditions, which comprise a temperature of about 37°C or less, a formamide concentration of less than about 50%, and a moderate to low salt (e.g. Standard Saline Citrate ("SSC") = 0.15 M sodium chloride; 0.15 M sodium citrate; pH 7) concentration.

Alternatively, a temperature of about 50°C or less and a high salt (e.g. "SSPE" 0.180 mM sodium chloride; 9 mM disodium hydrogen phosphate; 9 mM sodium dihydrogen phosphate; 1 mM sodium EDTA; pH 7.4). Preferably the screening is carried out at about 37°C, a formamide concentration of about 20%, and a salt concentration of about 5 X SSC, or a temperature of about 50°C and a salt concentration of about 2 X SSPE. These conditions will allow the identification of sequences which have a substantial degree of homology (similarity, identity) with the probe sequence, without requiring the perfect homology for the identification of a stable hybrid.

Suitable conditions include, e.g. for detection of sequences that are about 80-90% identical, hybridization overnight at 42°C in 0.25M Na_2HPO_4, pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 55°C in 0.1X SSC, 0.1% SDS. For detection of sequences that are greater than about 90% identical, suitable conditions include hybridization overnight at 65°C in 0.25M Na_2HPO_4, pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 60°C in 0.1X SSC, 0.1% SDS.

It is well known in the art to increase stringency of hybridisation gradually until only a few positive clones remain. Suitable conditions would be achieved when a large number of hybridising fragments were obtained while the background hybridisation was low.

Using these conditions nucleic acid libraries, e.g. cDNA libraries representative of expressed sequences, may be searched. Those skilled in the art are well able to employ suitable conditions of the desired stringency for selective hybridisation, taking into account factors such as oligonucleotide length and base composition, temperature and so on. One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology is (Sambrook et al., 1989):

T_m = 81.5°C + 16.6Log [Na+] + 0.41 (% G+C) - 0.63 (% formamide) - 600/#bp in duplex. As an illustration of the above formula, using [Na+] = [0.368] and 50-% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C. Such a sequence would be

considered substantially homologous to the nucleic acid sequence of the present invention.

Binding of a probe to target nucleic acid (e.g. DNA) may be measured using any of a variety of techniques at the disposal of those skilled in the art. For instance, probes may be radioactively, fluorescently or enzymatically labelled. Other methods not employing labelling of probe include amplification using PCR (see below) or RN'ase cleavage. The identification of successful hybridisation is followed by isolation of the nucleic acid which has hybridised, which may involve one or more steps of PCR or amplification of a vector in a suitable host.

Thus one embodiment of this aspect of the present invention is nucleic acid including or consisting essentially of a sequence of nucleotides complementary to a nucleotide sequence hybridisable with any encoding sequence provided herein. Another way of looking at this would be for nucleic acid according to this aspect to be hybridisable with a nucleotide sequence complementary to any encoding sequence provided herein. Of course, DNA is generally double-stranded and blotting techniques such as Southern hybridisation are often performed following separation of the strands without a distinction being drawn between which of the strands is hybridising. Preferably the hybridisable nucleic acid or its complement encode a product able to influence a resistance characteristic of a plant, particularly an *Rpi*-resistance response.

In a further embodiment, hybridisation of nucleic acid molecule to a variant may be determined or identified indirectly, e.g. using a nucleic acid amplification reaction, particularly the polymerase chain reaction (PCR)(see "PCR protocols; A Guide to Methods and Applications", Eds. Innis et al, Academic Press, New York, (1990)).

The methods described above may also be used to determine the presence of one of the nucleotide sequences of the present invention within the genetic context of an individual plant. This may be useful in plant breeding programmes e.g. to directly select plants containing alleles which are responsible for desirable traits in that plant species, either in parent plants or in progeny (e.g hybrids, F1, F2 etc.).

As used hereinafter, unless the context demands otherwise, the term "*Rpi* nucleic acid" is intended to cover any of the nucleic acids of the invention described above, including functional variants.

In one aspect of the present invention, the *Rpi* nucleic acid described above is in the form of a recombinant and preferably replicable vector. "Vector" is defined to include, inter alia, any plasmid, cosmid, phage or *Agrobacterium* binary vector in double or single stranded linear or circular form which may or may not be self

transmissible or mobilizable, and which can transform prokaryotic or eukaryotic host either by integration into the cellular genome or exist extrachromosomally (e.g. autonomous replicating plasmid with an origin of replication). Specifically included are shuttle vectors by which is meant a DNA vehicle capable, naturally or by design, of replication in two different host organisms, which may be selected from actinomycetes and related species, bacteria and eucaryotic (e.g. higher plant, yeast or fungal cells).

A vector including nucleic acid according to the present invention need not include a promoter or other regulatory sequence, particularly if the vector is to be used to introduce the nucleic acid into cells for recombination into the genome.

Preferably the nucleic acid in the vector is under the control of, and operably linked to, an appropriate promoter or other regulatory elements for transcription in a host cell such as a microbial, e.g. bacterial, or plant cell. The vector may be a bi-functional expression vector which functions in multiple hosts. In the case of genomic DNA, this may contain its own promoter or other regulatory elements and in the case of cDNA this may be under the control of an appropriate promoter or other regulatory elements for expression in the host cell.

By "promoter" is meant a sequence of nucleotides from which transcription may be initiated of DNA operably linked downstream (i.e. in the 3' direction on the sense strand of double-stranded DNA). "Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter. DNA operably linked to a promoter is "under transcriptional initiation regulation" of the promoter.

Thus this aspect of the invention provides a gene construct, preferably a replicable vector, comprising a promoter operatively linked to a nucleotide sequence provided by the present invention, such as SEQ. ID. 1a, 1b, 2a, 2b or 3 or a variant thereof.

Generally speaking, those skilled in the art are well able to construct vectors and design protocols for recombinant gene expression. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual:* 2nd edition, Sambrook *et al,* l989, Cold Spring Harbor Laboratory Press (or later editions of this work).

Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis (see above discussion in respect of variants), sequencing, introduction of DNA into cells and

gene expression, and analysis of proteins, are described in detail in *Current Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992. The disclosures of Sambrook et al. and Ausubel et al. are incorporated herein by reference.

In one embodiment of this aspect of the present invention, there is provided a gene construct, preferably a replicable vector, comprising an inducible promoter operatively linked to a nucleotide sequence provided by the present invention. The term "inducible" as applied to a promoter is well understood by those skilled in the art. In essence, expression under the control of an inducible promoter is "switched on" or increased in response to an applied stimulus. The nature of the stimulus varies between promoters. Some inducible promoters cause little or undetectable levels of expression (or no expression) in the absence of the appropriate stimulus. Other inducible promoters cause detectable constitutive expression in the absence of the stimulus. Whatever the level of expression is in the absence of the stimulus, expression from any inducible promoter is increased in the presence of the correct stimulus.

Particular of interest in the present context are nucleic acid constructs which operate as plant vectors. Specific procedures and vectors previously used with wide success upon plants are described by Guerineau and Mullineaux (1993) (Plant transformation and expression vectors. In: Plant Molecular Biology Labfax (Croy RRD ed) Oxford, BIOS Scientific Publishers, pp 121-148).

Suitable promoters which operate in plants include the Cauliflower Mosaic Virus 35S (CaMV 35S). Other examples are disclosed at pg.120 of Lindsey & Jones (1989) "Plant Biotechnology in Agriculture" Pub. OU Press, Milton Keynes, UK. The promoter may be selected to include one or more sequence motifs or elements conferring developmental and/or tissue-specific regulatory control of expression. Inducible plant promoters include the ethanol induced promoter of Caddick et al (1998) Nature Biotechnology 16: 177-180.

Purely by way of example, SEQ. ID. 1c, 2c and 2d show the nucleotide sequences of oka1, mcq1.1 and mcq1.2 and include promoter and terminator sequences that may be used in contstructs used to transform both potato and tomato.

It may be desirable to use a strong constitutive promoter. If desired, selectable genetic markers may be included in the construct, such as those that confer selectable phenotypes such as resistance to antibiotics or herbicides (e.g. kanamycin, hygromycin, phosphinotricin, chlorsulfuron, methotrexate, gentamycin, spectinomycin, imidazolinones and glyphosate).

The present invention also provides methods comprising introduction of such a construct into a host cell, particularly a plant cell.

In a further aspect of the invention, there is disclosed a host cell containing a heterologous construct according to the present invention, especially a plant or a microbial cell. The term "heterologous" is used broadly in this aspect to indicate that the gene/sequence of nucleotides in question (an *Rpi* gene) have been introduced into said cells of the plant or an ancestor thereof, using genetic engineering, i.e. by human intervention. A heterologous gene may replace an endogenous equivalent gene, i.e. one which normally performs the same or a similar function, or the inserted sequence may be additional to the endogenous gene or other sequence.

Nucleic acid heterologous to a plant cell may be non-naturally occurring in cells of that type, variety or species. Thus the heterologous nucleic acid may comprise a coding sequence of or derived from a particular type of plant cell or species or variety of plant, placed within the context of a plant cell of a different type or species or variety of plant. A further possibility is for a nucleic acid sequence to be placed within a cell in which it or a homolog is found naturally, but wherein the nucleic acid sequence is linked and/or adjacent to nucleic acid which does not occur naturally within the cell, or cells of that type or species or variety of plant, such as operably linked to one or more regulatory sequences, such as a promoter sequence, for control of expression.

The host cell (e.g. plant cell) is preferably transformed by the construct, which is to say that the construct becomes established within the cell, altering one or more of the cell's characteristics and hence phenotype e.g. with respect to *P. infestans.*

Nucleic acid can be transformed into plant cells using any suitable technology, such as a disarmed Ti-plasmid vector carried by Agrobacterium exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, NAR 12(22) 8711 - 87215 1984), particle or microprojectile bombardment (US 5100792, EP-A-444882, EP-A-434616) microinjection (WO 92/09696, WO 94/00583, EP 331083, EP 175966, Green *et al.* (1987) *Plant Tissue and Cell Culture*, Academic Press), electroporation (EP 290395, WO 8706614 Gelvin Debeyser) other forms of direct DNA uptake (DE 4005152, WO 9012096, US 4684611), liposome mediated DNA uptake (e.g. Freeman *et al. Plant Cell Physiol.* 29: 1353 (1984)), or the vortexing method (e.g. Kindle, *PNAS U.S.A.* 87: 1228 (1990d) Physical methods for the transformation of plant cells are reviewed in Oard, 1991, *Biotech. Adv.* 9: 1-11.

Purely by way of example, transformation strategies for potato, tomato, and tobacco are set out in Example 6 hereinafter. Other strategies, particularly those applicable to the genus *Solanum,* are well known to those skilled in the art (see e.g.

Mansure and Magioli, Acta Botanica Brasilica, 2005 (Vol. 19) (No. 1) 139-148). The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the experience and preference of the person practising the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce nucleic acid into plant cells is not essential to or a limitation of the invention, nor is the choice of technique for plant regeneration. Thus a further aspect of the present invention provides a method of transforming a plant cell involving introduction of a construct as described above into a plant cell and causing or allowing recombination between the vector and the plant cell genome to introduce a nucleic acid according to the present invention into the genome.

The invention further encompasses a host cell transformed with nucleic acid or a vector according to the present invention especially a plant or a microbial cell. In the transgenic plant cell (i.e. transgenic for the nucleic acid in question) the transgene may be on an extra-genomic vector or incorporated, preferably stably, into the genome. There may be more than one heterologous nucleotide sequence per haploid genome.

Generally speaking, following transformation, a plant may be regenerated, e.g. from single cells, callus tissue or leaf discs, as is standard in the art. Almost any plant can be entirely regenerated from cells, tissues and organs of the plant. Available techniques are reviewed in Vasil et al., *Cell Culture and Somatic Cell Genetics of Plants, Vol I, II and III, Laboratory Procedures and Their Applications*, Academic Press, 1984, and Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academic Press, 1989.

Plants which include a plant cell according to the invention are also provided.

In addition to the regenerated plant, the present invention embraces all of the following: a clone of such a plant, selfed or hybrid progeny and descendants (e.g. F1 and F2 descendants) and any part of any of these. The invention also provides parts of such plants e.g. any part which may be used in reproduction or propagation, sexual or asexual, including cuttings, seed and so on, or which may be a commodity *per se* e.g. tuber.

The invention further provides a method of influencing or affecting the degree of resistance of a plant to a pathogen, particularly *Phytophthora infestans*, more particularly to any of the isolates discussed herein, the method including the step of causing or allowing expression of a heterologous nucleic acid sequence as discussed above within the cells of the plant.

The step may be preceded by the earlier step of introduction of the nucleic acid into a cell of the plant or an ancestor thereof.

Preferred plants for transformation are of the family Solanaceae, more preferably genus *Solanum.* Optionally the plant may be S. tuberosum or S. lycopersicum

The methods may also include the manipulation of other genes e.g. which may be involved in transduction of the resistance signal, or in generating a resistance response.

Thus provided are methods of influencing or affecting the degree of resistance of a plant to *P. infestans,* the method including the step of causing or allowing expression of a heterologous nucleic acid as described above within the cells of the plant.

In preferred methods more than one Rpi gene is introduced into the plant. In other strategies, a plurality of plants is provided each having a different endogenous or heterologous Rpi gene (wherein at least one of said plants includes a heterologous Rpi gene of the present invention i.e. has been generated by the technical methods described above). The plurality of plants may be planted together in a single area such as to maximise the extent or durability of the crop's resistance to *P. infestans.* Alternatively the plurality of plants may be planted successively in the area (e.g. on a rotation) to achieve the same effect.

The foregoing discussion has been generally concerned with uses of the nucleic acids of the present invention for production of functional Rpi polypeptides in a plant, thereby increasing its pathogen resistance. Purely for completeness it is noted that the information disclosed herein may also be used to reduce the activity or levels of such polypeptides in cells in which it is desired to do so (e.g. in an experimental model). Nucleic acids and associated methodologies for carrying out down-regulation (e.g. complementary sequences) form one part of the present invention.

As noted above the present invention also encompasses the expression product of any of the *Rpi* (particularly functional *Rpi*) nucleic acid sequences disclosed above, plus also methods of making the expression product by expression from encoding nucleic acid therefore under suitable conditions, which may be in suitable host cells.

A preferred polypeptide includes the amino acid sequence shown in SEQ. ID. 4a, 4b, 5a, 5b, or 6. However a polypeptide according to the present invention may be a variant (allele, fragment, derivative, mutant or homologue etc.) of these polypeptides.

Also encompassed by the present invention are polypeptides which although clearly related to a functional Rpi polypeptides (e.g. they are immunologically cross reactive with the polypeptide, or they have characteristic sequence motifs in common with the polypeptide) no longer have *Rpi* function.

Following expression, the recombinant product may, if required, be isolated from the expression system. Generally however the polypeptides of the present invention will be used *in vivo* (in particular *in planta*).

Purified Rpi or variant proteins of the invention, produced recombinantly by expression from encoding nucleic acid therefor, may be used to raise antibodies employing techniques which are standard in the art. Methods of producing antibodies include immunising a mammal (e.g. mouse, rat, rabbit, horse, goat, sheep or monkey) with the protein or a fragment thereof. Antibodies may be obtained from immunised animals using any of a variety of techniques known in the art, and might be screened, preferably using binding of antibody to antigen of interest. For instance, Western blotting techniques or immunoprecipitation may be used (Armitage et al, 1992, Nature 357: 80-82). Antibodies may be polyclonal or monoclonal. As an alternative or supplement to immunising a mammal, antibodies with appropriate binding specificity may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, e.g. using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047.

Antibodies raised to a polypeptide or peptide can be used in the identification and/or isolation of homologous polypeptides, and then the encoding genes. Thus, the present invention provides a method of identifying or isolating a polypeptide with *Rpi* function (in accordance with embodiments disclosed herein), including screening candidate peptides or polypeptides with a polypeptide including the antigen-binding domain of an antibody (for example whole antibody or a fragment thereof) which is able to bind an Rpi peptide, polypeptide or fragment, variant or variant thereof or preferably has binding specificity for such a peptide or polypeptide, such as having an amino acid sequence identified herein.

Specific binding members such as antibodies and polypeptides including antigen binding domains of antibodies that bind and are preferably specific for polypeptides of the sequence SEQ. ID. 4a, 4b, 5a, 5b, or 6 or a mutant, variant or derivative thereof represent further aspects of the present invention, as do their use and methods which employ them.

The above description has generally been concerned with the translated and coding parts of *Rpi* genes. Also embraced within the present invention are untranscribed parts (UTRs) of the genes.

Thus a further aspect of the invention is an isolated nucleic acid molecule encoding the promoter, or other UTR (3' or 5'), of an *Rpi* gene described herein.

As noted above, SEQ. ID. 1c, 2c and 2d show the nucleotide sequences of oka1, mcq1.1 and mcq1.2 and include promoter and terminator sequences that may be used in contstructs used to transform both potato and tomato.

In summary, it can be seen that the present inventors have isolated, identified and characterised several different late blight *R* genes derived from the potato wild species *S. okadae* plus also from S. *mochiquense* and *S. neorossii.* Accordingly this invention provides novel gene sequences, compositions and methods for enhancing the resistance in crops, in particular but not limited to, potato, to late blight caused by the oomycete pathogen *Phytophthora infestans.*

In order to clone these late blight *R* genes a variety of methodologies were innovatively combined. As set out in more detail in the Examples below we constructed two BAC libraries from genomic DNA of the two species. In this patent disclosure, we describe the construction and analysis of the two BAC libraries. Furthermore, we identify and characterize BAC clones linked to late blight *R* genes using PCR-based markers developed during preliminary mapping experiments. This process has facilitated fine-scale mapping of the *R* genes, chromosome walking toward the target genes, physical mapping and finally gene cloning.

Construction of libraries with large genomic DNA inserts is one of the essential steps for map-based gene cloning strategies. Several methods have been developed for the construction of libraries, including yeast artificial chromosome (YAC), P1-derived artificial chromosome (PAC), plasmid-based clone (PBC), plant transformation-competent artificial chromosome (TAC), bacterial artificial chromosome (BAC) and binary bacterial artificial chromosome (BIBAC) (cited in Feng et al. 2006). During the last few years, BAC libraries have been constructed from a wide variety of plant species including the staple crops rice, wheat and potato (Tao et al. 2002; Nilmalgoda et al. 2003; Chen et al. 2004), and other species such as peach, garlic, banana, sugar beet, soybean, peanut and sunflower (Georgi et al. 2002; Lee et al. 2003; Vilarinhos et al. 2003; McGrath et al. 2004; Wu et al. 2004; Yüksel et al. 2005; Bouzidi et al. 2006; Feng et al. 2006).

We have been working to isolate genes from wild species *S. okadae* and *S. mochiquense* conferring resistance to late blight in potato using a map-based gene cloning approach. The gene derived from *S. mochiquense* has already been reported,

albeit that it was not previously isolated (Smilde et al. 2005) and recently the genes derived from *S. okadae* have been identified (Foster et al. unpublished data). As a step towards map-based cloning of these *R* genes, we constructed two BAC libraries from K39 containing *Rpi-oka1* and *Rpi-oka2* and K182 containing *Rpi-mcq1*. To construct high-quality BAC libraries, it is crucial to optimize partial digestion conditions and to accurately size-select partially digested DNA fragments. Smaller fragments often produce smaller insert clones with higher transformation efficiency, but larger fragments often result in higher percentages of clones lacking inserts and lower transformation efficiency (Feng et al. 2006). In this study, fragments of 100-200 Kb were selected for the BAC libraries.

The size of the haploid *Solanum* species ranges from 800 Mb to 1,200 Mb depending on species. Arumuganathan and Earle (1991) reported that the haploid genome size of *S. berthaultii* is 840 Mb and that of *S. tuberosum* is 800-930 Mb. In the present study, a total of 105,216 and 100,992 BAC clones with average insert sizes of 103.5 Kb and 85.5 Kb were obtained for the K39 and K182 libraries, respectively. Assuming a potato haploid genome size of 1,000 Mb, we estimate that these libraries contain approximately 10.9 and 8.6 genome equivalents for the K39 and K182 libraries, respectively. Although we selected the DNA fragments in the range of 100-200 Kb, the average insert sizes of both libraries is smaller than expected. This discrepancy has been observed by others (Danesh et al. 1998; Meksem et al. 2000; Yüksel and Paterson 2005) and could be caused by the presence of smaller fragments that were not fully removed in the size-selection steps as suggested by Frijters et al. (1997). Given the genome coverage, we expected that all regions of the genome should be well represented. We tested this using PCR-based markers known to be linked to the *R* genes *Rpi-oka1, Rpi-oka2* and *Rpi-mcq1*.

In order to minimize the number of PCR reactions required, we used a pooling strategy for screening of the libraries. Previously several different pooling strategies have been employed for screening BAC libraries (Klein et al. 2000; Ozdemir et al. 2004; Bouzidi et al. 2006). In our study, we used a plate pooling strategy combined with a column and row pooling strategy within plate pools. Each 384-well plate was pooled and plasmid DNA from each pool was prepared. Based on the genome equivalents of each library, theoretically we expected that 11 and 9 pools would be positive to a particular marker and that half of these pools, after digestion with restriction enzymes to identify resistant allele-specific markers would contain BAC clones from the haplotype corresponding to each gene.

The positive BAC clones in the K39 and K182 libraries to PCR-based markers were consistent with or slightly better than estimated genome equivalents and were

identified with average numbers of 15 and 12.5, respectively. Both are slightly more than numbers expected based on estimate of the genome equivalents. These could be caused by over-estimation of the potato haploid genome size or under-estimation of the average insert sizes of the BAC clones obtained. On the other hand, the BAC libraries we constructed could be biased due to an over- or under-representation of *Hind*III sites within our region of interest. In order to achieve better representation, others have used two or three different restriction enzymes, rich in either A/T or G/C when they constructed BAC libraries (Chang et al. 2001; Tao et al. 2002; Chen et al. 2004).

Based on the results of BAC screening with PCR markers linked to the *Rpi* genes, we sequenced the BAC-ends of eight single BAC clones for each library. Of these, one identified from each of the K39 and K182 libraries was similar to *Tm-2*^{2}, the tomato mosaic virus *R* gene on tomato chromosome 9 (Lanfermeijer et al. 2003). Additionally two other BAC end sequences from the K182 library were similar to several different resistance proteins. These results combined with the genetic linkage maps of *Rpi-oka1*, *Rpi-oka2* and *Rpi-mcq1* constructed in our complementary researches (Foster et al. unpublished; Zhu et al. unpublished) indicated that we had identified BAC clones that covered the genomic region containing the genes.

Large-insert BAC libraries are a valuable tool for chromosome walking, BAC contig construction and physical mapping in regions containing *R* genes. Although we haven't yet identified the precise physical location of the *R* genes, as shown in the results of BAC screening by the PCR-based approach and BAC-end sequences of selected BAC clones, the construction of BAC libraries covering 10.9- and 8.6-fold of the potato haploid genome from *S. okadae* and *S. mochiquense* has facilitated the cloning of the *Rpi* genes and will be of value for further potato genomic studies which require map-based cloning steps.

Having generally disclosed this invention, including methods of making and using compositions useful in conferring late blight resistance, the following examples are provided to further the written description and fully enable this invention, including its best mode and equivalents thereof. However, those skilled in the art will appreciate that the invention which these examples illustrate is not limited to the specifics of the examples provided here. Rather, for purposes of apprehending the scope of this invention, attention should be directed to the claims appended to this disclosure.

EXAMPLES

EXAMPLE 1

CONSTRUCTION OF BAC LIBRARIES FROM THE WILD POTATO SPECIES *SOLANUM OKADAE* AND *SOLANUM MOCHIQUENSE* AND THE IDENTIFICATION OF CLONES NEAR LATE BLIGHT RESISTANCE LOCI

a. Plant materials

The pedigrees of the plants used to construct the two BAC libraries are shown in Fig. 1. The *S. okadae* plant K39 is a transheterozygote carrying both *Rpi-oka1* originally from the parent A618 and *Rpi-oka2* from A624 (Foster et al. unpublished data). The *S. mochiquense* plant K182 is heterozygous for *Rpi-mcq1* (formerly named *Rpi-moc1*; Smilde et al. 2005) and was obtained from a BC1 population.

b. Preparation of high-molecular-weight insert DNA

A method used for high-molecular-weight (HMW) DNA preparation was slightly modified from Liu and Whittier (1994) and Chalhoub et al. (2004). Plant materials were grown on Murashige and Skoog (MS) medium without sucrose *in vitro* and young leaf tissues were harvested and stored at -80 °C. Twenty grams frozen leaf tissue was used to prepare DNA plugs containing HMW DNA. The DNA plugs were prepared in 0.7 % inCert agarose (Biozym, Oldendorf, Germany), washed in lysis buffer solution (1 % sodium lauryl sarcosine, 0.2 mg/ml proteinase K and 3.8 mg/ml sodium diethyldithiocarbamate dissolved in 0.5 M EDTA, pH 8.5) and stored at 4 °C in 0.5 M EDTA until required without decreasing DNA quality as suggested by Osoegawa et al. (1998). The stored plugs were soaked in TE buffer, chopped into small pieces and partially digested with 5 units of *Hind*III for 1 hour based on the results of prior optimisation experiments which showed that these conditions generated DNA of a size range 50-300 kb.

Triple size selection was used to improve the size and uniformity of the inserts as described in Chalhoub et al. (2004). The first size selection was performed on 1 % Seakem LE agarose (Biozym, Oldendorf, Germany) using clamped homogeneous electric field (CHEF) pulsed field gel electrophoresis (Bio-rad, Hercules, USA) at 1-40 seconds, 120°, 16 hours and 200 V in 0.25x TBE buffer directly followed by the second size selection in the same gel at 4-5 seconds, 120°, 6 hours and 180 V in the same buffer. The regions of gel containing partially digested DNA between 100 and 200 Kb were excised and divided into two. For the third size selection, the excised gel slices were separately run on 1 % Sea Plaque GTG Low-

melting point agarose (Biozym, Oldendorf, Germany) at 3-4.5 seconds, 120°, 14 hours and 180 V. Size-selected DNA fragments were excised from the gel and stored at 4 °C in 0.5 M EDTA (pH 8). DNA was recovered in 40 µl 1x TAE buffer by electro-elution using a BioRad Electro-elution system (Bio-rad, Hercules, USA).

c. BAC library construction

Ligation and transformation were performed according to the methods described in Allouis et al. (2003) and Chalhoub et al. (2004) with some modification. The total eluted DNA from the size-selected DNA fragment was ligated in a 100 µl reaction with 10 ng pIndigoBAC-5 vector (EpiCentre Biotechnologies, Madison, USA) and 800 U T4 DNA ligase (New England Biolabs, Ipswich, USA). The ligation was dialysed on 0.5 x TE buffer for 3 hours using Millipore membrane (Millipore, Billerica, USA). Three microliter of dialysed ligation was mixed with 20 µl ElectroMax DH10B electrocompetent cells (Invitrogen, Paisley, UK), incubated for 1 minute on ice and electroporated at 180 V, 200 ohms and 25 µF. Transformed cells were recovered in 1 ml of SOC medium (Invitrogen, Paisley, UK), incubated at 37 °C for 1 hour, plated on selective LB medium with 17 µg/ml chloramphenicol, 125 µg/ml IPTG (isopropylthio-β-D-galactoside) and 100 µg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and grown at 37 °C overnight. White colonies were picked into 384-well microtiter plates (Genetix Ltd., Dorset, UK) containing Freezing broth (1 % Tryptone, 0.5 % Yeast Extract, 0.5 % NaCl, 0.63 % K_2HPO_4, 0.045 % Sodium Citrate, 0.009 % $MgSO_4$, 0.09 % $(NH_4)_2SO_4$, 0.18 % KH_2PO_4, 4.4 % Glycerol and 17 µg/ml chloramphenicol, pH 7.2) using a Q-Pix instrument (Genetix Ltd., Dorset, UK), incubated at 37 °C overnight and stored at -80 °C.

d. BAC insert sizing

To determine the insert size of the BAC clones, randomly selected BAC clones were cultured in 3 ml LB containing 17 µg/ml chloramphenicol at 37 °C overnight. BAC DNA was isolated using the method slightly modified from the Qiagen plasmid midi kit (Qiagen Ltd, Crawley, UK) and digested with *Not*I for 3 hours to release the insert DNA from the vector. Digested DNA was separated on a 1 % agarose gel using CHEF gel electrophoresis (Bio-rad, Hercules, USA) at 5-15 seconds, 120°, 16 hours and 200 V in 0.5x TBE buffer.

e. BAC library screening and BAC clone characterization

The BAC clones stored in separate 384-well plate were pooled and plasmid DNA from each pool was prepared. The pooled-DNA was screened with eight PCR-based markers (Table 1a) known to be linked to the identified *Rpi* genes. Once positive pools were identified using particular marker primers, the original 384-well library plate of the library was replicated onto solid LB medium using a high density replicator tool and rows and columns of clones were screened by PCR using the same primers to select single positive clones. Selected positive clones were BAC-end sequenced using Big Dye v. 3.1 cycle sequencing reagents (Applied Biosystems, Foster City, USA). Sequencing reactions were run on an ABI 3730 at the John Innes Centre Genome Laboratory (Norwich, UK).

In addition, the pooled plasmid DNA from the BAC pools of the K39 library was spot-blotted onto Hybond-N+ membrane and probed by hybridisation with ^{32}P-labelled okaNBS-Hae marker as a probe. The 384-well BAC plates corresponding to the pools identified using this probe were then double spotted onto Hybond-N+ membrane and hybridised to the same probe to identify individual BAC clones from the pools. BAC DNA was isolated from identified BAC clones and subjected to SNaPshot fingerprinting to construct contigs from BACs containing sequences homologous to the probe. Selected BAC clones which were positive by PCR using selected marker primers (TG551 and TG35) were also included in the SNaPshot analysis.

f. Results

BAC library construction and characterization

With the goal of isolating potato late blight *R* genes, we constructed two BAC libraries from two plants, K39 and K182 (Fig. 1). Results of outcrosses with a susceptible *S. okadae* genotype indicate that K39 is transheterozygous for *Rpi-oka1* and *Rpi-oka2.* Analysis of the phenotype and genotypes of plants from the K182 pedigree indicate that K182 is heterozygous for *Rpi-mcq1.*

Two BAC libraries were constructed from the *Hin*dIII partially digested potato DNA. The libraries from K39 and K182 consisted of 105,216 and 100,992 clones stored in 274 x 384- and 263 x 384-well microtiter plates, respectively. Average insert sizes were estimated based on pulsed-field gel analysis of *Not*I digested DNA from 38 and 40 randomly selected clones from the K39 and K182 libraries, respectively. The patterns of *Not*I digested clones from the two libraries are shown in Fig. 2. The

estimated insert sizes ranged from 60 to 165 Kb with an average of 103.5 Kb for the K39 library and from 50 to 130 Kb with an average of 85.5 Kb for the K182 library. The haploid genome size of potato is estimated to be about 1,000 Mb, therefore the genome equivalents are predicted to be 10.9 X and 8.6 X for the K39 and K182 libraries, respectively.

EXAMPLE 2

IDENTIFICATION, MAPPING AND CLONING OF *Rpi* GENES FROM *Solanum okadae* and *S. neorossii*

a. Plant growth conditions

Seed of 12 *Solanum okadae* and 4 *S. neorossii* accessions (Table 1b) was obtained from the Centre for Genetics Resources in Wageningen, the Netherlands (CGN). Seed was surface sterilised in 70 % ethanol for 1 minute, disinfected with 1.5 % hypochlorite for 5 minutes, rinsed 3 times in sterile distilled water and placed on solid MS (murashige and Skoog) medium (2 % agarose) containing 3 % sucrose for germination. Germinated seedlings were transferred to glasshouse facilities and treated regularly with fungicides and pesticides to control thrips, aphids, spider mites, powdery mildew and early blight (*Alternaria solani*).

b. *Phytophthora infestans* strains, inoculation and pathotest scoring

P. infestans isolate 98.170.3 (race 1.3.4.10.11; Smilde et al. 2005) was provided by Dr David Shaw at Bangor University, UK. Isolates 90128 (race 1.3.4.7.8.9.10.11), IPO-complex (race 1.2.3.4.6.7.10.11), IPO-0 (virulence spectrum unknown) and EC1 (race 3.4.7.11) were provided by Dr Edwin van der Vossen at Plant Research International, Wageningen, The Netherlands. The 'SuperBlight' isolate was provided by Dr Paul Birch, SCRI, Dundee, UK and is an isolate currently virulent on a large number of commercially grown potato cultivars in the UK and Europe. Isolates MP324, MP717, MP778, MP674, MP622, MP618 and MP650 were obtained from IHAR, Poland.

The isolates were maintained at 18°C on Rye B agar. Fresh sporangia were produced in a two-weekly cycle by sub-culturing to fresh plates. Periodically, the ability of isolates to infect host material was confirmed on detached leaves of a suitable, sensitive plant. Mature, fresh sporangia were harvested after 10 days growth on Rye B medium by flooding the plate with sterile deionised water and

allowing the harvested spore suspension to stand for 20 minutes in a fresh Petri dish. After this time most sporangia are stuck to the plastic surfaces of the dish. Water from the original suspension was replaced by fresh cold water, the sporangia re-suspended and incubated at 4 °C for 1 to 4 hours to induce zoospore release.

A detached leaf assay was used to screen for resistance to *P. infestans* (modified from (Vleeshouwers et al. 1999)). Two leaves per plant were detached, inserted in a small portion of wet florist sponge and placed in a 9 cm Petri dish. Leaves were inoculated with 10 µl droplets of a zoospore suspension (20,000 to 50,000 zoospores ml^{-1}) and the inoculum gently spread over the abaxial leaf surface with an artist's brush. Petri dishes were wrapped in plastic film and incubated for 7 to 12 days under controlled environmental conditions (18°C; 18 h light/6 h dark cycle) before scoring phenotypes. Plants with leaves showing sporulating lesions were scored as susceptible; plants with leaves showing no visible symptoms or necrosis in the absence of sporulation were scored as resistant. When the two leaves did not show the same reaction, the plant phenotype was considered intermediate (weak resistance). To confirm these intermediate phenotypes, at least three independent inoculations were carried out. For clear cut phenotypes (either both leaves resistant or both sensitive), two independent rounds of inoculations were considered sufficient.

c. DNA isolation

DNA was isolated from plant material using either the DNeasy 96 Plant kit (Qiagen) or the protocol of (Park et al. 2005). Briefly approximately 50 mg of leaf material was harvested into 250 µl of nuclear lysis buffer (200 mM Tris-HCl pH 7.5, 50 mM EDTA, 2 M NaCl, 2 % CTAB) to which 200 µl of DNA extraction buffer (100 mM Tris-HCl pH 7.5, 350 mM sorbitol, 20 mM sodium bisulfite) was added. The leaf material was then disrupted using a Retsch MM300 milling machine with two 3 mm steel ball bearings for each sample and incubated at 65 °C for 1 hour. Two hundred and fifty microlitres of ice cold chloroform was added, the samples mixed and centrifuged at 3500 rpm for 10 minutes. The supernatant was transferred to a fresh tube and the DNA precipitated by the addition of an equal volume of isopropanol followed by centrifugation at 3500 rpm for 60 min. Precipitated DNA was air dried and resuspended in 100 µl TE.

d. AFLP and SSR analysis and PCR-based mapping

AFLP was performed essentially as described in (Thomas et al. 1995) and (Vos et al. 1995) on *Pst*I/*Mse*I-digested template DNA using a pre-amplification step with *Pst*I+0 and *Mse*I+1 primers and a selective amplification step using *Pst*I+2 and *Mse*I+3 primers. AFLP reaction products were denatured and separated by electrophoresis on a 4.5 % acrylamide/7.5 M urea/0.5 x TBE (45 mM Tris-borate, 1 mM EDTA) gel run at 100 W for 2.5 h. After electrophoresis, gels were transferred to Whatman 3 MM paper, dried without fixing and exposed to X-ray film (X-OMAT AR, Kodak) for 1-7 days.

Informative AFLP bands were cut from the gel and rehydrated in TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). The gel slices were then transferred to fresh TE, crushed and the debris removed by centrifugation at 14000g for 1 min. For cloning, AFLP fragments were first re-amplified by PCR using 2 µl of the supernatant and the same cycling conditions and primers as for the original amplification. Resulting products were cloned into pGEM-T Easy (Promega, Madison, Wisc.) following the manufacturer's instructions and sequenced using the ABI PRISM Big Dye (v. 3.1) Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems) according to the manufacturer's instructions.

SSR PCR reactions were done in 25 µl reaction volumes containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.4 mM each of dCTP, dTTP and dGTP, 0.012 mM non-labelled dATP, 370 kbq [γ-^{33}P)]dATP (Amersham Biosciences,), 0.4 µM of each primer, 1 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) and 100 ng template DNA. Thermal cycling conditions consisted of an initial denaturation step at 94 °C for 4 min, followed by a primer annealing step (either 50 °C or 55 °C depending upon the primer pair used; see Table 2) for 2 min and an extension step at 72 °C for 90s. Subsequent cycles were as follows: 29 cycles of 94 °C for 1 min, primer annealing temperature for 2 min, 72 °C for 90s, followed by a final extension step of 72 °C for 5 min. Amplification products were denatured by the addition of an equal amount of stop solution (95 % formamide containing bromophenol blue and xylene cyanol) and heated to 98 °C for 10 min. Two to five microlitres of the reaction were run on 6 % denaturing polyacrylamide gels containing 6 M urea at 100 W for 2-4 hours. Gels were dried and exposed to X-ray film as for AFLP reactions.

Conventional PCRs were done in 15µl reaction volumes containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each dNTP, 0.4 µM of each primer, 0.5 U *Taq* polymerase (Invitrogen) and 10-100 ng template DNA. Thermal cycling conditions typically consisted of an initial denaturation step of 94 °C for 2 min

followed by 35 cycles of 94 °C for 15 s, primer annealing temperature (Table 2) for 30 s, 72 °C for 1 min per kb of amplified product followed by a final extension step of 72 °C for 10 min. For sequencing, primers and dNTPs were removed from PCR products by incubation with 1.2 U Exonuclease I and x 1.2 U SAP at 37 °C for 30 min followed by incubation at 80 °C for 20 min to denature the enzymes. Sequencing was done using the ABI PRISM Big Dye (v. 3.1) Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems) according to the manufacturer's instructions. Sequences were examined for single nucleotide polymorphisms (SNPs) between resistant and sensitive haplotypes that could be used to develop CAPS (cleaved amplified polymorphic sequences) markers for mapping in segregating populations.

e. Results

Variation for resistance to *P. infestans* in CGN accessions

Screening of 12 *S. okadae* accessions using *P. infestans* isolate 98.170.3 in detached leaf assays showed phenotypic variation for resistance in six of them (Table 1b). The remaining six accessions were all sensitive to this particular isolate, despite CGN data indicating that at least three of these accessions were moderately or very resistant to *P. infestans*. Resistance was evident as a complete lack of sporulation on leaf tissue whereas extensive mycelial growth was evident on leaves of sensitive individuals from 4 days post inoculation (Fig. 1.). Sensitive leaves often turned completely black by seven days post inoculation.

Development of *S. okadae* and *S. neorossii* mapping populations

Resistant individuals from five of the *S. okadae* accessions were crossed with sensitive individuals from either the same or different accessions (Table 3). In each of the crosses, resistance to *P. infestans* segregated 1:1 in the resulting progeny indicating the presence of potentially five heterozygous *Rpi* genes in the resistant parents. An interspecific cross between a susceptible *S. okadae* plant and a resistant *S. neorossii* plant was made. Analysis of resistance in the resulting progeny indicated the presence of a dominant *Rpi* gene that segregated in a 1:1 ratio.

Mapping *Rpi* genes in *S. okadae*

Rpi-oka1

A total of 72 AFLP primer combinations were used to screen the Soka014 resistant and sensitive pools to obtain fragments linked to *Rpi-oka1*. Primer combination *Pst*I+CT/*Mse*I+AGA produced a 97 bp fragment that was amplified only from the resistant parent and resistant pools of the Soka014 population. The sequence of this fragment was similar to the expressed sequence tag (EST) SGN-U214221 from the Lycopersicon combined EST library (http://www.sgn.cornell.edu). PCR primers were designed from the sequence of SGN-U214221 and used to amplify a band of approximately 2 kb from *Solanum lycopersicum* (formerly *Lycopersicon esculentum*, Le) and *S. pennellii* (formerly *L. pennellii*, Lp) which showed polymorphism between the two species when digested with *Alu*I. Analysis of the polymorphism in Le/Lp introgression lines (Eshed and Zamir 1994) located this marker in IL 9.2, indicating that the marker could be in either arm of chromosome IX. The polymorphism was not in IL 9.1, which overlaps IL 9.2 substantially, suggesting that the marker is situated proximal to the centromere on either chromosome arm. Using different PCR primers (SokaM2.9LF5 and SokaM2.9LR5) and *Dde*I digestion, this marker (SokaM2.9L) was mapped in the Soka014 population, giving a distance of approximately 6 cM (3 recombinants out of 48 segregants) from *Rpi-oka1* (Fig. 3).

Additional markers were developed by designing PCR primers from known chromosome IX RFLP marker sequences within the SGN database, sequencing the PCR products amplified from both resistant and sensitive parental DNA and identifying SNPs that could be used to develop CAPS markers (Table 2). In this way, *Rpi-oka1* was mapped to a 6.0 cM region of chromosome IX, delimited by markers C2_At4g02680 and TG186. The markers TG551 and TG35 were found to co-segregate with *Rpi-oka1* (Fig. 3).

Rpi-oka3

The resistant and sensitive pools of the Soka040 population were screened with a total of 48 AFLP primer combinations. Primer combination *Pst*I+AT/*Mse*I+GCT produced a linked fragment of 108 bp. No significant sequence similarities were found for this fragment and although it was possible to amplify a fragment from Le and Lp using PCR primers designed from the sequence, no polymorphisms were found that could be used to map the marker in the introgression lines. However, a *Dde*I polymorphism was present between the parents of the Soka040 population, which enabled the converted PCR marker (M6.44) to be mapped approximately 23 cM from *Rpi-oka3* (Fig. 3).

AFLP primer combination *Pst*I+AA/*Mse*I+GTC produced a linked fragment of approximately 260 bp. Primers designed from this sequence amplified a fragment from Le and Lp that gave a polymorphism when digested with either *Hae*III or *Sau*3AI. This polymorphism was present in introgression line IL 9-2. No polymorphisms were found between the parents of the Soka040 population and so the marker could not be mapped in relation to *Rpi-oka3*.

To confirm the placing of *Rpi-oka3* on chromosome IX, four SSR markers (Stm0010, Stm 0017, Stm 1051 and Stm 3012; (Milbourne et al. 1998)) were investigated for linkage to *Rpi-oka3*. Stm0017 did not amplify successfully from the sensitive parent and thus could not be used for mapping. Stm0010, Stm1051 and Stm3012 which all map to the short arm of chromosome IX (Milbourne et al. 1998) all showed polymorphism between resistant and sensitive parents and pools and thus provided further evidence that the gene was on chromosome IX (Fig. 3).

Rpi-oka2

A total of 72 AFLP primer combinations were used to screen the Soka013 resistant and sensitive pools to obtain fragments linked to *Rpi-oka1*. AFLP primer combination *Pst*I+AA/*Mse*I+CAT produced a linked fragment which was converted into PCR based allele specific marker of approximately 200 bp (Soka13M5.17). This marker mapped a distance of about 6.5 cM from *Rpi-oka2* (Fig 3).

Additionally, 3 further AFLP markers (P12M44_103, P13M42_228 and P17M33_472) were placed on the *Rpi-oka2* linkage map (Figure 3).

As *Rpi-oka1* and *Rpi-oka3* were shown to be closely linked to the chromosome IX markers TG551 and TG35, these markers were also investigated for linkage to *Rpi-oka1*. In the Soka013 population, TG551 and TG35 mapped 0.7 cM centromeric of *Rpi-oka2* on chromosome IX (Figure 3), flanked by marker T1421.

Mapping *Rpi* genes in *S. neorossii*

Rpi-nrs1

A total of 11 AFLP markers were placed on the *Rpi-nrs1* linkage map (Figure 3). Attempts were made to convert these markers into SCAR markers to investigate polymorphisms that could be used to place these markers on the Le/Lp introgression lines (Eshed and Zamir 1994). However, none of these markers were informative and thus a chromosomal location for *Rpi-nrs1* could not be confirmed using these

markers. Marker TG551 did show tight linkage to *Rpi-nrs1* and thus we concluded that *Rpi-nrs1* is also situated on chromosome IX, probably at the same locus as *Rpi-oka1-3*.

Use of an NBS marker closely linked to *Rpi-oka1, 2, 3* and *Rpi-nrs1* for mapping

The NBS marker NBS3B (see EXAMPLE 3) was converted to a PCR-based SCAR marker which could be amplified using the primers okaNBSHae-F and okaNBSHae-R (Table 2). These primers amplified a 555 bp fragment (marker okaNBSHae) from resistant plants containing *Rpi-oka1, Rpi-oka2* and *Rpi-nrs1*. In each population, this marker was shown to co-segregate with the resistance gene (Fig 3). For *Rpi-oka3*, a PCR product was amplified from both resistant and susceptible plants. However, the marker was converted into a CAPS marker by digestion with *Mae*III. This CAPS marker was shown to co-segregate with *Rpi-oka3* in the Soka040 population (Fig 3).

BAC library screening and contig construction

We used nine PCR markers linked to *Rpi-oka1, Rpi-oka2* and *Rpi-mcq1* for screening the BAC libraries. As shown in Table 1a, TG551, TG35 and TG186 are linked to *Rpi-oka1*. TG551 is also linked to *Rpi-oka2*. The marker okaNBSHae is linked to both *Rpi-oka1* and *Rpi-oka2*. Although TG551 is linked to both genes from *S. okadae*, the alleles of this marker from the *Rpi-oka1* and *Rpi-oka2* haplotypes can be distinguished by restriction digestion as indicated in Table 1a. U282757, U296361, TG591 and U279465 are all linked to *Rpi-mcq1*. The number of BAC pools shown to be positive for these markers varied from 11 to 17 for the K39 library and from 9 to 14 for the K182 library (Table 1a).

In the K39 library, the TG186 marker was amplified from 17 pools. It was not possible to determine from which haplotype (*Rpi-oka1* or *Rpi-oka2*) this marker was amplified. TG186, despite being a CAPS marker, is linked to *Rpi-oka1* in repulsion, and the allele present in the *Rpi-oka1* haplotype is indistinguishable from that in the *Rpi-oka2* haplotype. The marker okaNBSHae was amplified from 18 pools. As this marker is not polymorphic between the *Rpi-oka1* and *Rpi-oka2* haplotypes, it was not possible to assign haplotype to these BACs based on this marker. TG551 was amplified by PCR from 16 pools and restriction enzyme digestion showed there to be six pools positive for the *Rpi-oka1* haplotype and seven for the *Rpi-oka2* haplotype. TG35 was amplified by PCR from 11 pools, eight of which were shown to be from the

Rpi-oka1 haplotype by restriction digestion, suggesting that the remaining three were from the *Rpi-oka2* haplotype. Gel images showing the PCR-based markers and their restriction patterns (where relevant) are shown in Fig. 4a-4c.

From hybridisation of the okaNBSHae probe to the pooled BAC DNA, a total of 67 pools were identified as containing BAC clones with homologus sequences. From screening high density double-spotted membranes containing each individual BAC clone from the pools identified, a total of 85 BAC clones were identified, DNA isolated and subjected to BAC SNaPshot fingerprinting, along with an additional 10 selected clones which were positive for the TG551 and/or TG35 markers. From the contigs generated, one contig contained BAC clones identified by a) PCR based screening using the linked markers TG551, TG35 and the co-segregating marker okaNBSHae and b) by hybridisation using the okaNBSHae marker as a probe. This BAC contig is shown in Fig 5.

Table 1a Screening of BAC pools with PCR-based markers linked to late blight resistance loci

Marker	Linked gene	Hits by PCR[a]	RE[b]	Hits after Digestion[c]
TG551	*Rpi-oka2*	16	*Taq*I	7
TG551	*Rpi-oka1*	16	*Mwo*I	6
okaNBSHae	*Rpi-oka1* & *Rpi-oka2*	18	n.a.[d]	n.a.
TG186	*Rpi-oka1*	17	n.a.[d]	n.a.
TG35	*Rpi-oka1*	11	*Hha*I	8
U282757	*Rpi-mcq1*	14	*Xho*I	6
U296361	*Rpi-mcq1*	13	*Hinc*II	3
TG591	*Rpi-mcq1*	14	*Hae*III	7
U279465	*Rpi-mcq1*	9	n.a.	n.a.

[a] The number of positive pools to the marker by PCR

[b] Restriction enzyme causing polymorphic between resistant and susceptible alleles

[c] The number of pools positive to the marker after digestion with a certain enzyme

[d] not applicable because the marker is linked in repulsion or not polymorphic between resistant and susceptible alleles

Pools from the K39 library which were positive to either TG551, TG35 or okaNBSHae, markers which are closely linked to or, in the case of okaNBSHae, co-segregate with *Rpi-oka1* or *Rpi-oka2,* were randomly chosen. The original 384-well plates for each of the BAC libraries were replicated onto solid LB medium. Colonies

from each plate were scraped by rows and columns and screened for the presence of the relevant marker. Single clones from 384-well plates were selected.

Following selection of single clones, BAC DNA was isolated and BAC-ends were sequenced. BLAST homology searches (http://www.ncbi.nlm.nih.gov/BLAST/) showed that two of the clones from the K39 library (K39_272N11 and K39_256M23) had BAC-end sequences which were highly similar to each other and to the tomato mosaic virus *R* gene *Tm-2*^{2} (Lanfermeijer et al. 2003).

PCR Primers were designed from each of the BAC end sequences obtained and used to amplify products from the parental genotypes of *Rpi-oka1* and *Rpi-oka2.* PCR products were sequenced and analysed for the presence of SNPs that allowed use of the PCR products as markers in the respective populations. Successfully converted markers were placed on a higher resolution genetic map for *Rpi-oka1* (1213 individuals) and *Rpi-oka2* (1706 individuals). The position of these markers in relation to *Rpi-oka1* and *Rpi-oka2* is shown in Fig 6.

High resolution mapping and cloning of *Rpi-oka1*

The *Rpi-oka1* population Soka014 consisting of 1214 individuals was screened for recombinants between the markers SokaM2.9L and TG186. A total of 169 recombinants were identified, covering a genetic interval of 14 cM. Initial screening of these recombinants for disease phenotype indicated that the resistance locus was located south of marker TG35. Hence, a subset of 53 recombinants between the markers TG35 and TG186 were selected from the larger subset of recombinants. These recombinants were screened for resistance or susceptibility to *P. infestans* and with markers developed from the BAC end sequences. The results indicated that *Rpi-oka1* was located within a genetic interval of 0.33 cM delimited by the markers TG35 and 185L21R (BAC end marker) (Fig 5, 6). By reference to the physical map constructed from PCR and fingerprinting analysis of BAC clones from the K39 library (Fig 5) *Rpi-oka1*was predicted to be present on a physical region covered by the two BAC clones K39_148P20 and K39_266I9. These two BAC clones were sequenced and one candidate ORF was identified for *Rpi-oka1*.

High resolution mapping and cloning of *Rpi-oka2*

To construct a high resolution map, we used two flanking PCR based markers (TG551 and T1421; Figure 1a) to screen an expanded population for selecting recombinants around the resistance locus. 46 recombinants from the expanded

population of 1706 genotypes were selected representing an interval of 2.9 cM between the two PCR markers. These recombinants were phenotyped for late blight resistance and genotyped using the BAC end markers from the BAC contig. The results indicated that *Rpi-oka2* was located within a genetic interval of 0.12 cM delimited by the BAC-end markers 266I9F and 185L21R (Fig 5, 6). By reference to the physical map constructed from PCR and fingerprinting analysis of BAC clones from the K39 library *Rpi-oka2* was therefore predicted to be present on the same physical region as that identified for *Rpi-oka1*. However, other than the BAC clone K39_272N11 (for which one end contained a partial *Tm2*^{2} homologue) clones which covered the same physical region as *Rpi-oka1* could not be identified within the library (Fig 5). As an alternative approach, primers were designed to amplify the complete *Rpi-oka1* ORF were used in a PCR reaction with DNA from the parent plant of the *Rpi-oka2* population. The resulting PCR product was cloned into pGEM-T Easy and 4 clones were sequenced to obtain a consenus sequence for the *Rpi-oka2* candidate.

High resolution mapping of *Rpi-nrs1*

To construct a high resolution map, we used three flanking PCR based markers (SneoM2.9a, TG551 and TP25; Figure 3) to screen an expanded population and select recombinants around the *Rpi-nrs1* resistance locus. TP25 was converted from an AFLP marker, P13M34_370[R]. Initially 323 recombinants from the expanded population of 1402 genotypes were selected resulting in an interval of 23 cM genetic distance between SneoM2.9a and TP25. At the same time, closer PCR markers to *Rpi-nrs1* were developed (U317500 and U270442). Consequently 40 recombinants were selected and these recombinants were phenotyped for late blight resistance. The RGA marker designated okaNBSHae mapped to the same genetic location as that of *Rpi-nrs1* and several BAC-end sequence based PCR markers were developed from the *Rpi-oka1/2* contig from the K39 BAC library allowing construction of a fine scale genetic map around *Rpi-nrs1* (Figure 6). Additionally the recombinants were tested for resistance using three different isolates and the phenotypes were differently segregated indicating that there are multiple genes in the population. The second gene designated *Rpi-nrs1b* is expected to be located to the south of *Rpi-nrs1a* (Figure 6).

Analysis of *Rpi-oka1, Rpi-oka2* and *Rpi-oka3*

The *Rpi-oka1* ORF is 2673 bp long and translates into a protein sequence of 891 amino acids with a calculated molecular weight of 102 kDa and a pI of 8.05. The *Rpi-oka2* ORF comprises 2715 bp and translates into a protein sequence of 905 amino acids with a calculated molecular weight of 103.6 kDa and a pI of 8.16. The sequence of the PCR product amplified from material containing *Rpi-oka3* was identical to that of *Rpi-oka2*. The Rpi-oka1 protein contained all the features characteristic of the coiled coil-nucleotide binding region-leucine rich repeat (CC-NB-LRR)-class of resistance proteins. Within the first 215 amino acids of the N-terminal part of the protein were 4 regions each with 3 predicted heptad repeat motifs typical of coiled coil domains (Fig. 7). All NB-ARC domains (van der Biezen and Jones 1998) were present in the amino acid sequence from 216-505. Following the NB-ARC domain was a region comprising of a series of 15 irregular LRR motifs that could be aligned according to the consensus sequence LxxLxxLxxLxLxxC/N/Sx(x)LxxLPxx (where L can be L, I, M, V, Y or F and x is any amino acid) (McHale et al. 2006).

The sequence of *Rpi-oka2* differs from *Rpi-oka1* by an insertion of 42 nucleotides in the 5' end of the gene (Fig. 7). The resulting additional 14 amino acids present in the corresponding region of Rpi-oka2 do not affect any of the predicted coiled coil domains. There are also 3 single nucleotide polymorphisms (SNPs) between *Rpi-oka1* and *Rpi-oka2*; A1501T, T1767C and G2117A (Fig. 7). These nucleotide differences result in two amino acid differences between Rpi-oka1 and Rpi-oka2 (Fig. 7). The difference at position 501 is at the end of the NB-ARC domain, just prior to the LRR region and results in the change of an asparagine in Rpi-oka1 to a tyrosine in Rpi-oka2. This amino acid change does not affect any of the characterised NB-ARC domains. At position 706, within the 9th LRR, an arginine in Rpi-oka1 becomes a lysine in Rpi-oka2; both of these residues are positively charged polar amino acids and hence this can be considered a synonymous change.

Rpi-oka1 and *Rpi-oka2* share 80.9% and 79.7%, identity, respectively, with *Tm-2*^{2} at the nucleic acid level. At the amino acid level, this translates to 72.1% and 71.1% identity, respectively, at the amino acid level. As expected, given its role in recognition specificity, the percentage of similarity was lowest in the LRR domain where *Rpi-oka1/2* and *Tm-2*^{2} share only 57.5% similarity. In contrast, the sequence similarity across the coiled-coil and NB-ARC domains of *Rpi-oka1/2* and *Tm-2*^{2} was 81.8% and 79.7%; within the conserved domains of the NB-ARC region, *Tm-2*^{2} and *Rpi-oka1* differ by only 1 amino acid.

The primers oka1long-F and oka1long-R (Table 2) were used to amplify *Rpi-oka1* homologous sequences from the parental material containing *Rpi-oka3*. Resulting

PCR products were cloned into pGEM-T and sequenced. The sequences obtained were identical to *Rpi-oka2.*

It was not possible to amplify full-length *Rpi-oka1* paralogues from the susceptible *S. okadae* parent A613. This observation, together with the fact that the okaNBSHae marker could only be amplified from resistant genotypes suggests that the susceptible phenotype is caused by an absence of *Rpi-oka1* rather than a non-functional copy.

Rpi-oka1* is also present in resistant *S. neorossii* genotypes and is the orthologue of *Rpi-phu1.

Mapping of an *Rpi* gene in a segregating population derived from a resistant individual of *S. neorossii* accession CGN1800 also showed close linkage between the identified gene (*Rpi-nrs1*) and marker TG551, indicating that this gene was located in the same region as *Rpi-oka1.* Similarly, *Rpi-phu1* from *S. phureja* was also reported to map to this region (Sliwka et al. 2006). The *Rpi-oka1* marker okaNBSHae-F/R co-segregated with resistance in a population of 149 *S. tuberosum* plants segregating for *Rpi-phu1.* Full-length *Rpi-oka1* paralogues were amplified from DNA of 3 resistant genotypes containing *Rpi-phu1.* A single product was obtained and sequencing showed this to be identical to *Rpi-oka1.* Similarly, amplification from resistant *S. neorossii* material showed that *Rpi-oka2* was present in this material and the presence of this gene correlated with resistance in 40 pre-selected recombinants. Resistant plant material containing *Rpi-nrs1* or *Rpi-phu1* was also shown to be resistant to each of the *P. infestans* isolates used in this study, with the exception of EC1. Thus we conclude that *Rpi-oka1=Rpi-phu1* and *Rpi-oka2=Rpi-oka3=Rpi-nrs1.*

Table 1b Reaction to *Phytophthora infestans* of twelve *Solanum okadae* and four *S. neorossii* accessions

Accession[a]	Wild species	Reference data Phenotype	Source	Fine screening[b] R	MR	MS	S
CGN17998	*S. okadae*	Very resistant	CGN	2			7
CGN17999	*S. okadae*	Resistant	CGN	3			7
CGN18108	*S. okadae*	Very resistant	CGN	8		3	
CGN18109	*S. okadae*	Very resistant	CGN				10
CGN18129	*S. okadae*	Susceptible	CGN		2	2	6
CGN18157	*S. okadae*	Moderately resistant	CGN				10
CGN18269	*S. okadae*	Susceptible	CGN				10
CGN18279	*S. okadae*	Very resistant	CGN	4			5
CGN20599	*S. okadae*	Susceptible	CGN				10
CGN22703	*S. okadae*	Very susceptible	CGN	4	1		4
CGN22709	*S. okadae*	Very susceptible	CGN				8
BGRC27158	*S. okadae*	Moderately resistant	CGN				1
CGN17599	*S. neorossii*	Susceptible	CGN				10
CGN18000	*S. neorossii*	Very resistant	CGN	11			
CGN18051	*S. neorossii*	Susceptible	CGN				6
CGN18280	*S. neorossii*	Very susceptible	CGN				10

[a]CGN, Centre for Genetic Resources in the Netherlands (http://www.cgn.wageningen-ur.nl); BGRC, Braunschweig Genetic Resources Center.

[b]Number of plants showing resistant (R) or susceptible (S) phenotypes

Table 2. PCR based markers used for mapping of *Rpi-oka1*, *Rpi-oka2*, *Rpi-oka3* and *Rpi-nrs1*

Marker	Primer sequence (5'-3')	Tm (°C)	Type of marker[a]			
			Rpi-oka1	*Rpi-oka2*	*Rpi-oka3*	*Rpi-nrs1*
TG254	F: AGTGCACCAAGGGTGTGAC	60				
	R: AAGTGCATGCCTGTAATGGC					
At2g38025	F: ATGGGCGCTGCATGTTTCGTG	55			*Tsp509*I [R]	
	R: ACACCTTTGTTGAAAGCCATCCC					
Stm1051	F: TCCCCTTGGCATTTTCTTCTCC	55			SSR	
	R: TTTAGGGTGGGGTGAGGTTGG					
Stm3012	F: CAACTCAAACCAGAAGGCAAA	55			SSR	
	R: GAGAAATGGGCACAAAAAACA					
Stm0010	F: TCCTTATATGGAGCAAGCA	50			SSR [R]	
	R: CCAGTAGATAAGTCATCCCA					
M6.44	F: ATTGAAAGAATACACAAACATC	55			*Dde*I	
	R: ATTCATGTTCAGATCGTTTAC					
At3g63190	F: TTGGTGCAGCCGTATGACAAATCC	55		*Eco*RI	*Tsp509*I	
	R: TCCATCATTATTTGGCGTCATACC					
SneoM2.9a	F: TAGATCTATACTACACTTGGCAC	50				as
	R: TAATCTCTTCCATCTTCCC					
SokaM2.9L	F: ACAAACCTATGTTAGCCTCCCACAC	60	*Dde*I			
	R: GGCATCAAGCCAATGTCGTAAAG					
At2g29210	F: AGCAGGACACTCGATTCTCTAATAAGC	55	*Nco*I			
	R: TGCACTAAGTAGTAATGCCCAAAGCTC					
Soka13M5.17	F: CTGAGGTGCAGCCAATAAC	55		as		
	R: CCAGTGAGAAACAGCTTCTC					
U276927	F: GATGGGCAACGATGTTGTTG	60		*Hpy188*I		
	R: GCATTAGTACAGCGTCTTGGC					
At4g02680	F: GTGAAGAAGGTCTACAGAAAGCAG	55	*Mse*I		*Nhe*I	
	R: GGGCATTAATGTAGCAATCAGC					
TG551	F: CATATCCTGGAGGTGTTATGAATGC	60	*Mwo*I	*Taq*I	*Taq*I	*Taq*I
	R: CATATCCTGGAGGTGTTATGAATGC					
TG35	F: CACGGAGACTAAGATTCAGG	55	*Hha*I	*Alu*I	*Tsp509*I	
	R: TAAAGGTGATGCTGATGGGG					

T1421	F: CATCAATTGATGCCTTTGGACC	60		*Bsl*I	*Rsa*I	
	R: CTGCATCAGCTTCTTCCTCTGC					
TG186	F: AATCGTGCAGTTTCAGCATAAGCG	60	*Dra*I [R]			
	R: TGCTTCCAGTTCCGTGGGATTC					
TG429	F: CATATGGTGACGCCTACAG	55			*Mse*I	
	R: GGAGACATTGTCACAAGG					
T1190	F: GTTCGCGTTCTCGTTACTGG	55	as			
	R: GTTGCATGGTTGACATCAGG					
TG591A	F: CTGCAAATCTACTCGTGCAAG	60	as			
	R: CTCGTGGATTGAGAAATCCC					
okaNBSHae	F: CTTACTTTCCCTTCCTCATCCTCAC	60	as	as	*Mae*III	as
	R: TGAAGTCATCTTCCAGACCGATG					
oka1long	F: AGTTATACACCCTACATTCTACTCG	60	as	as	as	as
	R: CTTTGAAAAGAGGCTTCATACTCCC					
266I9F	F: GTATGTTTGAGTTAGTCTTCC	55		*Hinf*I		
	R: TATAATAGGTGTTCTTGGGG					
266I9R	F: AAGGTGTTGGGAGTTTTTAG	55	*Hind*III	*Hind*III		
	R: TATCTTCCTCATTTTGGTGC					
185L21R	F: GATTGAGACAATGCTAGTCC	55	*Bsl*I	*Rsa*I		
	R: AGAAGCAGTCAATAGTGATTG					
148P20R	F: AAGATTCTTTTTCCTCCTTAG	58	*Hpy*CH4IV			
	R: AAAGATGAAGTAGAGTTTTGG					

[a] Restriction enzymes indicate that marker is a CAPS marker, as indicates allele-specific markers, [R] indicates that marker is linked in repulsion phase, SSR indicates that marker is a simple sequence repeat marker, blank indicates that the marker was either not polymorphic or not tested for that *Rpi* gene.

Table 3 Crosses within *S. okadae* and late blight resistant (R) and sensitive (S) segregants in their progenies

Population identifier	Population parents[a]		Segregating progeny[b]	
	Female	Male	R	S
Soka014	A618, CGN18108, R	A613, CGN18108, S	26	24
Soka012	A622, CGN18279, S	A618, CGN18108, R	18	23
Soka013	A624, CGN18279, R	A613, CGN18108, S	59	80
Soka040	A606, CGN17998, R	A628, CGN18279, S	25	23
Soka241	D986, BGRC08237, R	B419, Soka012, S	24	26
Soka184	D403, CGN17999, R	D401, CGN17999, S	24	21
Sokaneo140	A613, CGN18108, S	A795, CGN18000, R	67	73

[a]Plant identifier number followed by its accession number and reaction to *P. infestans* inoculation: CGN Centre for Genetic Resources in the Netherlands; BGRC, Braunschweig Genetic Resources Center.

[b]Number of plants showing resistant (R) or sensitive (S) phenotypes

EXAMPLE 3

MAPPING AND CLONING *Rpi-oka1* AND *RPI-nrs1* USING A CANDIDATE GENE/ALLELE MINING APPROACH

To date, cloning of *R* genes is typically done through a positional cloning strategy. Once a functional gene is cloned from a specific *R* locus, one can try to clone functional alleles from the same or different species in order to determine allele frequency and allelic variation at a given locus. Here we demonstrate that NBS profiling (Linden *et al.*, 2004) when combined with bulked segregant analysis (BSA) (Michelmore *et al.*, 1991) is a powerful tool to generate candidate gene markers which can predict the position of the *R* locus under study and in doing so form a starting point for the cloning of the gene through a functional allele mining strategy.

Plant material

Accessions of *Solanum okadae* and *Solanum neorossii* were requested from the Centre of Genetic Resources (CGN) in Wageningen, The Netherlands. Following screening with *Phytophthora infestans*, resistant genotypes from specific accessions

were used to make inter- or intra-specific mapping populations. The *Rpi-oka1* mapping population 7698 was made by crossing OKA7014-9 (resistant F1 plant derived from a cross between OKA367-1 and OKA366-8, both derived from accession CGN18108) with the susceptible plant NRS735-2 (CGN18280). All *S. okadae* genotypes were derived from accession CGN18108. The *Rpi-nrs1* mapping population 7663 was generated by crossing the resistant plant NRS365-1 (CGN18000) with NRS735-2.

Disease assays

Detached leaf assays (DLA) on the *Solanum* species were carried out as described by Vleeshouwers et *al.* (1999). Leaves were inoculated with 10µl droplets of inoculum ($5x10^4$ zoospores/ml) on the abaxial side and incubated at 15°C for 6 days in a climate chamber with a photoperiod of 16h/8h day/night. At 6 days post inoculation, leaves showing sporulation were scored as susceptible whereas leaves showing no symptoms or necrotic lesions were scored as resistant.

Marker development

Markers from appropriate chromosomal positions were selected from the Solanaceae Genomics Network (SGN) database and subsequently developed into polymorphic markers in each of the relevant mapping populations. Additional candidate gene markers were developed through NBS profiling as described by van der Linden *et al.* (2004). Templates were generated by restriction digestion of genomic DNA using the restriction enzymes *MseI*, *HaeIII*, *AluI*, *RsaI* or *TaqI*. Adapters were ligated to restriction fragments. PCR fragments were generated by radioactive-labeled primers (nbs1, nbs2, nbs3, nbs5a6 or nbs9) designed on conserved domains of the NBS domain (P-loop, Kinase-2 and GLPL motifs (Calenge, 2005; Syed, 2006).

PCR amplification of candidate *R* genes

Long range PCR with Taq-polymerase or *Pfu* Turbo polymerase 50µl reaction-mixture was prepared containing 50ng of gDNA, 1µl of the forward primer (10µM), 1µl of the reverse primer (10µM), 0.8µl dNTPs (5mM each), 5µl 10X buffer, 5 units of Taq-polymerase (Perkin Elmer) or 1µl of *pfu* Turbo (Invitrogen). The following

PCR program was used: 94°C for 3mins, 94°C for 30 sec, 55°C for 30 sec, 72°C for 4mins, 72°C for 5mins during 29 cycles.

Genome walking

Marker sequences were extended by cloning flanking DNA fragments with the ClonTech Genome Walker kit according to the manufacturer's instructions using a blunt adapter comprising the complementary sequences: 5-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGA-3 and 5'-PO_4TCCAGCCC And the adapter specific primers AP1 (5'-TAATACGACTCACTATAGGGC) and AP2 (5'-ACTATAGGGCACGCGTGGT). A simultaneous restriction-ligation was performed followed by two rounds of PCR. A 50µl restriction-ligation (RL) mixture was prepared containing 250ng of genomic DNA, 5 units of blunt cutting enzyme (*Bsh1236I*, *AluI*, *DpnI*, *HaeIII*, *RsaI*, *HincII*, *DraI*, *ScaI*, *HpaI* or *SspI*), 1µl genome walker adapter (25µM), 10mM ATP, 10µl of 5X RL buffer, 1 unit of T4 DNA ligase (Invitrogen 1U/µl). The digestion mix was incubated at 37°C for 3 hours. Samples were diluted 50 times prior to PCR. For the first PCR round, a 20µl reaction-mixture was prepared containing 5µl of diluted RL DNA, 0.6µl specific forward primer 1 (10µM), 0.6µl AP1 (10µM), 0.8µl dNTPs (5mM each), 2µl 10X buffer (Perkin Elmer), 5 units *Taq*-polymerase (Perkin Elmer). The first PCR was performed using the following cycle program: 30-sec at 94°C as denaturation step, 30-sec at 56°C as annealing step and 60-sec at 72°C as extension step. 35cycles were performed. A second PCR using the same conditions as the first one was performed using specific primer 2 and AP2 and 5µl of 50 times diluted product from the first PCR. 5µl of the second PCR product was checked on gel (1% agarose) and the largest amplicons were cloned into the pGEM®-T Easy Vector from Promega and sequenced.

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

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

respectively, generating pENTR-Blb3P and pENTR-Blb3T. PCR amplicons generated with *Pfu* Turbo polymerase were cloned into pDONR221 generating pENTR-RGH clones, and subsequently cloned together with the *Rpi-blb3* promoter and terminator fragments into pKGW - MGW using the multiple Gateway® cloning kit (Invitrogen). The pENTR clones were made by carrying out a BP-Reaction II overnight (http://www.untergasser.com/lab/protocols/bp_gateway_reaction_ii_v1_0.shtml). DH5α competent cells (Invitrogen) were transformed by heat shock with 5µl of the BP Reaction II mixture. Cells were selected on LB medium containing 50mg/ml of Kanamycine. Colonies were checked for the presence of the relevant inserts by colony PCR. DNA of appropriate pENTR clones was extracted from *E. coli* and used to perform a multiple Gateway® LR cloning reaction to generate the final binary expression clones (http://www.untergasser.com/lab/protocols/lr_multiple_gateway_reaction_v1_0.shtml) .. DH5α competent cells (Invitrogen) were transformed by heat shock with 5µl of the LR reaction mixture. Cells were selected on LB medium containing 100mg/ml of spectinomycine. Colonies were checked by PCR for the presence of the correct inserts. Positive colonies were grown overnight in LB medium supplemented with 100mg/ml of spectinomycine to extract the final expression vector. The final expression vector was transferred to *Agrobacterium tumefaciens* strain COR308 through electropration. Colonies were selected on LB medium supplemented with 100mg/ml of spectinomycine and 12.5mg/ml of tetracycline overnight at 30°C.

Sequencing

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

RESULTS

Genetic basis and spectrum of late blight resistance in accessions of *S. okadae* and *S. neorossii*.

To determine the genetic basis of late blight resistance in *S. okadae* and *S. neorossii*, 14 and 5 accessions, respectively, were screened in detached leaf assays (DLA) with the complex *P. infestans* isolate IPO-C. Resistant genotypes selected from the oka accession CGN18108 and the nrs accession CGN18000 were used to generate the *S. okadae* and *S. neorossii* mapping populations 7698 [oka7014-9 (oka367-1 x oka366-8) x nrs735-2) and 7663 (nrs365-1 x nrs735-2), respectively. Following DLA's with 50 F1 progeny plants of population 7698, 30 were scored as resistant and 22 as susceptible, suggesting the presence of a single dominant *R*-gene, which we named *Rpi-oka1*. Of the 60 F1 progeny plants screened from population 7663, 24 were scored as resistant and 36 as susceptible, suggesting that also nrs365-1 contained a single dominant *R* gene, which we named *Rpi-nrs1*.

The resistance spectrum of both genes was analyzed by challenging them with several isolates of different complexity and aggressiveness (Table 4). *Rpi-oka1* and *Rpi-nrs1* appear to have the same specificity. Strain EC1 was the only one able to overcome both *R* genes.

Mapping of *Rpi-oka1* and *Rpi-nrs1* to chromosome IX

To determine whether the *Rpi-oka1* gene segregating in population 7698 was on chromosome IX, we tried to develop and map the chromosome IX specific markers TG35, TG551, TG186, CT183 and T1421 in the initial 50 F1 progeny plants of population 7698. Only TG35 and TG186 were found to be polymorphic between the parental genotypes and were indeed linked to *Rpi-oka1* (Fig 9). In an attempt to develop additional markers for *Rpi-oka1*, and also markers for *Rpi-nrs1*, we carried out a bulked segregant analysis (BSA) in combination with NBS profiling in both mapping populations. This led to the identification of 9 bulk specific markers for *Rpi-oka1* in 7698 and 8 for *Rpi-nrs1* in 7663. Finally, only two resistant bulk specific fragments, one generated with the NBS2/RsaI primer-enzyme combination and the other with NBS3/HaeIII, cosegregated with resistance in the initial 7698 and 7663 populations of 50 and 60 F1 progeny plants, respectively. These fragments were therefore cloned and sequenced. When subjected to a BLAST analysis, both sequences turned out to be highly similar to the *Tm-2*^{2} gene on chromosome IX of tomato (Lanfermeijer *et al.*, 2003; 2005). The cloned NBS2/RsaI and NBS3/HaeIII fragments were 350 and 115 bp in size and shared 88.3% and 80.3% DNA sequence identity with *Tm-2*^{2}. These findings suggested that *Rpi-nrs1* could be located at the same region on chromosome IX as *Rpi-oka1*. In an attempt to verify this, specific primers were designed for each fragment and used to develop SCAR

markers in both the *Rpi-oka1* and *Rpi-nrs1* mapping populations. In this way the two NBS-profiling derived markers NBS3A and NBS3B were developed for population 7663 and 7698, respectively. Subsequently, the positions of these markers relative to *Rpi-nrs1* and the chromosome IX specific markers TG35 and TG551 were determined in the *Rpi-nrs1* mapping 7698. Cosgregation of TG35, TG551 and NBS3B with *Rpi-nrs1* in the initial F1 population of 60 individuals, confirmed that *Rpi-nrs1* was indeed located on chromosome IX, in the same region as *Rpi-oka1* (Fig 9).

In order to develop flanking markers for a recombinant analysis markers linked to TG35 or TG551 were selected from SGN and screened in both populations. Despite low levels of polymorphism, EST based markers U276927 and U270442 I were developed and mapped in populations 7698 and 7663, respectively (Table 4 and Figure 9). U276927 was mapped 2cM north of *Rpi-oka1* whereas U270442 I was mapped 3.5cM south of *Rpi-nrs1*. Subsequently, a recombinant analysis was performed in 500 offspring of population 7698 and 1005 offspring of population 7663, using the flanking markers U276927 / TG186 and NBS3B / U270442, respectively. This resulted in the mapping of *Rpi-oka1* and *Rpi-nrs1* to genetic intervals of 4cM and 3.6cM, respectively (Figure 9).

$Tm\text{-}2^2$ based allele mining in *S. okadae* and *S. neorossi*

The present inventors adopted a homology based allele mining strategy to clone *Rpi-oka1* and *Rpi-nrs1*.

The first step was to design degenerated primers incorporating the putative start and stop codons of candidate *Tm-2* gene homologs (*Tm2*GH) at the *Rpi-oka1* and *Rpi-nrs1* loci. Based on an alignment of all the available potato and tomato derived $Tm\text{-}2^2$-like sequences in public sequence databases, we designed primers ATG-Tm2-F and TGA-Tm2-R (Table 5). However, no amplicons of the expected size were generated when this primer set was tested on the parental genotypes of both mapping populations. As the ATG-Tm2-F primer sequence was present in the cosegregating NBS profiling derived marker sequence, three new reverse primers (REV-A, -B and -C) were designed 100bp upstream of the initial TGA-Tm2-R primer site, in a region that was conserved in all the aligned $Tm\text{-}2^2$-like sequences. When combined with either ATG-Tm2-F or NBS3B-F, a single amplicon of approximately 2.5kb was specifically amplified only from the resistant parental genotypes, i.e., oka7014-9 and nrs365-1. These fragments were cloned into the pGEM®-T Easy vector and approximately 96 individual clones from each genotype were sequenced using a primer walk strategy. All the obtained sequences shared 75-80% similarity to

Tm-2^2. A total of 5 different classes could be distinguished within the oka7014-9 derived sequences whereas the nrs365-1 sequences fell into only 3 different classes. These different classes were subsequently named NBS3B-like or non-NBS3B-like based on the degree of homology to the NBS3B sequence (Table 6).

In an attempt to retrieve the missing C-terminal part of the amplified *Tm2*GH's we embarked on a 3'-genome walk using primers GSP1-1, GSP1-2 and GSP2 (Table 5), which were designed approximately 100bp upstream of the REV-A, -B and –C primers, in order to generate an overlap of 100bp between the cloned NBS3B-like sequences and clones generated with the genome walk. Three amplicons of ~200bp were obtained from oka7014-9 and a single one of ~1kb from nrs365-1. Following cloning, sequencing and alignment to the cloned *Tm2*GH's, all four clones seemed to fit to clone *Tm2*GH-nrs8bis, as the overlapping 100bp were an exact match. To be able to subsequently amplify full-length *Tm2*GH's from the *Rpi-oka1* and *Rpi-nrs1* loci we designed a novel reverse primer (TAA-8bis-R) (Table 5) based on the alignment of the full-length *Tm2*GH-nrs8bis sequence with the *Tm2*^{2} sequence from tomato (Figure 2). As the original TGA stop codon was not present in the *Tm2*GH-nrs8bis sequence we included the next in-frame stop-codon (TAA) which was situated 12bp downstream.

Full-length amplification of *Tm2*GH's from oka7014-9 and nrs365-1 was subsequently pursued with high fidelity *Pfu* Turbo polymerase using primers ATG-Tm2-F and TAA-8bis-R. Amplicons of ~2.6kb were cloned into the pGEM®-T Easy Vector and sequenced. Three different types of clones were obtained from OKA7014-9, one of which harbored an ORF of the expected size (*Tm2*GH-oka1bis). All the clones obtained from NRS365-1 were identical to each other and also contained the expected ORF. Clone *Tm2*GH-nrs1.9 was chosen together with *Tm2*GH-oka1bis for further genetic analysis.

Before targeting *Tm2*GH-oka1bis and *Tm2*GH-nrs1.9 for complementation analysis, we needed to confirm that the selected *Tm2*GH's indeed mapped to the *Rpi-oka1* and *Rpi-nrs1* loci. When tested as SCAR markers in the initial mapping populations, both markers cosegregated with resistance. Upon amplification of ATG-Tm2-F and TAA-8bis-R in the set of recombinants which defined the *Rpi-oka1* and *Rpi-nrs1* loci, amplicons of the expected size were indeed only generated from late blight resistant recombinants, confirming that both *Tm2*GA's were indeed good candidates for *Rpi-oka1* and *Rpi-nrs1.* However, there were resistant recombinants, 2 in the *Rpi-oka1* mapping population and 1 in the *Rpi-nrs1* mapping population, which did not give the expected PCR product, suggesting that both loci could in fact harbor a tandem of two functional *R* genes.

Analysis of the *Rpi-oka1* and *Rpi-nrs1* ORFs

Gene structure of *Rpi-oka1* and *Rpi-nrs1*

The 5'-terminal structure of *Rpi-oka1* and *Rpi-nrs1* was determined by comparing the amplicon sequences with cDNA fragments generated by 5' rapid amplification of cDNA ends (RACE). RACE identified 5' *Rpi-oka1* and *Rpi-nrs1* specific cDNA fragments comprising 5'-untranslated regions of 83 nucleotides (nt) for *Rpi-oka1* and 5 nt for *Rpi-nrs1*. Both genes are intron free. The open reading frames of *Rpi-oka1* and *Rpi-nrs1* encode predicted peptides of 891 and 905 amino acids, respectively. In addition to the 14 amino acid insertion in the N-terminal region of Rpi-nrs1, only two other amino acids differ between Rpi-oka1 and Rpi-nrs1. At position 548 and 753, Rpi-oka1 harbours an asparagine and arginine residue whereas the corresponding residues in Rpi-nrs1 are tyrosine and lysine, respectively (Figure 8). However, the substituted residues have the same characteristics. Asparagine and tyrosine belong to the group of hydrophobic residues whereas arginine and lysine are positively charged residues. The protein sequences of both genes harbor several conserved motifs of the CC-NBS-LRR class of R proteins (Figure 8). A coiled-coil (CC) domain is located in the N-terminal parts of the proteins between amino acids 1 and 183 for Rpi-oka1 and between 1 and 198 for Rpi-nrs1. In the first 183 or 198 residues 4 pairs of putative heptad motifs composed of hydrophobic residues could be recognized in Rpi-oka1 and Rpi-nrs1 sequences respectively. A NB-ARC (nucleotide-binding site, apoptosis, *R* gene products, CED-4) domain could be recognized in the amino acid stretch between residues 183 or 198 and 472 or 486 respectively (Ploop, Kinase-2, GLPL) (Van der Bierzen and Jones 1998). The C terminal half of Rpi-oka1 and Rpi-nrs1 comprises a series of 15 LRR motifs of irregular size that can be aligned according to the consensus sequence LxxLxxLxxLxLxxC/N/Sx(x)LxxLPxx (where x is any amino acid) (McHale et al. 2006). A PROSITE analysis (Hofmann et al. 1999) identified 4 N-glycosylation sites, 7 Casein kinase II phosphorylation sites, 10 protein kinase C phosphorylation sites, 6 N-myristoylation sites and 1 Camp- and Cgmp-dependent protein kinase phosphorylation site.

At the protein level, Rpi-oka1 and Rpi-nrs1 share 75% amino acid identity with the Tm-2^2 protein sequence. Interestingly, the lowest homology was found in the LRR domain where the Tm-2^2 shares only 62% identity with Rpi-oka1 and Rpi-nrs1. In contrast, the coiled-coil and NB-ARC domains of Rpi-oka1 andf Rpi-nrs1 share 87% amino acid sequence identity with the same regions of Tm-2^2.

Table 4: Characteristics of Phytophthora infestans isolates used to determine the specificity of *Rpi-oka1* and *Rpi-nrs1*

Isolate ID	Country of origin	Isolation year	Host	Mating type	RACE	Phenotype
90128	Geldrop, The Netherlands	1990	Potato	A1	1.3.4.7.(8)	Resistant
H30P04	The Netherlands		Potato		7	Resistant
IPO-C	Belgium	1982	Potato		1.2.3.4.6.7.10.11	Resistant
USA618	Toluca Valley, Mexico	unknown	Potato	A2	1.2.3.6.7.11	Resistant
VK98014	Veenkolonién, The Netherlands	1998	Potato	A1	1.2.4.11	Resistant
IPO-428-2	The Netherlands	1992	Potato		1.3.4.7.8.10.11	Resistant
NL00228	The Netherlands	2000	Potato		1.2.4	Resistant
Katshaar	Katshaar, The Netherlands		Potato		1.3.4.7.10.11	Resistant
F95573	Flevoland, The Netherlands	1995	Potato	A1	1.3.4.7.10.11	Resistant
89148-09	The Netherlands	1989	Potato		0	Resistant
EC1	Ecuador		Potato		3.4.7.11	Susceptible

Table 5A. Overview of markers used to map *Rpi-oka1* and *Rpi-nrs1*

Marker	Primer orientation	Primer sequence	annealing temperature	Enzyme
NBS3A	F	GAAGTTGGAGGCGATTCAAGG	56	cfr131 (c)
	R	GGCTTGTAGTGTATTGAAGTC		
NBS3B	F	CCTTCCTCATCCTCACATTTAG	65	a.s.
	R	GCATGCCAACTATTGAAACAAC		
TG35	F	CACGGAGACTAAGATTCAGG	60	HhaI[a] / XapI[b] (c)
	R	TAAAGGTGATGCTGATGGGG		
TG551	F	CCAGACCACCAAGTGGTTCTC	58	TaqI (c)
	R	AACTTTCAGATATGCTCTGCAG		
TG186	F	AACGGTGTACGAGATTTTAC	58	HphI (c)
	R	ACCTACATAGATGAACCTCC		
U270442 I	F	GGATATTATCTTGCAACATCTCG	55	XapI (r)
	R	CTTCTGATGGTATGCATGAGAAC		
U276927	F	GCATTAGCGCAATTGGAATCCC	58	HphI (c)
	R	GGAGAGCATTAGTACAGCGTC		

a.s.: allele specific

(c) : coupling phase

(r) : repulsing phase

Table 5b. Overview of primers used for genome walking based on NBS3B-like sequences, primers targeting the start and stop codons of *Rpi-oka1* and *Rpi-nrs1* and 5' RACE primers.

Primer pair	Primer orientation	Primer sequence	Annealing temperature
NBS-GSP1-1	F	tccaaatattgtcgagttggg	/
NBS-GSP2	F	gctttggtgcagacatgatgc	/
REV-A	R	ggttgtctgaagtaacgtgcac	55
REV-B	R	tgcacggatgatgtcagtatgcc	55
REV-C	R	caacttgaagttttgcatattc	55
ATG-Tm2F	F	atggctgaaattcttctcacagc	55
TAA-8bisR	R	ttatagtacctgtgatattctcaac	55
ATG2-Tm2F	F	atgaattattgtgtttacaagacttg	55
TGA-Tm2R	R	tgatattctcaactttgcaagc	55
GSP1-5race	R	gaacactcaaattgatgacagacatgcc	67
GSP2-5race	R	cccaaaccgggcatgccaactattg	67

Table 6. Classification of *Tm2* homologs, amplified from the resistant parents of S. neorossii (1a) and S.okadae (1b), according to a restriction pattern and NBS3B homology (marker closely linked to both *R* loci).

1a

S. neorossii		
Digestion pattern groups	Clone	NBS3B groups
1	24	non-NBS3B-like
2	22	
	23	
3	25	NBS3B-like
	27	
	28	
	29	
	30	
	31	
	8bis	

1b

S. okadae		
Digestion pattern groups	Clone	NBS3B groups
1	7	NBS3B-like
	8	
	9	
	10	
	11	
	12	
	13	
	14	
	16	
	17	
	20	
	21	
	1bis	
	6bis	
	7bis	
2	2	non-NBS3B-like
	3	
	4	
	3bis	
	4bis	
3	1	
	5	
	6	
	5bis	
4	19	
5	18	

EXAMPLE 4A

TRANSIENT COMPLEMENTATION IN NICOTIANA BENTHAMIANA

Depending on the resolution of relevant genetic mapping studies and the size of the candidate gene family, an allele mining approach can generate many candidate genes which need to be functionally analyzed. To date functional analyses of candidate R gene homologues (RGH) typically require stable transformation of a susceptible genotype for complementation purposes. This is a time consuming and inefficient approach as it takes several months at the least to generate transgenic plants that can functionally be analyzed. In the current study, we have exploited the finding that *Nicotiana benthamiana* is susceptible to *P. infestans,* despite previous reports (reference Kamoun et al., 1998), to develop a *Agrobacterium* transient complementation assay (ATCA) for *R* genes that confer resistance against *P. infestans.*

Agrobacterium transient transformation assays (ATTA)

Agrobacterium transient transformation assays (ATTA) were performed in *Nicotiana benthamiana* followed by detached leaf assays's using appropriate *P. infestans* isolates. Four week old plants were infiltrated with a solution of *Agrobacterium tumefaciens* strain COR308 (Hamilton et al., 1996), harboring putative *R* gene candidates. Two days before infiltration, *A. tumefaciens* was grown over night at 30°C in LB medium with tetracycline (12.5 mg/ml) and spectinomycine or kanamycine (100 mg/ml and 50 mg/ml respectively). After 16h growth, the OD_1 was measured and 50ml of YEB medium was inoculated with x µl of LB culture and grown overnight at 30°C in order to reach an OD_2 of 0.8 the next day [$x=z/OD_1$ with z=80000 (2power (delta t/2)]. The following day, 45 ml of YEB culture was centrifuged for 8mins at 4000 rpm. The pellet was resuspended with y ml of MMA containing 1ml/L of acetosyringone. $Y=22xOD_2$ enabled the standardization of the different cultures at an OD_3 of 2.0. Every resuspended pellet was incubated for an hour at room temperature. Then the lower side of the leaf was infiltrated with MMA culture at an OD_4 of 0.1 using a 2ml syringe. Two days post infiltration, a DLA was performed as mentioned above. Infection phenotypes (resistant or susceptible) were assessed from 4 to 7 days post inoculation.

Detached leaf assays were carried out as described by Vleeshouwers et *al.* (1999) using two *P. infestans* isolates, IPO-complex which is not virulent on *Rpi-oka1, 2* or *Rpi-nrs1* and isolate EC1 which is virulent on all three genes. Leaves were inoculated with 10µl droplets of *Phytophthora infestans* inoculum ($5x10^4$ zoospores/ml) on the abaxial side and incubated at 15°C for 6 days in a climate chamber with a photoperiod of 16h/8h day/night. At 6 days post inoculation, leaves showing sporulation were scored as susceptible whereas leaves showing no symptoms or necrotic lesions were scored as resistant. Three independent transient complementation assays were carried out in triplicate with both isolates. For each replicate, leaf numbers 4, 5 and 6 when counting from the bottom of the plant, were agro-infiltrated and subsequently challenged with *P. infestans.* Five days post inoculation with IPO-C, 60-70% of the leaves transiently expressing the candidate *Rpi-oka1* or *Rpi-nrs1* genes displayed a typical HR reponse, as did the positive control plants transiently expressing the functional *Rpi-sto1* gene (Vleeshouwers et al, 2008), although in the latter case complementation efficiency was significantly higher (80-90% of the challenged leaves showed an HR) . In contrast, leaves expressing *abpt*GH-a, a non-functional paralogue of *Rpi-abpt* (Lokossou et al., in preparation) were fully susceptible. In the case of EC1, all agro-infiltrated leaves were susceptible except for those infiltrated with *Rpi*-sto1, which confers resistance to EC1.

These data matched with the resistance spectrum of *Rpi-oka1* and *Rpi-nrs1* and therefore suggested that the candidate genes represented *Rpi-oka1* and *Rpi-nrs1*.

EXAMPLE 4B

Complementation analysis through stable transformation of cv. Desiree

To confirm the results obtained with the transient complementation assays in *N. benthamiana,* the binary Gateway constructs harbouring *Tm2*GH-oka1b and *Tm2*GH-oka1.9 were transferred to the susceptible potato cultivar Desiree through *Agrobacterium* mediated transformation. As a control we also transformed cv. Desiree with construct pSLJ21152, a binary construct harbouring a 4.3 kb fragment carrying the putative *Rpi-oka1* promoter, ORF and terminator sequence (see EXAMPLE 6). Primary transformants harbouring the transgenes of interest were tested for resistance to *P. infestans* in detached leaf assays. Surprisingly, only the genetic construct harbouring the 4.3 kb *Rpi-oka1* fragment was able to complement the susceptible phenotype; 8 out of 9 primary transformants were resistant. All 22 *Tm2*GH-oka1b and 17 *Tm2*GH-oka1.9 containing primary transformants were susceptible to *P. infestans*.

Alignment of the *Tm2*GH-oka1b and *Tm2*GH-oka1.9 sequences to the 4.4 kb *Rpi-oka1* fragment revealed the presence of an additional in-frame ATG start codon 99 nt upstream from the start codon that was initially used as basis for the allele mining experiments. This finding, together with the negative complementation results obtained with the *Tm2*GH -oka1b and *Tm2*GH-oka1.9 and the positive complementation result with 4.3 kb *Rpi-oka1* fragment suggested that the 5' most start codon represents the actual start of the functional *Rpi-oka1* and *Rpi-nrs1* ORFs.

Transient complementation assays using 5' extended allele mining products

In an attempt to mine the putatively full-length *Rpi-oka1* and *Rpi-nrs1* genes from oka7014-9 nrs365-1, respectively, genomic DNA of both genotypes was subjected to long range PCR using the primers ATG2-Tm2F and TAA-8bR (Table 2). Amplicons of the expected size were cloned into the pGEM®-T Easy vector and sequenced. Clones obtained from oka7014-9 were all the same and identical to the corresponding sequence in pSLJ21152 (see EXAMPLE 6). Clones obtained from nrs365-1 were also all identical but contained an insertion of 42 nt in the 5' extended region compared to those obtained from oka7014-9. Both sequences were subsequently inserted into the Gateway® binary expression vector in between the regulatory elements of the *Rpi-blb3* gene (Lokossou et al., in preparation) and

targeted for transient complementation analysis in *N. benthamiana*, together with the original *Tm2*GH-oka1b and *Tm2*GH-oka1.9 constructs and pSLJ21152. Both full-length genes and the 4.3 kb *Rpi-oka1* gene showed comparable resistance levels as the positive control *Rpi-sto1* (80-90% of the challenged leaves showed an HR response), whereas the shorter gene constructs again displayed significantly lower levels of resistance (60-70% HR), indicating that the full-length amplicons derived from oka7014-9 and nrs365-1 represent *Rpi-oka1* and *Rpi*-nrs1, respectively.

EXAMPLE 5 - IDENTIFICATION, MAPPING AND CLONING OF *Rpi* GENES FROM *S. mochiquense*

Mapping *Rpi* genes in *S. mochiquense*

Rpi-mcq1

Rpi-mcq1 was previously mapped generally to the bottom of the long arm of chromosome IX (Smilde et al, 2005) although no fine mapping or characterisation was disclosed. Flanking markers that span a distance of 20 cM were developed in addition to a marker (TG328) that co-segregated with *Rpi-mcq1* in a population of 68 individuals. To fine map *Rpi-mcq1*, a total of 72 AFLP primer combinations were used to look for more closely linked markers. One polymorphic band P13M32_472 was identified to map on the southern side of the gene. An additional 5 CAPS markers (Table 7) were developed from released sequence of the tomato BAC clones C09HBa0165P17 and other known RFLP markers from chromosome IX within the SGN database. In this way, *Rpi-mcq1* was mapped to a 11.6 cM region, flanked by markers T0156 and S1d11, and co-segregating with CAPS markers TG328, U286446, U296361, and TG591 (Fig. 10).

Table 7 CAPS markers used for mapping of *Rpi-mcq1*

Marker	Primer sequence (5'-3')	Tm (°C)	Restriction enzymes
T0156	F: AAGGCAGGAACAAGATCAGG	55	*Rsa*I
	R: TTGACAGCAGCTGGAATTG		
TG328	F: AATTAAATGGAGGGGGTATC	50	*Alu*I
	R: CCTTTGAATGTCTAGTACCAG		
U296361	F: CAGAAGCAGCTGACTCCAAA	55	*Hin*cII
	R: TTCAACAGTGAGAGAGCCACA		
U286446	F: GCACAAGCACAGTCTGGAAA	55	*Hae*III
	R: GCTGCATTAATAGGGCTTGC		
TG591	F: TACTCGTGCAAGAAGGAACG	55	*Hae*III, *Hpa*II
	R: CCAACTTGTTTGGCTATGTCA		
U272857	F: GTGGTCTTTTGAGGCAGAGC	55	*Xho*I
	R: AGATTCGCCGTCTGTGAAGT		
9C23R	F: TCTTGCCAAGCAGGTCTTTT	55	*Hin*fI
	R: CAGCCATTAGGCATTTGACA		
S1d11	F: CTGGTCCTATAGGGTTACCATT	55	*Apo*I
	R: AGAACCGCACCATCATTTCTTG		
T0521	F: CCACTTCACCCACCTGGTAT	55	*Hae*III
	R: AGCTTTGCAGACATTACATGG		

High resolution mapping and cloning of *Rpi-mcq1*

BAC library screening

In the K182 library, the marker U279456 was amplified from nine pools (Table 1a). This marker is allele specific and is linked to the susceptible haplotype in K182. The other three markers (U282757, U296361 and TG591) are CAPS markers and thus restriction digestion was used to assign haplotype to the identified BACs. Analysis of these three markers showed that between three and seven BAC pools contained BAC clones with the marker alleles from the resistant haplotype (Table 1a, Fig. 4d-4e).

High resolution mapping and cloning of *Rpi-mcq1*

Eight BAC pools from the K182 library which were positive to U282757, U296361, TG591 or U279465, markers which are closely linked to *Rpi-mcq1* were randomly chosen. The original 384-well plates for each of the BAC libraries were replicated onto solid LB medium. Colonies from each plate were scraped by rows and columns and screened for the presence of the relevant marker. Single clones from 384-well plates were selected. One of the BAC-end sequences from the clones selected from the K182 library was highly similar to the gene *Tm-2*^{2}. Additionally two of the BAC-end sequences for the K182 library were similar to several different resistance proteins identified in *Solanum tuberosum*, *Malus baccata*, *Populus balsamifera*, *Populus trichocarpa*, *Medicago truncatula* and *Lens culinaris*.
An expanded population consisting of 2502 individuals was used to identify recombinants between the flanking markers T0156 and S1d11. A total of 163 recombinants were found and used for analysis the co-segregating markers TG328, U286446, U296361, and TG591. As a result, *Rpi-mcq1* was mapped to a 0.32 cM region of chromosome IX, flanked by markers U286446 and 9C23R (marker developed from a BAC end sequence), and co-segregating with markers U272857 and TG591 (Fig 10). Based on the order of the CAPS markers, the corresponding BAC clones were ordered and used to construct a contig which was spanned from 9C23R to TG328 (Fig 11). The region predicted to contain *Rpi-mcq1* was covered by two overlapping BAC clones, 9C23 and 43B09, which were identified from the resistant haplotypes. These two BAC clones were sequenced and subcloned into the binary cosmid vector pCLD04541 by partial digestion using *Sau*3AI. Analysis of the BAC sequence indicated that it contains 2 complete ORFs and 2 incomplete ORFs which are similar to the Tm2^2 resistance gene against ToMV. The 2 complete ORFs were predicted to be the candidates for *Rpi-mcq1* and cosmid clones containing these two ORFs (*Rpi-mcq1.1* and *Rpi-mcq1.2*) were identified and introduced into the susceptible potato variety Desiree, tomato cultivar Moneymaker and *N. benthamiana* by *Agobacterium*-mediated transformation.

Analysis of the *Rpi-mcq1* ORFs

We sequenced the two candidate BAC clones 9C23 and 43B09. BAC clone K182_43B09 is 103,863 bp long and has been completely sequenced. BAC clone K182_9C23 has been sequenced to 3 contigs, K182_9C23_2699 (62,389 bp), K182_9C23_2732 (22,072 bp), and K182_9C23_2737 (10,119 bp). After alignment between the BAC sequences, K182_43B09 was found to contain all of

K182_9C23_2737 and approximately half of K182_9C23_2732 which indicates the overlapping regions of the two BAC clones.

K182_9C23_2699 was found to contain 3 ORFs and K182_9C23_2732 and K182_9C23_2737 each contained 1 ORF longer than 300 bp. The ORFs from K182_9C23_2732 and K182_9C23_2737 were identical to the first and second ORFs from K182_43B09. The first ORF of K182_9C23_2699 encoded a putative NAD dependent epimerase (same gene as CAPS marker U272857). The second encoded a complete CC-NBS-LRR type plant resistance gene protein which is highly similar to ToMV resistance gene *Tm2*^{2} and thus is a candidate gene for *Rpi-mcq1* (*Rpi-mcq1.1*). The third ORF encoded an incomplete *Tm2*^{2}-like protein which contained a partial NBS motif and complete LRR motif. Also there are 5 additional ORFs predicted from the sequence of K182_43B09. The first and third ORFs were predicted to encode RNA-directed DNA polymerases and retrotransponson proteins, the fifth ORF encoded the same gene as CAPS marker U296361. These are not considered to be *Rpi-mcq1* candidates as such proteins are not associated with a plant resistance gene function. The second and fourth ORFs are similar to the resistance gene *Tm2*^{2}. The second ORF encoded a complete gene in which can be found all CC, NB and LRR domains; this was regarded as a second candidate for *Rpi-mcq1* (*Rpi-mcq1.2*). The fourth ORF was predicted to encode a truncated protein due to an early stop codon at amino acid 110.

The two candidate genes for Rpi-mcq1 were subcloned into binary cosmid vector pCLD04541. *Rpi-mcq1.1* is 2,589 bp long and predicted to encode a CC-NB-LRR protein of 862 amino acids with a calculated molecular weight of 98.2 kDa and a pI of 7.75. The coiled-coil (CC) domain is located in the N-terminal part of the protein between amino acids 1 and 173. In the first 173 residues 12 putative heptad motifs composed of hydrophobic residues could be recognized. A NB-ARC domain which contains all the characterized motifs was present in the amino acid sequence from 173 to 478 (van der Biezen & Jones, 1998). The LRR domain was present in the amino acid sequence from 479 to 862 which comprises a series of 15 LRR motifs of irregular size that can be aligned according to the consensus sequence LxxLxxLxxLxLxxC/N/Sx(x)LxxLPxx (where x is any amino acid) (McHale *et al.*, 2006). The NB-ARC motif 5 (amino acid postions 481-485) overlaps with the start of the LRR domain.

Rpi-mcq1.2 is 2,571 bp long and predicted to encode a CC-NBS-LRR protein of 856 amino acids with a calculated molecular weight of 98.0 kDa and a pI of 7.81. The coiled-coil (CC) domain is located in the N-terminal part of the protein between amino acids 1 and 170. The NB-ARC domain is present in the amino acid sequence

from 171 to 476 and contains all characterized motifs (van der Biezen & Jones, 1998). The LRR domain was present in the amino acid sequence from 477 to 856 which comprises a series of 15 LRR motifs of irregular size that can be aligned according to the consensus sequence LxxLxxLxxLxLxxC/N/Sx(x)LxxLPxx (where x is any amino acid) (McHale *et al.*, 2006). The NB-ARC motif 5 overlaps with the start of the LRR domain.

Rpi-mcq1.1 and *Rpi-mcq1.2* are approximately 77% and 75% identical, respectively, to the $Tm2^2$ protein at the amino acid level, and 81% identical to each other.

EXAMPLE 6

INTRODUCTION OF NOVEL RESISTANCE GENES INTO POTATO AND TOMATO GENOTYPES SUSCEPTIBLE TO PHYOPHTHORA INFESTANS AND INTO N. BENTHAMIANA

Binary vector with the *Rpi-oka1* gene under the control of its own promoter and terminator (the original gene)

A 4.3kb fragment (SEQ. ID. No. 1c) carrying the *Rpi-oka1* promoter, open reading frame (ORF) and terminator was amplified by PCR using the primers oka1longF (5'-AGTTATACACCCTACATTCTACTCG-3') and oka1longR (5'-CTTTGAAAAGAGGCTTCATACTCCC-3') from the BAC clone K39_26619. This fragment was cloned into pGEM-T Easy (Promega) and sequenced to confirm no mistakes had been introduced during PCR. The resulting plasmid was digested with *Eco*RI and the fragment containing the original gene cloned into the *Eco*RI site of pBin19. The resulting plasmid was named pSLJ21152. Plasmid pSLJ21152 was introduced into *Agrobacterium tumefaciens* strain AGL1.

Binary vector with the *Rpi-mcq1.1*and *Rpi-mcq1.2* genes under the control of their own promoters and terminators (the original genes)

The BAC clones K182_9C23 and K182_43B09 are subcloned by partial digestion using *Sau*3AI into the *Bam*HI site of the binary cosmid vector pCLD04541. The recombinant cosmid vector is packaged using GigaPack Gold (Stratagene) and introduced into *E. coli* strain DH5α. Resulting clones are screened by PCR using primers TG591-F and TG591-R to identify clones containing *Rpi-mcq1* candidates and positive clones are selected for end-sequencing. Clones carrying the two *Rpi-*

mcq1 candidates are identified by reference to the full BAC sequences of BAC clones K182_9C23 and K182_43B09. Clone pSLJ21153 carries the full sequence of *Rpi-mcq1.1* including the promoter and terminator sequences (SEQ. ID. No 2c). This clone also contains an additional resistance gene homologue which lacks a coiled-coil domain and part of the NBS domain and is therefore presumend to be non-functional. To confirm this, an additional clone designated D5 which contains this truncated gene in the absence of *Rpi-mcq1.1* is also identified. Clone pSLJ21148 carries the full sequence of *Rpi-mcq1.2* including the promoter and terminator sequences (SEQ. ID. No 2d). The clones carrying the full-length candidate *Rpi-mcq1* genes and the truncated gene are introduced into *Agrobacterium tumefaciens* strains AGL1 and LBA4404. To ensure no rearrangements of the plasmids have occurred, plasmid is isolated from resulting transconjugants, transformed back into *E. coli* strain DH10-β, digested and compared with digests of the original plasmid stocks.

Potato Transformation

Agrobacterium tumefaciens culture(s) with the appropriate antibiotic selection regime are set up and grown for 24 hours with shaking at 28 °C. Stem internode sections (without nodes) are harvested from 4-6 week old potato cv. Desiree plants grown in aseptic culture on MS medium (2% sucrose). The internodes are sliced into 1cm sections and placed into 20ml of LSR broth. 100ul of overnight *Agrobacterium tumefaciens* culture is added to stem sections and incubated for 20 minutes at 40rpm in the dark at 24 °C. The stem sections are removed from the *Agrobacterium tumefaciens* suspension, blotted dry and incubated under low light conditions at 18C for 3 days on LSR1 solid media (around 15-20 explants are plated per dish). Co-cultivated stem sections are then transferred to LSR1 medium with selection antibiotics at around 10 explants per dish. Stem explants are subcultured onto fresh LSR1 media every 7-10 days for around 3-6 weeks or until the appearance of the first small calli. Once the calli have sufficiently developed the stem sections are transferred onto LSR2 media with selection antibiotics. Stem sections are subcultured every 7-10 days until shoots start to develop. Shoots appear within 2 months from the start of transformation. Shoots are removed with a sharp scalpel and planted into MS2R solid media with selection antibiotics. Transgenic plants harbouring appropriate antibiotic or herbicide resistance genes start to root normally within 2 weeks and are weaned out of tissue culture into sterile peat blocks before being transplanted to the glasshouse.

Media

MS Medium for Potato Plantlets

1X Murashige and Skoog medium
2% Sucrose
0.6% Agarose
100mg/L casein acid hydrolysate
pH 5.7

LSR Broth

1X Murashige and Skoog medium
3% Sucrose
pH 5.7

LSR1 Medium

1X Murashige and Skoog medium
3% Sucrose
2.0mg/L zeatin riboside
0.2mg/l NAA
0.02mg/L GA_3
0.6% Agarose
pH 5.7

LSR2 Medium

1X Murashige and Skoog medium
3% Sucrose
2.0mg/L zeatin riboside
0.02mg/l NAA
0.02mg/L GA_3
0.6% Agarose
pH 5.7

MS2R

1X Murashige and Skoog medium
2% Sucrose
100mg/L myo-inositol
2.0mg/L glycine

0.2% Gelrite

pH 5.7

Media	***Agrobacterium tumefaciens* strain**	**Antibiotics**	**T-DNA marker**	**Selection antibiotic/ herbicide**
LSR1/LSR2/ MS2R	GV3101/ LBA4404	Cefotaxime/ Augmentin at 250mg/L	*nptII*	Kanamycin at 100mg/L
	AgI1	Timentin at 320mg/L	*bar*	Phosphinothricin at 2.5mg/L

Tomato Transformation

Tomato seeds are surface sterilised for 2 minutes in 70 % ethanol to loosen gelatinous seed coat and then rinsed once with sterile water. The seeds are then sterilised in 10 % domestic bleach (e.g. Domestos/Vortex) solution for 3 hours with shaking and washed 4 times in sterile water. Seeds are put into tubs (20-30 seeds / tub) containing germination medium and incubated at 25 °C in a culture room (16 hour photoperiod, supplemented with Gro-Lux or incandescent light). The seedlings are grown for 7-10 days and used at a stage when cotyledons are young and still expanding and no true leaf formation is visible. Ten millilitres of minimal A medium containing the appropriate antibiotics is inoculated with *A. tumefaciens* strain LBA4404 and grown with shaking at 28C. One millilitre of fine tobacco suspension culture is placed onto plates containing the cell suspension medium solidified with 0.6% agarose or MS medium amended with 0.5mg/L 2,4-D and 0.6% agarose. Cells are spread around to give an even layer and plates are placed unsealed and stacked in the culture room at 25 °C in 1ow light until the following day. A piece of Whatman no.1 filter paper is placed on top of the feeder plates, taking care to exclude any air bubbles and ensuring that the paper is completely wetted. Cotyledons are used for transformation as hypocotyls give rise to a high number of tetraploids. In a petri dish, the tips are cut off cotyledons and then two more transverse cuts are made to give two explants of about 0.5 cm long. Explants are transferred to a new petri dish of water to prevent any damage during further cutting. Once a number of explants are collected in the pool, they are blotted dry on sterile filter paper and placed about 30-40 on a feeder plate, abaxil surface uppermost (upside down). Petri-dishes are

placed unsealed and stacked at 25 °C under low light intensity for 8 hours. The *Agrobacterium* culture is spun down and resuspended in MS medium containing 3% sucrose to an OD_{600} of 0.4-0.5. The bacterial suspension is transferred to a petri dish and the explants from one feeder plate are immersed in the suspension. These are then removed and blotted on sterile filter paper before returning them to the original feeder plate, again taking care not to damage the tissue. No particular period of time is required in the bacteria, just enough time to ensure that the pieces have been completely immersed. Plates are returned to the same conditions as used in the pre-incubation phase (25 °C under low light intensity and co-cultivated for 40 hours. The explants from the feeder layers are placed (12 explants per Petri dish) onto tomato regeneration plates containing Augmentin or carbenicillin at 500ug/ml and kanamycin at 100ug/ml to select for the T-DNA transformation marker. The cotyledons are placed right side upwards so that they curl into the medium ensuring good contact between the cut edges of the leaf and the nutrients and antibiotics in the medium. Agargel is used as the setting agent as it produces a soft medium into which the pieces can be pushed gently. Plates are left unsealed and returned to the previous culture conditions (25 °C under low light intensity). Explants are transferred to fresh medium every 2-3 weeks. Once regenerating material is too large for petri dishes it is put into larger screw capped glass jars. Shoots are cut from the explants and put into rooting medium with Augmentin at 200ug/ml and kanamycin at 50ug/ml. To transfer to soil, as much of the medium as possible is removed by washing the roots gently under running water. Plant are transferred carefully to hydrated, autoclaved Jiffy pots (peat pots) and kept enclosed to maintain high humidity while in the growth room. Humidity is gradually decreased. Once roots can be seen growing through the Jiffy-pots the plants are transferred to the glasshouse.

REGENERATION

/Litre	
MS salts	1x
myo-inositol	100mg
Nitsch's vitamins	1ml of 1000X stock
Sucrose	20g
Agargel	4g
pH 6.0 (KOH)	
Autoclave	
Zeatin Riboside (trans isomer)	2mg
(Filter sterilise and add after autoclaving)	

Nitsch's Vitamins

	mg/l	Final conc. 1000x stock (mg/100ml)
Thiamine	0.5	50
Glycine	2.0	200
Nicotinic acid	5.0	500
Pyridoxine HCl	0.5	50
Folic acid	0.5	50
Biotin	0.05	5

At 1000x not all vitamins go into solution. Keep at 4°C and shake before using.

Rooting

	/Litre
MS medium	0.5X
Sucrose	5g
Gelrite	2.25g
pH 6.0 (KOH)	

Media

Seed Germination

	/Litre
MS medium	1x
Glucose	10g
Agarose	6g
pH 5.8	

Pour into round Sigma 'margarine' tubs.

Minimal A	/Litre
K_2HPO_4	10.5g
KH_2PO_4	4.5g
$(NH_4)_2SO_4$	1.0g
Na citrate.$2H_2O$	0.5g

Autoclave in 990ml

Before use add; 1.0ml of 1M $MgSO_4.H_2O$

10ml of 20% Glucose

For plates;
Make the above in 500ml and autoclave.
Separately autoclave 15g Bactoagar in 490 ml H_2O
Add $MgSO_4$ and glucose and combine.

Nicotiana benthamiana Transformation

N. benthamiana plants are grown until they are 10-20cm high, but before they start to flower. *Agrobacterium tumefaciens* cultures are initiated with the appropriate antibiotic selection regime and grown for 24 hours with shaking at 28 °C. The following day, the *A. tumefaciens* cultures are spun down and resuspended in Murashige and Skoog medium containing 3% sucrose. Young *N. benthamiana* leaves (up to 10cm in diameter) are harvested and surface sterilised in 1% fresh sodium hypochlorite containing a few drops of Tween 20 to act as a surfactant for 20 minutes. The leaves are then washed well in sterile water, cut into 1-2cm squares with a sharp scalpel and immersed into the *Agrobacterium tumefaciens* suspension. Ensuring that all the leaves have been fully wetted, they are then briefly blotted dry and placed onto co-cultivation medium for 3 days. Following this, co-cultivated leaf pieces are transferred onto selection medium with appropriate antibiotics at around 10 explants per dish. Explants are subcultured onto fresh media every 7-10 days for around 1-2 months until the appearance of the first shoots. Shoots are removed with a sharp scalpel and planted into rooting media with selection antibiotics.Transgenic plants harbouring appropriate antibiotic or herbicide resistance genes start to root normally within 2 weeks and can be weaned out of tissue culture into sterile peat blocks before being transplanted to the glasshouse.

MS Broth
1X Murashige and Skoog medium
3% Sucrose
pH 5.7

Co-cultivation Medium
1X Murashige and Skoog basal salt mixture
1X Gamborg's B5 vitamins
3% Sucrose
0.59g/L MES

1.0mg/L BAP
0.1mg/l NAA
0.6% Agarose
pH 5.7

Selection Medium

1X Murashige and Skoog basal salt mixture
1X Gamborg's B5 vitamins
3% Sucrose
0.59g/L MES
1.0mg/L BAP
0.1mg/l NAA
0.4% Agargel
pH 5.7

Rooting Medium

½ strength Murashige and Skoog medium
0.5% Sucrose
0.25% Gelrite
pH 5.8

Media	*Agrobacterium tumefaciens* strain	Antibiotics		T-DNA marker	Selection antibiotic/ herbicide
Selection Medium/ Rooting Medium	GV3101/ LBA4404	Cefotaxime / Augmentin at 500mg/L		*nptII*	Kanamycin at 100mg/L
	Agl1	Timentin at 320mg/L		*bar*	Phosphinothricin at 2.0mg/L
				hgh	Hygromycin at 10mg/L

Complementation analysis (*Rpi-oka1*).

A total of 37 *S. tuberosum* cv. Desiree plants capable of growth on kanamycin were selected as putative *Rpi-oka1* transformants. Following transfer to the glasshouse, leaves were excised and used in a detached leaf assay with *P. infestans* isolates 90128 and 'Superblight' to determine whether the transgene conferred blight resistance. Of the 37 transformants, 31 were confirmed as being resistant and did not show any signs of blight infection. Some plants exhibited signs of a hypersensitive response localised to the inoculation site. The remaining 6 plants were susceptible to both isolates, as was the control (non-transformed Desiree). The phenotype of the transgenic plants correlated exactly with amplification of the *Rpi-oka1* ORF by PCR, all plants from which the *Rpi-oka1* could be amplified were confirmed as resistant. The *Rpi-oka1* transgene also conferred resistance to a range of *P. infestans* isolates as detailed in Table 6.1. All transgenic plants tested were susceptible to isolate EC1, showing that the specificity of *Rpi-oka1* was retained in the transgenic plants and that the resistance phenotype was not due to constitutive activation of defence pathways by the transgene.

Transgenic tomato cv. Moneymaker plants carrying *Rpi-oka1* were also shown to be resistant to *P. infestans* isolate 90128. A total of 21 *S. lycopersicum* cv. Moneymaker plants capable of growth on kanamycin were selected as putative *Rpi-oka1* transformants. Following transfer to the glasshouse, leaves were excised and used in a detached leaf assay with *P. infestans* isolate 90128 to determine whether the transgene conferred blight resistance. Of the 21 transformants, 13 were confirmed as being resistant and did not show any signs of blight infection. Some plants exhibited signs of a hypersensitive response localised to the inoculation site. The remaining 8 plants were susceptible isolates 90128, as was the control (non-transformed Moneymaker). The phenotype of the transgenic plants was generally correlated with amplification of the *Rpi-oka1* ORF by PCR, most plants from which the *Rpi-oka1* could be amplified were confirmed as resistant. However, two plants contained *Rpi-oka1* as determined by PCR, yet were susceptible indicating that the transgene had either been silenced or was inserted into a transcriptionally inactive region of the recipient tomato genome. All plants from which *Rpi-oka1* could not be amplified were susceptible to *P. infestans.*

Spectrum of *P. Infestans* isolates against which *Rpi-oka1* confers resistance

Detached leaves of transgenic potato cv. Desiree carrying *Rpi-oka1* were inoculated with a range of *P. infestans* isolates (Table 6.1) to determine the range of isolates against which *Rpi-oka1* confers resistance. Of the 11 isolates tested, only isolate EC1 from Ecuador was able to overcome *Rpi-oka1* and cause disease on the inoculated plants.

Complementation analysis (*Rpi-mcq1.1* and *Rpi-mcq1.2*).

A total of 22 and 20 putative transgenic lines of *S. tuberosum* cv Desiree were obtained following transformation with, pSLJ21153 (*Rpi-mcq1.1*) and pSLJ21148 (*Rpi-mcq1.2*), respectively. Following transfer to the glasshouse, detached leaf assays were done using *P. infestans* isolates 90128, EC1, Hica and IPO-complex. For construct pSLJ21153, 12 transgenic lines were shown to be resistant to isolates 90128 and EC1, but susceptible to Hica and IPO-complex. For construct pSLJ21148, 10 transgenic lines showed enhanced resistance to Hica when inoculated at low concentrations ($1x10^4$ zoospores ml^{-1}), but were susceptible to 90128, EC1 and IPO-complex. *Rpi-mcq1.2* present in this construct also conferred partial resistant to the isolates 'Superblight' and MP618 (Table 6). Transgenic potato lines transformed with cosmid D5 which contained the truncated resistance gene honolog also present on pSLJ21153 were shown to be susceptible to all *P. infestans* isolates tested.

The two constructs (pSLJ21153 and pSLJ21148) carrying *Rpi-mcq1.1* and *Rpi-mcq1.2,* respectively were transformed into tomato cv. Moneymaker. Two lines positive for *Rpi-mcq1.1* and 11 lines positive for *Rpi-mcq1.2* were identified by PCR using gene specific primers. The two transgenic lines carrying *Rpi-mcq1.1* conferred resistant to 90128 and EC1. Transgenic Moneymaker lines carrying *Rpi-mcq1.2* or the truncated R gene homolog which is also present on construct pSLJ21153 with *Rpi-mcq1.1* (cosmid D5) were susceptible to 90128 and EC1.

Spectrum of *P. Infestans* isolates against which *Rpi-mcq1.1* and *Rpi-mcq1.2* confer resistance

A wider range of *P. infestans* isolates were tested to determine their virulence/avirulence on *Rpi-mcq1.1* and *Rpi-mcq1.2* (Table 6.1). Of 12 isolates tested, *Rpi-mcq1.1* conferred resistance to 6 isolates, and *Rpi-mcq1.2* conferred partial resistance to 3 isolates (Table 6.1).

Table 6.1. Response of *Rpi-oka1*and *Rpi-mcq1* transgenic potato plants against a range of *P. infestans* isolates

Isolate	Country of Origin	Race (if known)	*Rpi-oka1* phenotype	*Rpi-mcq1.1* phenotype	*Rpi-mcq1.2* phenotype
90128	The Netherlands	1.3.4.7.8.9.10.11	Resistant	Resistant	Susceptible
IPO-0			Resistant	Resistant	Susceptible
IPO-Complex	Belgium	1.2.3.4.6.7.10.11	Resistant	Susceptible	Susceptible
'Superblight'	United Kingdom		Resistant	Susceptible	Partially resistant
Hica	United Kingdom		Resistant	Susceptible	Partially resistant
MP717	Poland	1.2.3.4.5.6.7.9.10.11	Resistant	Resistant	Susceptible
MP778	Poland	1.3.4.5.6.7.9.10.11	Resistant	Resistant	Susceptible
MP674	Poland	1.2.3.4.5.6.7.10.11	Resistant	Susceptible	Susceptible
MP622	Poland	1.3.4.7.8.10.11	Resistant	Susceptible	Susceptible
MP618	Poland	1.2.3.4.6.7.11	No data	Susceptible	Partially resistant
MP650	Poland	1.2.3.4.5.7.8.10.11	Resistant	Resistant	Susceptible
EC1	Ecuador	2.4.10.11	Susceptible	Resistant	Susceptible

EXAMPLE 7

METHODS AND COMPOSITIONS TO AVOID DEVELOPMENT OF RESISTANCE TO NOVEL GENES

Resistance genes in wild populations are usually highly polymorphic (Jones 2001, Dangl and Jones 2001, Bergelson *et al*, 2001) and this heterogeneity is probably critical for their effectiveness, because they are subject to frequency-dependent selection (if any one *R* gene predominates, selection is intensified for pathogen races that can overcome it). In agriculture, monocultures are the norm, which facilitates an epidemic of any pathogen that can grow and rapidly reproduce on a particular crop variety. We propose that if enough *R* genes could be identified and deployed in mixtures, in genetic backgrounds that are otherwise uniform for agronomic and consumer traits, then durable resistance might be achieved in crops (Pink and Puddephat 1999, Jones 2001). For this strategy to work and to address questions related to overcoming *R* gene based resistance and evolution, it is essential to isolate as many new *Rpi* genes as possible.

This is achieved according to the present invention by isolating multiple *Rpi* genes from wild relatives of potato, introducing those genes separately into one variety, and the resulting lines mixed and planted. This strategy circumvents the problem that varietal monocultures become completely susceptible to any race of

blight that can overcome the specific *Rpi* gene in that variety, and the resulting race then dominates the parasite population. Using 3 *Rpi* genes in a mixture, any blight race that overcomes one of these *Rpi* genes could only grow on 33% of the plants in the field. An alternate strategy according to this invention comprises using the same variety, but carrying a different *Rpi* gene, each year. In this scenario, pathogen races that are successful one year are not successful or as successful the next year, resulting in reduced losses. We propose that specifically:

1. Epidemics are slower

If a strain of the *P. infestans* that is virulent on one of the *R* genes enters the crop then the spread of that strain is limited to the plants which carry that *R* gene, other plants harbouring different *R* genes will remain resistant, thus limiting the spread of the pathogen and the overall losses. It is more difficult for the pathogen to develop virulence against multiple *R* genes and thus the development of strains able to cause disease on more than one *R* gene will be minimised. As the plants with different *R* genes will be mixed there will be a spatial separation between plants with the same *R* gene that will limit the rate of spread within the field.

2. Virulence on more than 1 *Rpi* gene leads to reduced fitness compared with those that are virulent on only 1 gene

Plant *R* genes function by recognising molecules produced by particular strains of plant pathogens. The currently accepted hypothesis is that a pathogen gains virulence by loss of a gene that encodes an effector molecule that is normally required by the pathogen for overcoming host defence responses and is normally recognised by a plant *R* gene. Thus to become virulent on more than one *R* gene involves the loss by the pathogen of more than one effector molecule. Such a strain of the pathogen would be inherently less fit when competing with strains that had lost none or a single effector when growing on hosts that contain different *R* gene complements.

3. Incompatible races trigger systemic acquired resistance

In addition to *R* gene-mediated resistance responses, plants possess the ability to mount a non-specific defense response known as systemic acquired resistance (SAR). Following the recognition of a pathogen, the plant can initiate defence responses that are active against a wide range of pathogens and is not limited to the pathogen strain that was recognised by the *R* gene. Thus the recognition of a

particular pathogen strain by an introduced *R* gene will also aid in defence against other strains that may not necessarily be recognised by any introduced R genes.

4. There will be weak selection for virulence on a rare *Rpi* gene

An *Rpi* gene that is deployed infrequently within a population will exert less selection pressure on the pathogen to overcome this resistance. Such a strategy could be used to protect valuable *R* genes and ensure that their longevity is maximised by deploying them in a rational manner so that the pathogen population is not exposed to large amount of crop with the particular *R* gene. Such rational deployment will be aided by knowledge about the frequency of the corresponding virulence allele within the pathogen population.

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CLAIMS

1. An isolated Rpi resistance gene having a sequence which is at least about 85% homologous to the nucleic acid sequence provided herein as SEQ. ID. 1a, 1b, 2a, 2b or 3.

2. An isolated Rpi resistance gene as claimed in claim 1 having a sequence which is at least about 90% homologous to the nucleic acid sequence provided herein as SEQ. ID. 1a.

3. An isolated Rpi resistance gene as claimed in claim 2 which comprises the nucleic acid sequence provided herein as SEQ. ID. 1a, 1b, or 3.

4. An isolated Rpi resistance gene as claimed in claim 3 which comprises the nucleic acid sequence provided herein as SEQ. ID. 1c

5. An isolated Rpi resistance gene as claimed in claim 1 having a sequence which is at least about 86% homologous to the nucleic acid sequence provided herein as SEQ. ID. 2a, and which is optionally obtainable from *S. mochiquense*.

6. An isolated Rpi resistance gene as claimed in claim 5 which comprises the nucleic acid sequence provided herein as SEQ. ID. 2a or 2b.

7. An isolated Rpi resistance gene as claimed in claim 6 which comprises the nucleic acid sequence provided herein as SEQ. ID. 2c or 2d.

8. An isolated protein having an amino acid sequence which is at least 80% homologous to the amino acid sequence provided herein as SEQ. ID. 4a, 4b, 5a, 5c, or 6 and which is capable of mediating a response against *P. infestans*.

9. An isolated protein as claimed in claim 8 having a sequence which is at least about 90% homologous to the amino acid sequence provided herein as SEQ. ID. 4a.

10. An isolated protein as claimed in claim 9 having a sequence which comprises the amino acid sequence provided herein as SEQ. ID. 4a, 4b or 6.

11. An isolated protein as claimed in claim 8 having a sequence which is at least about 80% homologous to the amino acid sequence provided herein as SEQ. ID. 5a and which is optionally obtainable from *S. mochiquense*.

12. An isolated protein as claimed in claim 11 having a sequence which comprises the amino acid sequence provided herein as SEQ. ID. 5a or 5b.

13. An isolated gene encoding a protein as claimed in any one of claims 8 to 12.

14. A plant comprising the gene according to any one of claims 1 to 7 or claim 13 which has been introduced into said plant.

15. The plant according to claim 14 which is a potato.

16. Progeny of the plant according to claim 15.

17. A method of making a transgenic plant having enhanced late blight resistance which comprises introducing into a cell a of said plant or into a portion of said plant, a gene according to any one of claims 1 to 7 or claim 13 and generating a whole plant from said cell or from said portion of said plant.

18. A composition comprising the gene according to any one of claims 1 to 7 or claim 13 and appropriate regulatory sequences operatively linked to said gene to achieve expression thereof when placed into an appropriate *in vitro* or *in vivo* system.

19. A method for providing durable disease resistance in potato which comprises isolating multiple *Rpi* genes from wild relatives of potato, introducing said genes separately into a commercial line or variety of potato, and mixing and planting the resulting mixture of lines thus produced.

20. A method for providing durable disease resistance in potato which comprises isolating multiple *Rpi* genes from wild relatives of potato, introducing said genes separately into a commercial line or variety of potato, and each year, planting a variety carrying a different *Rpi* gene.

21 A method as claimed in claim 19 or claim 20 wherein the *Rpi* genes are as defined in any one of claims 1 to 7 or claim 13

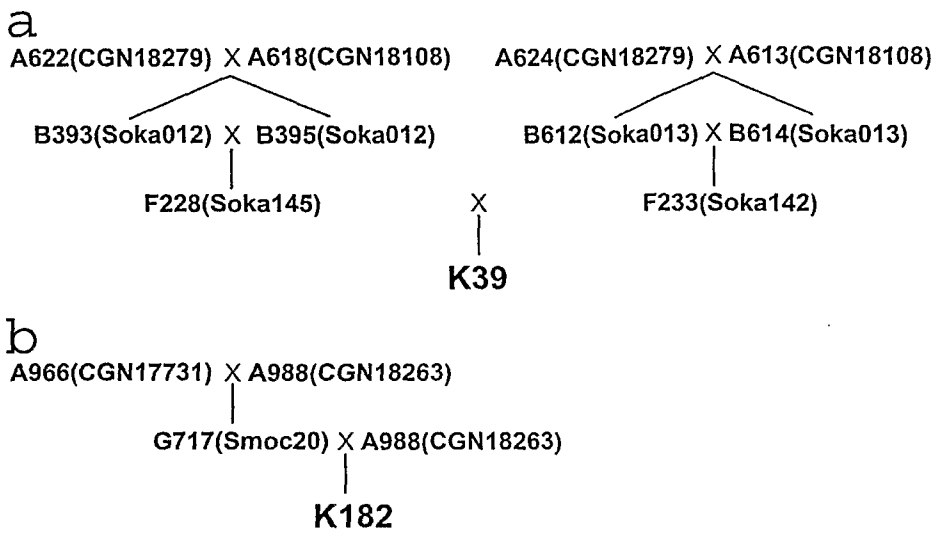

a
A622(CGN18279) X A618(CGN18108)
A624(CGN18279) X A613(CGN18108)
B393(Soka012) X B395(Soka012)
B612(Soka013) X B614(Soka013)
F228(Soka145)
X
F233(Soka142)
K39
b
A966(CGN17731) X A988(CGN18263)
G717(Smoc20) X A988(CGN18263)
K182

FIGURE 1

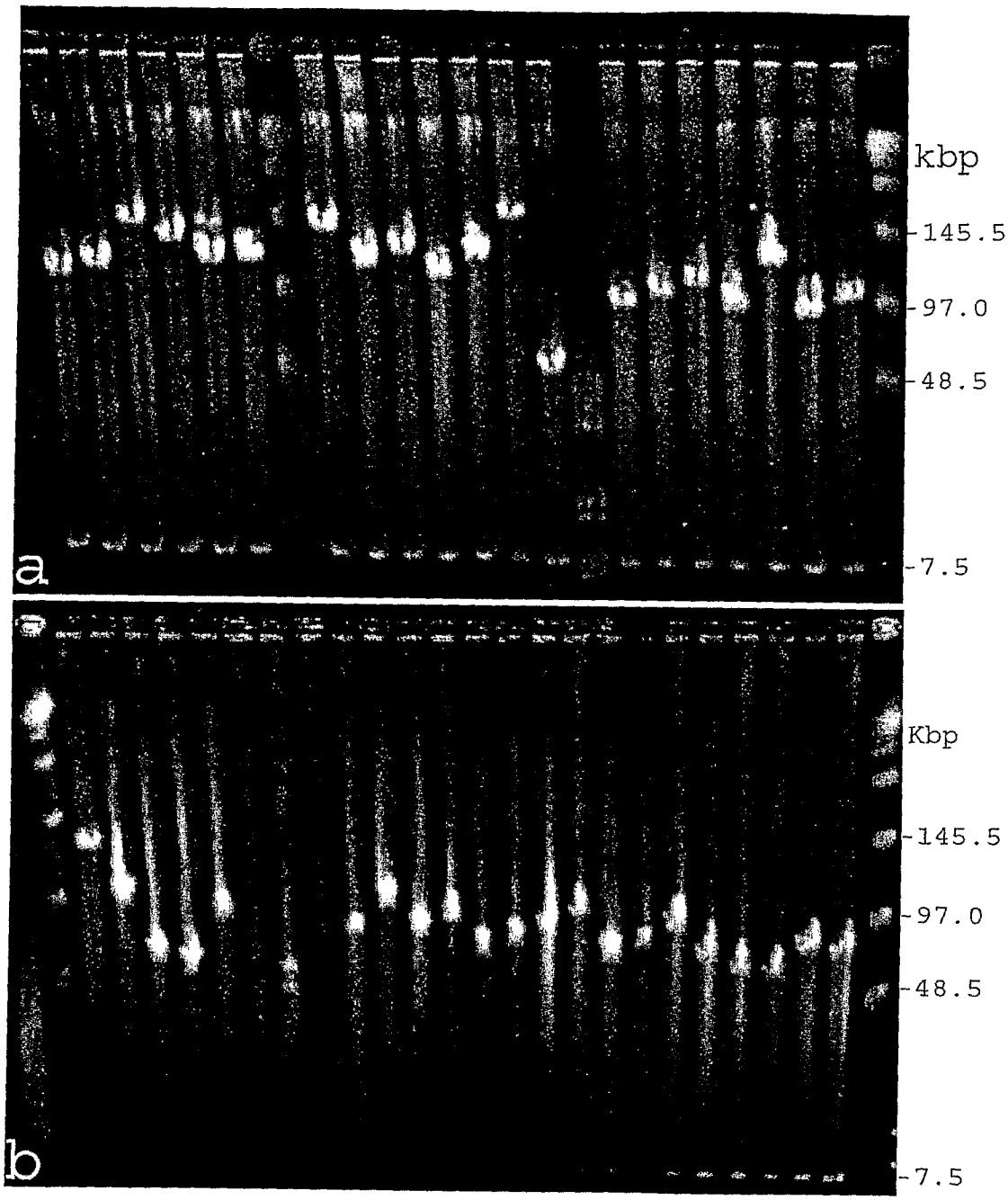
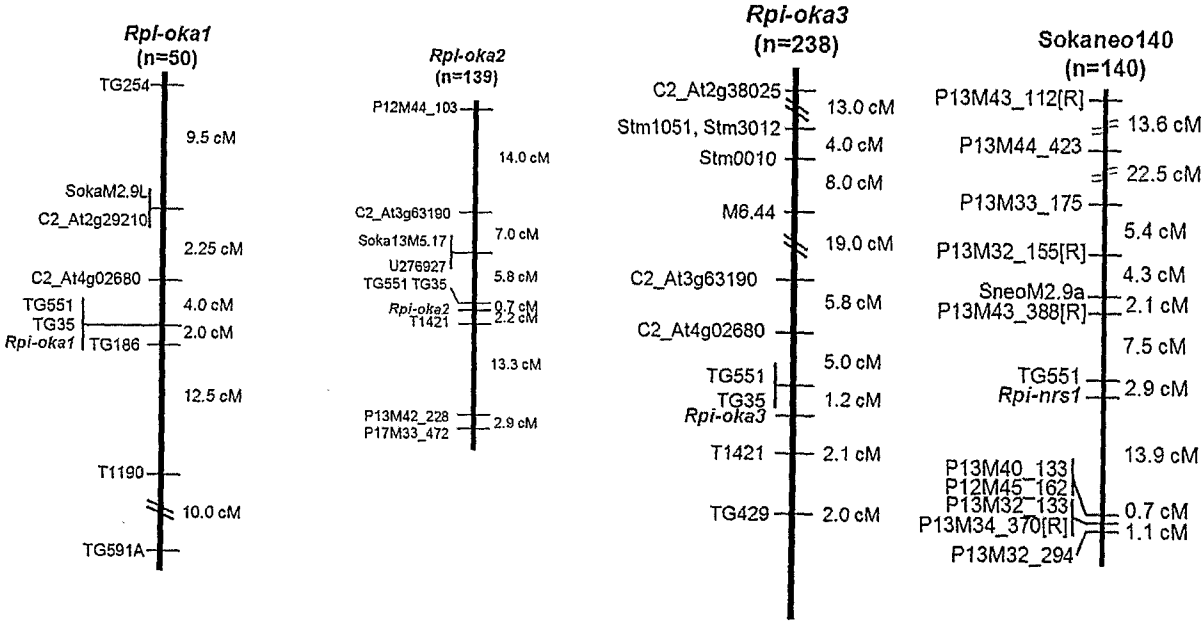


FIGURE 2

FIGURE 3


Rpi-oka1
(n=50)
TG254
9.5 cM
SokaM2.9L
C2_At2g29210
2.25 cM
C2_At4g02680
TG551
TG35
Rpi-oka1
TG186
4.0 cM
2.0 cM
12.5 cM
T1190
10.0 cM
TG591A
Rpi-oka2
(n=139)
P12M44_103
14.0 cM
C2_At3g63190
Soka13M5.17
U276927
TG551 TG35
Rpi-oka2
T1421
7.0 cM
5.8 cM
0.7 cM
2.2 cM
13.3 cM
P13M42_228
P17M33_472
2.9 cM
Rpi-oka3
(n=238)
C2_At2g38025
Stm1051, Stm3012
Stm0010
M6.44
C2_At3g63190
C2_At4g02680
TG551
TG35
Rpi-oka3
T1421
TG429
13.0 cM
4.0 cM
8.0 cM
19.0 cM
5.8 cM
5.0 cM
1.2 cM
2.1 cM
2.0 cM
Sokaneo140
(n=140)
P13M43_112[R]
P13M44_423
P13M33_175
P13M32_155[R]
SneoM2.9a
P13M43_388[R]
TG551
Rpi-nrs1
P13M40_133
P12M45_162
P13M32_133
P13M34_370[R]
P13M32_294
13.6 cM
22.5 cM
5.4 cM
4.3 cM
2.1 cM
7.5 cM
2.9 cM
13.9 cM
0.7 cM
1.1 cM

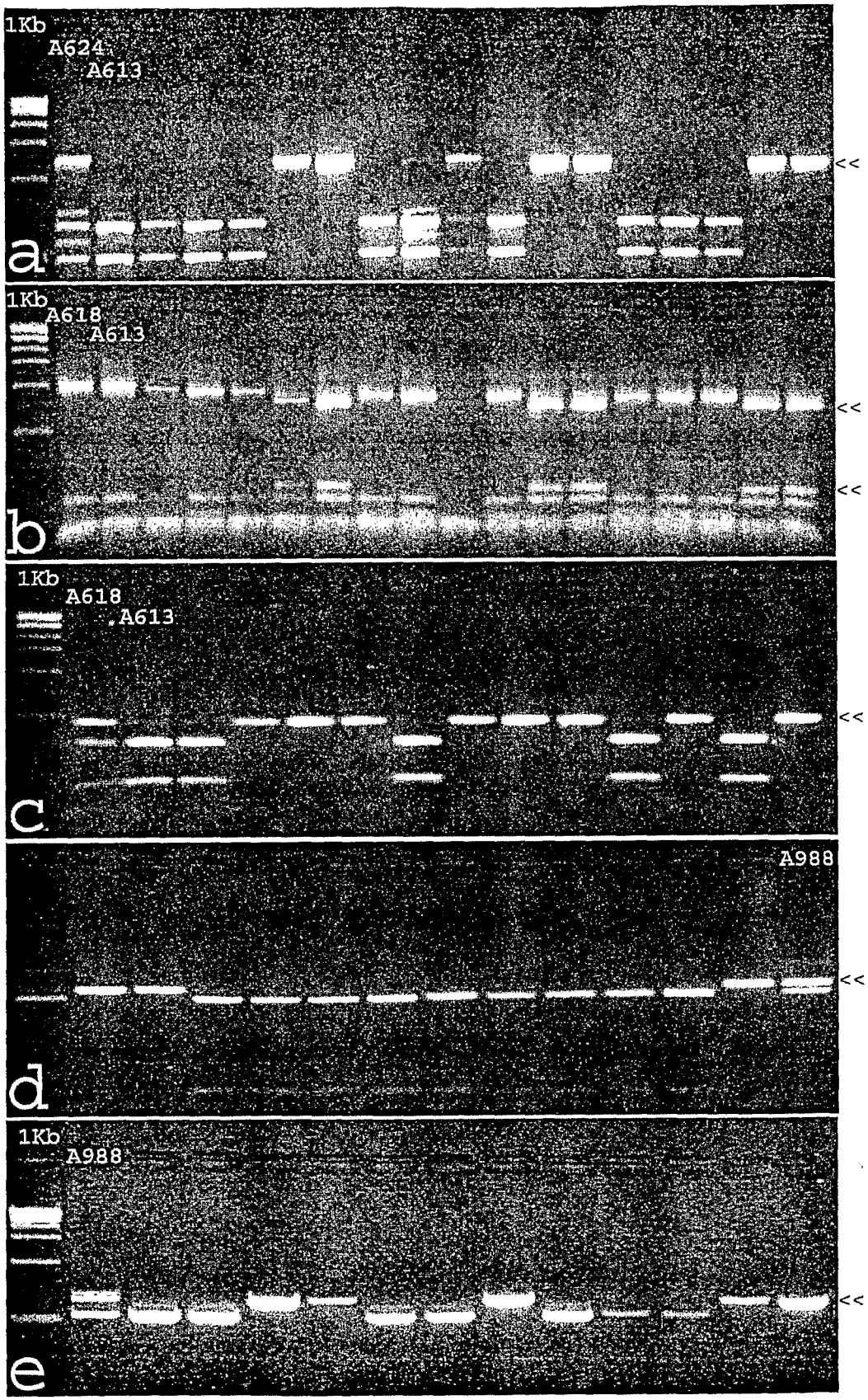


FIGURE 4

FIGURE 5

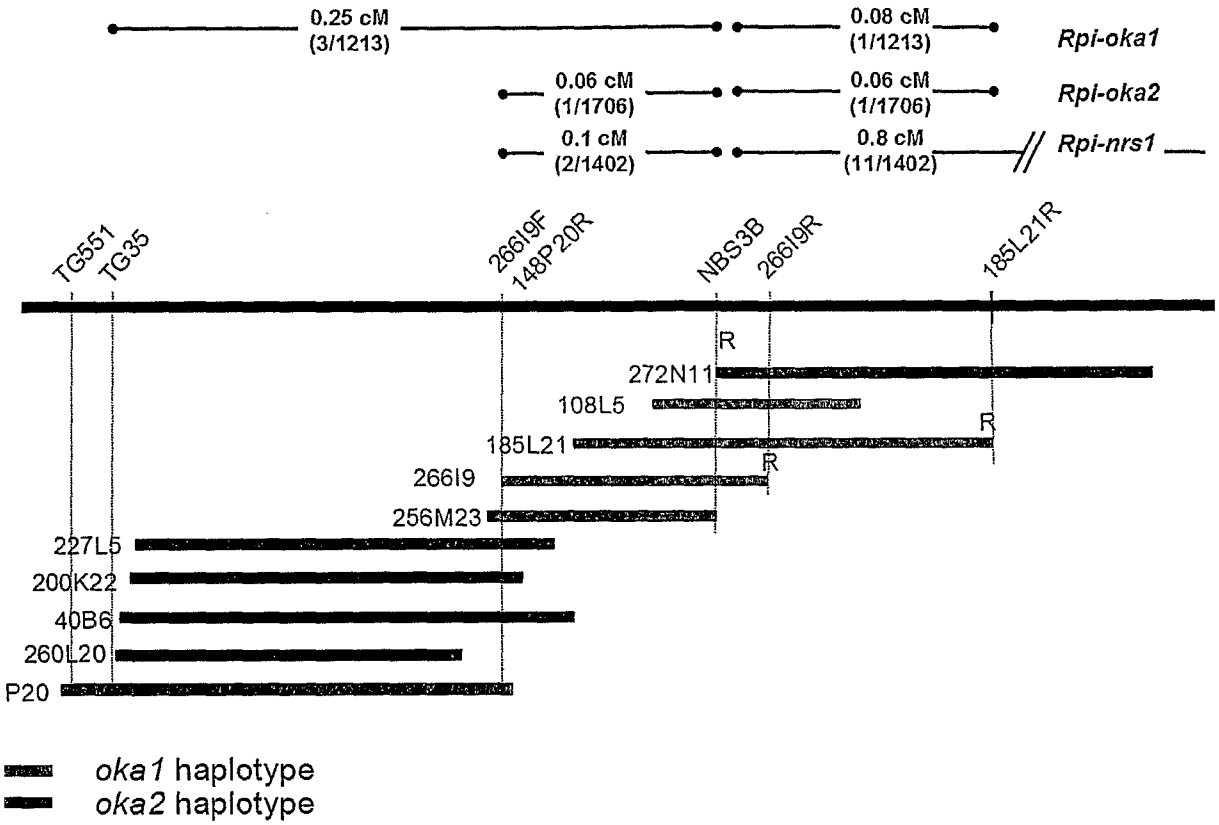

0.25 cM
(3/1213)
0.08 cM
(1/1213)
Rpi-oka1
0.06 cM
(1/1706)
0.06 cM
(1/1706)
Rpi-oka2
0.1 cM
(2/1402)
0.8 cM
(11/1402)
Rpi-nrs1
TG551
TG35
266I9F
148P20R
NBS3B
266I9R
185L21R
272N11
108L5
185L21
266I9
256M23
227L5
200K22
40B6
260L20
P20
R
oka1 haplotype
oka2 haplotype

FIGURE 6

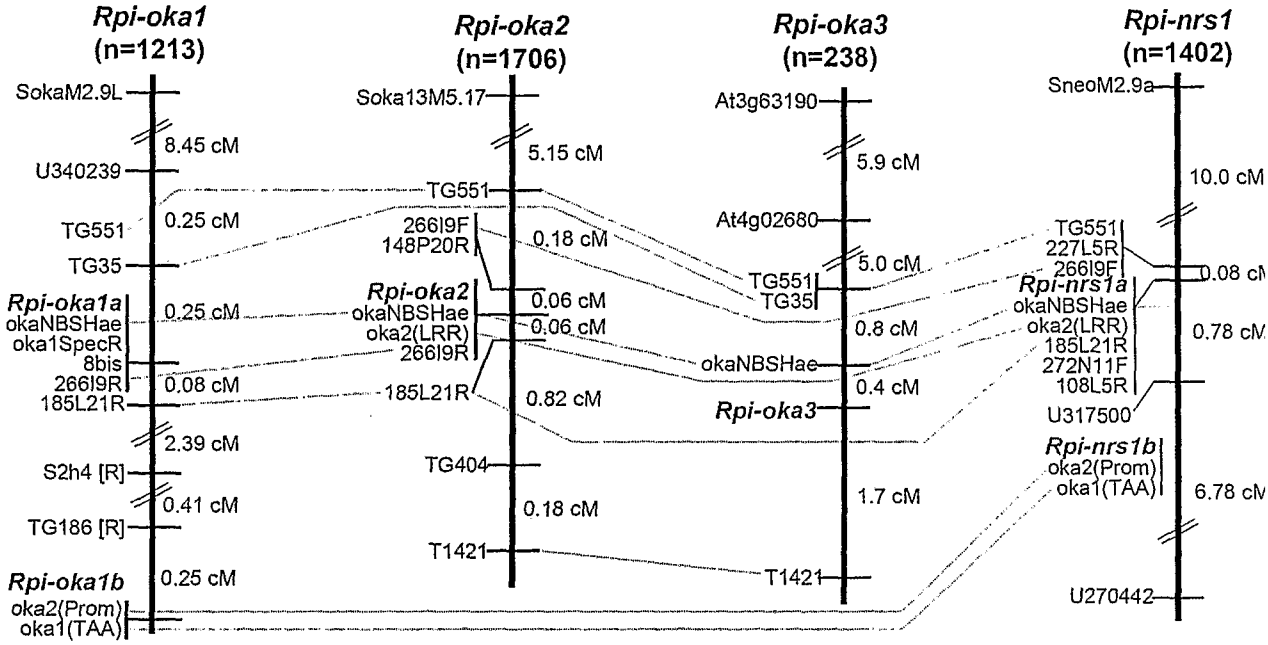

Rpi-oka1
(n=1213)
SokaM2.9L
8.45 cM
U340239
TG551
0.25 cM
TG35
Rpi-oka1a
okaNBSHae
oka1SpecR
8bis
0.25 cM
266I9R
0.08 cM
185L21R
2.39 cM
S2h4 [R]
0.41 cM
TG186 [R]
Rpi-oka1b
0.25 cM
oka2(Prom)
oka1(TAA)
Rpi-oka2
(n=1706)
Soka13M5.17
5.15 cM
TG551
266I9F
148P20R
0.18 cM
Rpi-oka2
okaNBSHae
0.06 cM
oka2(LRR)
0.06 cM
266I9R
185L21R
0.82 cM
TG404
0.18 cM
T1421
Rpi-oka3
(n=238)
At3g63190
5.9 cM
At4g02680
TG551
5.0 cM
TG35
0.8 cM
okaNBSHae
0.4 cM
Rpi-oka3
1.7 cM
T1421
Rpi-nrs1
(n=1402)
SneoM2.9a
10.0 cM
TG551
227L5R
266I9F
0.08 cM
Rpi-nrs1a
okaNBSHae
oka2(LRR)
0.78 cM
185L21R
272N11F
108L5R
U317500
Rpi-nrs1b
oka2(Prom)
oka1(TAA)
6.78 cM
U270442

FIGURE 7

```
Rpi-oka1 MNYCVYKTWAVDS----------YFPFLILTFRKKKFNEKLKEMAEILLTAVINKS :  46
Rpi-oka2 .............NTKANSTSFLSFFS............................. :  60
Tm2-2    ------------------------------------------------.......S..... :  13

Rpi-oka1 IEIAGNVLFQEGTRLYWLKEDIDWLQREMRHIRSYVDNAKAKEVGGDSRVKNLLKDIQQL : 106
Rpi-oka2 ..................................................... : 120
Tm2-2    V.....L.I...K.............................A.............E. :  73

Rpi-oka1 AGDVEDLLDEFLPKIQQSNKFICCLKTVSFADEFAMEIEKIKRRVADIDRVRTTYSITDT : 166
Rpi-oka2 ..................................................... : 180
Tm2-2    .........D...........NY...RS.................V....I.K..N.I.. : 133

Rpi-oka1 SNNNDDCIPLDRRRLFLHADETEVIGLEDDFNTLQAKLLDHDLPYGVVSIVgmpglgktt : 226
Rpi-oka2 ..................................................... : 240
Tm2-2    D......VL..............I...D....M......NQ..H.............. : 193

Rpi-oka1 laKKLYRHVCHQFECSGLVYVSQQPRAGEILHDIAKQVGLTEEERKENLENNLRSLLKIK : 286
Rpi-oka2 ..................................................... : 300
Tm2-2    .......LIRD.................S...L.....I....QKM.....D......... : 253

Rpi-oka1 ryvilldiwDVEIWDDLKLVLPECDSKIGSRIIITSRNSNVGRYIGGDFSIHVLQPLDS : 346
Rpi-oka2 ..................................................... : 360
Tm2-2    ...F.....................V..................ES.L.A....E. : 313

Rpi-oka1 EKSFELFTKKIFNFVNDN-WANASPDLVNIGRCIVERCGgiplaivvtagMLRARGRTEH : 405
Rpi-oka2 ................-..................................... : 419
Tm2-2    ..............DDN.S.............N..G..................E.... : 373

Rpi-oka1 AWNRVLESMAHKIQDGCGKVLALSYNDLPIALrpcflyfglYPEDHEIRAFDLTNMWIAE : 465
Rpi-oka2 ..................................................... : 479
Tm2-2    .........G..V....A.............S.....................I...... : 433

Rpi-oka1 KLIVVNTGNGREAESLADDVLNDLVSRNLIQVAKRTYDGRISSCRIHDLLHSLCVDLAKE : 525
Rpi-oka2 ..................................................... : 539
Tm2-2    .F....S..R....D..E.............L.....N..................... : 493

                     <---------------------><----------------------
Rpi-oka1 SNFFHTEHNAFGDPSNVARVRRITFYSDDNAMNEFFHLNPKPMKLRSLFCFTKDRCIFSQ : 585
Rpi-oka2 ........Y..................................................... : 599
Tm2-2    ......A.D.....G....L........N-V.I...RS...LE...V....A..PS...H : 552

         -><------------------------><---------------------><-------
Rpi-oka1 MAHLNFKLLQVLVVVMSQKGYQHVTFPKKIGNMSCLRYVRLEGAIRVKLPNSIVKLKCLE : 645
Rpi-oka2 ..................................................... : 659
Tm2-2    ..YFD....HT.......SFQAY..I.S.F...T....L....N.CG.........TR.. : 612

         -------------><-------------------------------><-----------
Rpi-oka1 TLDIFHSSSK-LPFGVWESKILRHLCY------TEECYCVSFASPFCRIMPPNNLQTLMW : 698
Rpi-oka2 ..........................------.......................... : 712
Tm2-2    .I..DRR.LIQP.S......H......RDYGQACNS.FSI.SFY.NIYSLH......... : 672

         --------><------------------------><---------------------><
Rpi-oka1 VDDKFCEPRLLHRLINLRTLCIMDVSGSTIKILSALSPVPRALEVLKLRFFKNTSEQINL : 758
Rpi-oka2 ....................................K..................... : 772
Tm2-2    IP...F.............K.G.LG..N..V.M..IF...LK.......S.SSDP....K. : 732

         -------------------><----------------------><--------------
Rpi-oka1 SSHPNIVELGLVGFSAMLLNIEAFPPNLVKLNLVGLMVDGHLLAVLKKLPKLRILILLWC : 818
```

```
Rpi-oka2 ..................................................................... : 832
Tm2-2    ..Y.H.AK.H.NVNRT.A..SQS.....I..TLANFT..RYI.....TF....K.KMFI. : 792

         ---------><--------------------------><--------------------
Rpi-oka1 RHDAEKMDLSGDS----FPQLEVLYIEDAQGLSEVTCMDDMSMPKLKKLFLVQGPNISPI : 874
Rpi-oka2 .............----..................................................... : 888
Tm2-2    KYNE.......EANGYS.......HIHSPN.......T..V........L.TG----FHC : 848

         ><--------------->
Rpi-oka1 SLRVSERLAKLRISQVL : 891
Rpi-oka2 ................. : 905
Tm2-2    RISL....K..SK---- : 861
```

FIGURE 8

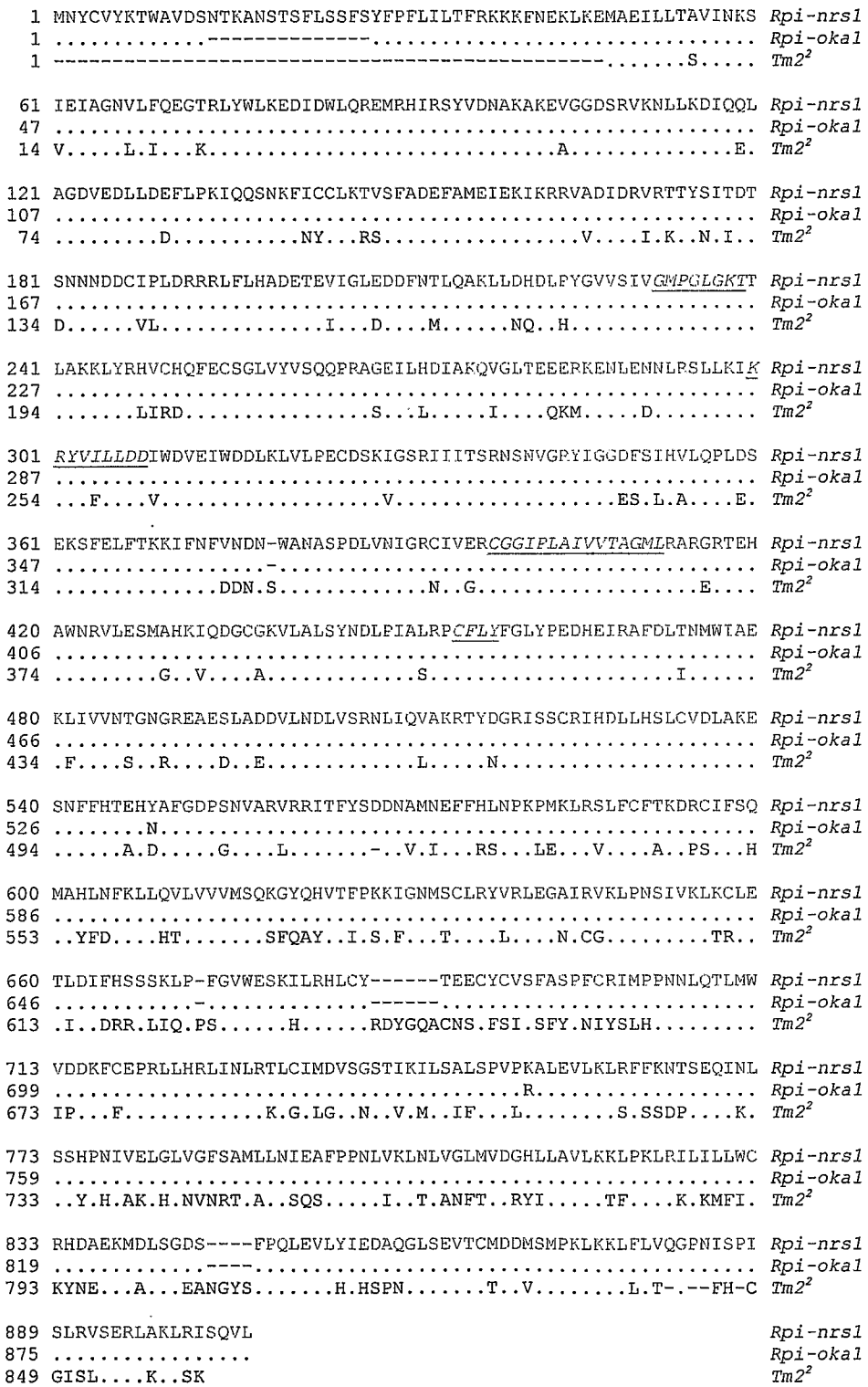

1 MNYCVYKTWAVDSNTKANSTSFLSSFSYFPFLILTFRKKKFNEKLKEMAEILLTAVINKS Rpi-nrs1
1 .....---------.....Rpi-oka1
1 ----------S..... Tm2²
61 IEIAGNVLFQEGTRLYWLKEDIDWLQREMRHIRSYVDNAKAKEVGGDSRVKNLLKDIQQL Rpi-nrs1
47 ..... Rpi-oka1
14 V.....L.I...K.....A.....E. Tm2²
121 AGDVEDLLDEFLPKIQQSNKFICCLKTVSFADEFAMEIEKIKRRVADIDRVRTTYSITDT Rpi-nrs1
107 ..... Rpi-oka1
74 .....D.....NY...RS.....V....I.K..N.I.. Tm2²
181 SNNNDDCIPLDRRRLFLHADETEVIGLEDDFNTLQAKLLDHDLFYGVVSIVGMPGLGKTT Rpi-nrs1
167 ..... Rpi-oka1
134 D.....VL.....I...D....M.....NQ..H..... Tm2²
241 LAKKLYRHVCHQFECSGLVYVSQQPRAGEILHDIAKQVGLTEEERKENLENNLRSLLKIK Rpi-nrs1
227 ..... Rpi-oka1
194 .....LIRD.....S...L.....I....QKM.....D..... Tm2²
301 RYVILLDDIWDVEIWDDLKLVLPECDSKIGSRIIITSRNSNVGRYIGGDFSIHVLQPLDS Rpi-nrs1
287 ..... Rpi-oka1
254 ...F....V.....V.....ES.L.A....E. Tm2²
361 EKSFELFTKKIFNFVNDN-WANASPDLVNIGRCIVERCGGIPLAIVVTAGMLRARGRTEH Rpi-nrs1
347 .....-..... Rpi-oka1
314 .....DDN.S.....N..G.....E.... Tm2²
420 AWNRVLESMAHKIQDGCGKVLALSYNDLPIALRPCFLYFGLYPEDHEIRAFDLTNMWIAE Rpi-nrs1
406 ..... Rpi-oka1
374 .....G..V....A.....S.....I..... Tm2²
480 KLIVVNTGNGREAESLADDVLNDLVSRNLIQVAKRTYDGRISSCRIHDLLHSLCVDLAKE Rpi-nrs1
466 ..... Rpi-oka1
434 .F....S..R....D..E.....L.....N..... Tm2²
540 SNFFHTEHYAFGDPSNVARVRRITFYSDDNAMNEFFHLNPKPMKLRSLFCFTKDRCIFSQ Rpi-nrs1
526 .....N..... Rpi-oka1
494 .....A.D.....G....L.....-..V.I...RS...LE...V....A..PS...H Tm2²
600 MAHLNFKLLQVLVVVMSQKGYQHVTFPKKIGNMSCLRYVRLEGAIRVKLPNSIVKLKCLE Rpi-nrs1
586 ..... Rpi-oka1
553 ..YFD....HT.....SFQAY..I.S.F...T....L....N.CG.....TR.. Tm2²
660 TLDIFHSSSKLP-FGVWESKILRHLCY------TEECYCVSFASPFCRIMPPNNLQTLMW Rpi-nrs1
646 .....-.....------..... Rpi-oka1
613 .I..DRR.LIQ.PS.....H.....RDYGQACNS.FSI.SFY.NIYSLH..... Tm2²
713 VDDKFCEPRLLHRLINLRTLCIMDVSGSTIKILSALSPVPKALEVLKLRFFKNTSEQINL Rpi-nrs1
699 .....R..... Rpi-oka1
673 IP...F.....K.G.LG..N..V.M..IF...L.....S.SSDP....K. Tm2²
773 SSHPNIVELGLVGFSAMLLNIEAFPPNLVKLNLVGLMVDGHLLAVLKKLPKLRILILLWC Rpi-nrs1
759 ..... Rpi-oka1
733 ..Y.H.AK.H.NVNRT.A..SQS.....I..T.ANFT..RYI.....TF....K.KMFI. Tm2²
833 RHDAEKMDLSGDS----FPQLEVLYIEDAQGLSEVTCMDDMSMPKLKKLFLVQGPNISPI Rpi-nrs1
819 .....----..... Rpi-oka1
793 KYNE...A...EANGYS.....H.HSPN.....T..V.....L.T-.--FH-C Tm2²
889 SLRVSERLAKLRISQVL Rpi-nrs1
875 ..... Rpi-oka1
849 GISL....K..SK Tm2²

FIGURE 9

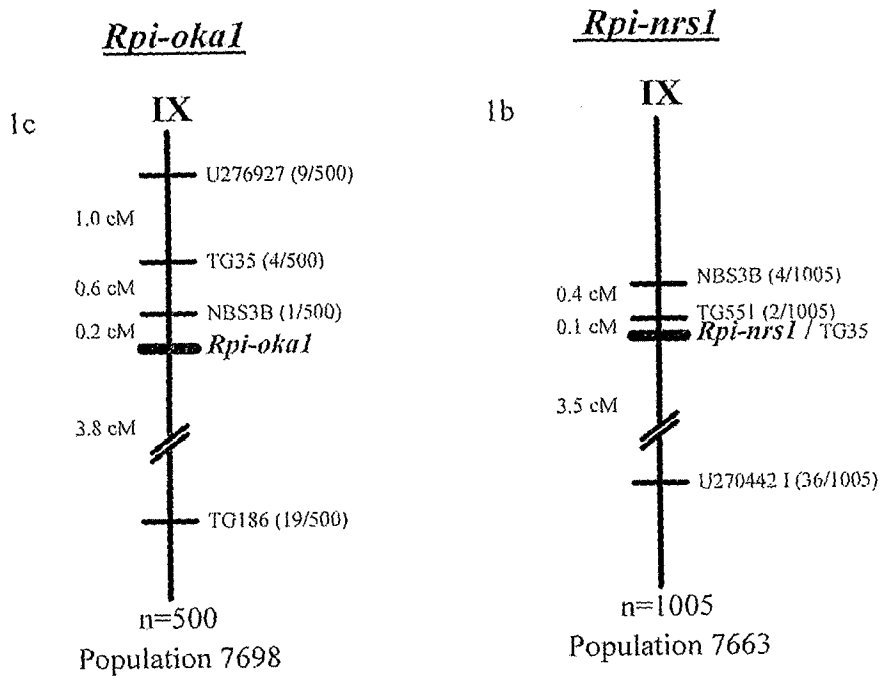

Rpi-oka1
1c
IX
U276927 (9/500)
1.0 cM
TG35 (4/500)
0.6 cM
NBS3B (1/500)
0.2 cM
Rpi-oka1
3.8 cM
TG186 (19/500)
n=500
Population 7698
Rpi-nrs1
1b
IX
NBS3B (4/1005)
0.4 cM
TG551 (2/1005)
0.1 cM
Rpi-nrs1 / TG35
3.5 cM
U270442 I (36/1005)
n=1005
Population 7663

FIGURE 10

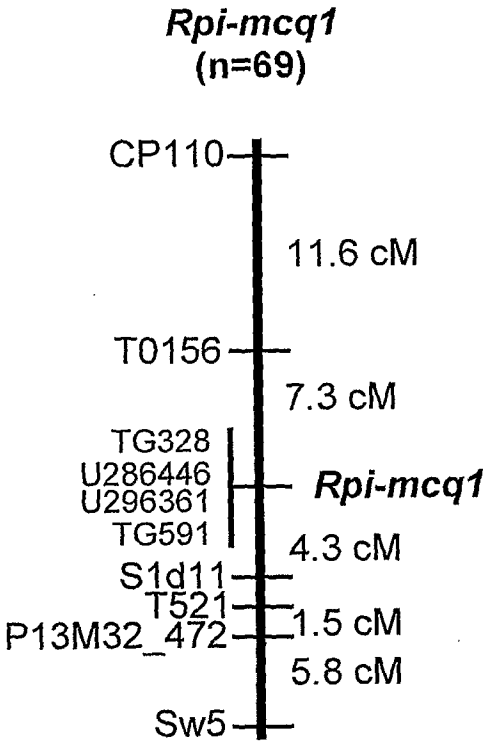

Rpi-mcq1
(n=69)
CP110
11.6 cM
T0156
7.3 cM
TG328
U286446
U296361
Rpi-mcq1
TG591
4.3 cM
S1d11
T521
1.5 cM
P13M32_472
5.8 cM
Sw5

FIGURE 11

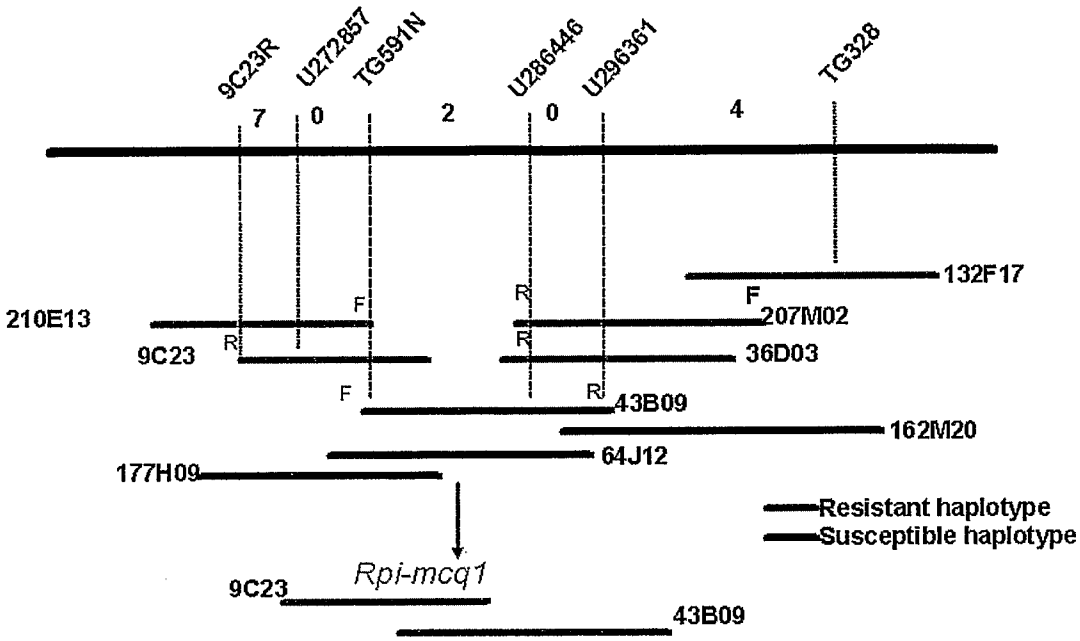

9C23R
U272857
TG591N
U286446
U296361
TG328
7
0
2
0
4
132F17
F
R
F
210E13
207M02
R
R
9C23
36D03
F
R
43B09
162M20
64J12
177H09
Resistant haplotype
Susceptible haplotype
Rpi-mcq1
9C23
43B09

FIGURE 12

SEQUENCE LISTING

SEQ. ID. 1A = NUCLEIC ACID SEQUENCE OF *Rpi-oka1*

```
   1 atgaattatt gtgtttacaa gacttgggcc gttgactctt actttccctt cctcatcctc
  61 acatttagaa aaaagaaatt taacgaaaaa ttaaaggaga tggctgaaat tcttctcaca
 121 gcagtcatca ataaatcaat agaaatagct ggaaatgtac tctttcaaga aggtacgcgt
 181 ttatattggt tgaaagagga catcgattgg ctccagagag aaatgagaca cattcgatca
 241 tatgtagaca atgcaaaggc aaaggaagtt ggaggcgatt caagggtgaa aaacttatta
 301 aaagatattc aacaactggc aggtgatgtg gaggatctat tagatgagtt tcttccaaaa
 361 attcaacaat ccaataagtt catttgttgc cttaagacgg tttcttttgc cgatgagttt
 421 gctatggaga ttgagaagat aaaaagaaga gttgctgata ttgaccgtgt aaggacaact
 481 tacagcatca cagatacaag taacaataat gatgattgca ttccattgga ccggagaaga
 541 ttgttccttc atgctgatga aacagaggtc atcggtctgg aagatgactt caatacacta
 601 caagccaaat tacttgatca tgatttgcct tatggagttg tttcaatagt tggcatgccc
 661 ggtttgggaa aaacaactct tgccaagaaa ctttataggc atgtctgtca tcaatttgag
 721 tgttcgggac tggtctatgt ttcacaacag ccaagggcgg gagaaatctt acatgacata
 781 gccaaacaag ttggactgac ggaagaggaa aggaaagaaa acttggagaa caacctacga
 841 tcactcttga aaataaaaag gtatgttatt ctcttagatg acatttggga tgttgaaatt
 901 tgggatgatc taaaacttgt ccttcctgaa tgtgattcaa aaattggcag taggataatt
 961 ataacctctc gaaatagtaa tgtaggcaga tacataggag gggatttctc aatccacgtg
1021 ttgcaacccc tagattcaga gaaaagcttt gaactcttta ccaagaaaat ctttaatttt
1081 gttaatgata attgggccaa tgcttcacca gacttggtaa atattggtag atgtatagtt
1141 gagagatgtg gaggtatacc gctagcaatt gtggtgactg caggcatgtt aagggcaaga
1201 ggaagaacag aacatgcatg gaacagagta cttgagagta tggctcataa aattcaagat
1261 ggatgtggta aggtattggc tctgagttac aatgatttgc ccattgcatt aaggccatgt
1321 ttcttgtact ttggtcttta ccccgaggac catgaaattc gtgcttttga tttgacaaat
1381 atgtggattg ctgagaagct gatagttgta aatactggca atgggcgaga ggctgaaagt
1441 ttggcggatg atgtcctaaa tgatttggtt tcaagaaact tgattcaagt tgccaaaagg
1501 acatatgatg gaagaatttc aagttgtcgc atacatgact tgttacatag tttgtgtgtg
1561 gacttggcta aggaaagtaa cttctttcac acggagcaca atgcatttgg tgatcctagc
1621 aatgttgcta gggtgcgaag gattacattc tactctgatg ataatgccat gaatgagttc
1681 ttccatttaa atcctaagcc tatgaagctt cgttcacttt tctgtttcac aaaagaccgt
1741 tgcatatttt ctcaaatggc tcatcttaac ttcaaattat tgcaagtgtt ggttgtagtc
1801 atgtctcaaa agggttatca gcatgttact ttccccaaaa aaattgggaa catgagttgc
1861 ctacgttatg tgcgattgga gggggcaatt agagtaaaat tgccaaatag tattgtcaag
1921 ctcaaatgtc tagagaccct ggatatattt catagctcta gtaaacttcc ttttggtgtt
1981 tgggagtcta aaatattgag acatctttgt tacacagaag aatgttactg tgtctctttt
2041 gcaagtccat tttgccgaat catgcctcct aataatctac aaactttgat gtgggtggat
2101 gataaatttt gtgaaccaag attgttgcac cgattgataa atttaagaac attgtgtata
2161 atggatgtat ccggttctac cattaagata ttatcagcat tgagccctgt gcctagagcg
2221 ttggaggttc tgaagctcag atttttcaag aacacgagtg agcaaataaa cttgtcgtcc
2281 catccaaata ttgtcgagtt gggtttggtt ggtttctcag caatgctctt gaacattgaa
2341 gcattccctc caaatcttgt caagcttaat cttgtcggct tgatggtaga cggtcatcta
2401 ttggcagtgc ttaagaaatt gcccaaatta aggatactta tattgctttg gtgcagacat
2461 gatgcagaaa aaatggatct ctctggtgat agctttccgc aacttgaagt tttgtatatt
2521 gaggatgcac aagggttgtc tgaagtaacg tgcatggatg atatgagtat gcctaaattg
2581 aaaaagctat ttcttgtaca aggcccaaac atttccccaa ttagtctcag ggtctcggaa
2641 cggcttgcaa agttgagaat atcacaggta ctataa
```

SEQ. ID. 1B = NUCLEIC ACID SEQUENCE OF *Rpi-oka2*

```
   1 atgaattatt gtgtttacaa gacttgggcc gttgactcta acactaaagc aaatagtaca
  61 tctttcttat ccttttctc ttactttccc ttcctcatcc tcacatttag aaaaaagaaa
 121 tttaacgaaa aattaaagga gatggctgaa attcttctca cagcagtcat caataaatca
 181 atagaaatag ctggaaatgt actctttcaa gaaggtacgc gtttatattg gttgaaagag
 241 gacatcgatt ggctccagag agaaatgaga cacattcgat catatgtaga caatgcaaag
 301 gcaaaggaag ttggaggcga ttcaagggtg aaaaacttat taaaagatat tcaacaactg
 361 gcaggtgatg tggaggatct attagatgag tttcttccaa aaattcaaca atccaataag
 421 ttcatttgtt gccttaagac ggtttctttt gccgatgagt ttgctatgga gattgagaag
 481 ataaaaagaa gagttgctga tattgaccgt gtaaggacaa cttacagcat cacagataca
 541 agtaacaata atgatgattg cattccattg gaccggagaa gattgttcct tcatgctgat
 601 gaaacagagg tcatcggtct ggaagatgac ttcaatacac tacaagccaa attacttgat
 661 catgatttgc cttatggagt tgtttcaata gttggcatgc ccggtttggg aaaaacaact
 721 cttgccaaga aactttatag gcatgtctgt catcaatttg agtgttcggg actggtctat
 781 gtttcacaac agccaagggc gggagaaatc ttacatgaca tagccaaaca agttggactg
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 901 aggtatgtta ttctcttaga tgacatttgg gatgttgaaa tttgggatga tctaaaactt
 961 gtccttcctg aatgtgattc aaaaattggc agtaggataa ttataacctc tcgaaatagt
1021 aatgtaggca gatacatagg aggggatttc tcaatccacg tgttgcaacc cctagattca
1081 gagaaaagct ttgaactctt taccaagaaa atctttaatt ttgttaatga taattgggcc
1141 aatgcttcac cagacttggt aaatattggt agatgtatag ttgagagatg tggaggtata
1201 ccgctagcaa ttgtggtgac tgcaggcatg ttaagggcaa gaggaagaac agaacatgca
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1261 tggaacagag tacttgagag tatggctcat aaaattcaag atggatgtgg taaggtattg
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1381 taccccgagg accatgaaat tcgtgctttt gatttgacaa atatgtggat tgctgagaag
1441 ctgatagttg taaatactgg caatgggcga gaggctgaaa gtttggcgga tgatgtccta
1501 aatgatttgg tttcaagaaa cttgattcaa gttgccaaaa ggacatatga tggaagaatt
1561 tcaagttgtc gcatacatga cttgttacat agtttgtgtg tggacttggc taaggaaagt
1621 aacttctttc acacggagca ctatgcattt ggtgatccta gcaatgttgc tagggtgcga
1681 aggattacat tctactctga tgataatgcc atgaatgagt tcttccattt aaatcctaag
1741 cctatgaagc ttcgttcact tttctgtttc acaaaagacc gttgcatatt ttctcaaatg
1801 gctcatctta acttcaaatt attgcaagtg ttggttgtag tcatgtctca aaagggttat
1861 cagcatgtta ctttccccaa aaaaattggg aacatgagtt gcctacgcta tgtgcgattg
1921 gaggggggcaa ttagagtaaa attgccaaat agtattgtca agctcaaatg tctagagacc
1981 ctggatatat ttcatagctc tagtaaactt ccttttggtg tttgggagtc taaaatattg
2041 agacatcttt gttacacaga agaatgttac tgtgtctctt ttgcaagtcc attttgccga
2101 atcatgcctc ctaataatct acaaactttg atgtgggtgg atgataaatt ttgtgaacca
2161 agattgttgc accgattgat aaatttaaga acattgtgta taatggatgt atccggttct
2221 accattaaga tattatcagc attgagccct gtgcctaaag cgttggaggt tctgaagctc
2281 agatttttca agaacacgag tgagcaaata aacttgtcgt cccatccaaa tattgtcgag
2341 ttgggtttgg ttggtttctc agcaatgctc ttgaacattg aagcattccc tccaaatctt
2401 gtcaagctta atcttgtcgg cttgatggta gacggtcatc tattggcagt gcttaagaaa
2461 ttgcccaaat taaggatact tatattgctt tggtgcagac atgatgcaga aaaaatggat
2521 ctctctggtg atagctttcc gcaacttgaa gttttgtata ttgaggatgc acaagggttg
2581 tctgaagtaa cgtgcatgga tgatatgagt atgcctaaat tgaaaaagct atttcttgta
2641 caaggcccaa acatttcccc aattagtctc agggtctcgg aacggcttgc aaagttgaga
2701 atatcacagg tactataa
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SEQ. ID. 1C = NUCLEIC ACID SEQUENCE OF *Rpi-oka1* TRANSGENE FROM PSLJ21152 (INCLUDES OWN PROMOTER AND TERMINATOR)

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   1 agttatacac cctacattct actcgagtca ttatgatgat gtctcacgac caaatcaaat
  61 caaagttaaa taaatatcga accgaacgcc cactctgtat gagtatggca aaagattttg
 121 agagaatcaa gttgcataaa agcctaattt tcatggaaca tacaaattga gtctcataat
 181 agcccaaact cacagccatg aacccaaatt gggtaaagtt ttgcaagacg ttcatcaaac
 241 agttaggaaa cataaaatgg cgctagatat ataataaatt tttttaacat atggtgtgat
 301 tgatagttat atactaaaga tgtttgctta gttacgtaat tttttcaaaa aaaaaaggta
 361 cattatcaat catcagtcac aaaatattaa aagttactgt ttgtttttta aattccatgt
 421 cgaatttaat tgaatgacac ttaaattggg acgaacggtg taatttcttt tgactattct
 481 actagtatct atccacagca cgtgttgttc ctttcttctt tcgtttttca tttacttgac
 541 attattagga gacttggccc tgaactccaa ctattctaag ctgacctttc ttttccttta
 601 ccaattatct tcttctttct aatttcgttt tacgcgtagt actgcctgaa ttttctgact
 661 ttcaacgttt gttattcatg cttgaaaacg aaataccagc taacaaaaga tgaattattg
 721 tgtttacaag acttgggccg ttgactctta ctttcccttc ctcatcctca catttagaaa
 781 aaagaaattt aacgaaaaat taaaggagat ggctgaaatt cttctcacag cagtcatcaa
 841 taaatcaata gaaatagctg gaaatgtact ctttcaagaa ggtacgcgtt tatattggtt
 901 gaaagaggac atcgattggc tccagagaga aatgagacac attcgatcat atgtagacaa
 961 tgcaaaggca aaggaagttg gaggcgattc aagggtgaaa aacttattaa aagatattca
1021 acaactggca ggtgatgtgg aggatctatt agatgagttt cttccaaaaa ttcaacaatc
1081 caataagttc atttgttgcc ttaagacggt ttcttttgcc gatgagtttg ctatggagat
1141 tgagaagata aaaagaagag ttgctgatat tgaccgtgta aggacaactt acagcatcac
1201 agatacaagt aacaataatg atgattgcat tccattggac cggagaagat tgttccttca
1261 tgctgatgaa acagaggtca tcggtctgga agatgacttc aatacactac aagccaaatt
1321 acttgatcat gatttgcctt atggagttgt ttcaatagtt ggcatgcccg gtttgggaaa
1381 aacaactctt gccaagaaac tttataggca tgtctgtcat caatttgagt gttcgggact
1441 ggtctatgtt tcacaacagc caagggcggg agaaatctta catgacatag ccaaacaagt
1501 tggactgacg gaagaggaaa ggaaagaaaa cttggagaac aacctacgat cactcttgaa
1561 aataaaaagg tatgttattc tcttagatga catttgggat gttgaaattt gggatgatct
1621 aaaacttgtc cttcctgaat gtgattcaaa aattggcagt aggataatta taacctctcg
1681 aaatagtaat gtaggcagat acataggagg ggatttctca atccacgtgt tgcaacccct
1741 agattcagag aaaagctttg aactctttac caagaaaatc tttaattttg ttaatgataa
1801 ttgggccaat gcttcaccag acttggtaaa tattggtaga tgtatagttg agagatgtgg
1861 aggtataccg ctagcaattg tggtgactgc aggcatgtta agggcaagag gaagaacaga
1921 acatgcatgg aacagagtac ttgagagtat ggctcataaa attcaagatg gatgtggtaa
1981 ggtattggct ctgagttaca atgatttgcc cattgcatta aggccatgtt tcttgtactt
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2101 tgagaagctg atagttgtaa atactggcaa tgggcgagag gctgaaagtt tggcggatga
2161 tgtcctaaat gatttggttt caagaaactt gattcaagtt gccaaaagga catatgatgg
2221 aagaatttca agttgtcgca tacatgactt gttacatagt ttgtgtgtgg acttggctaa
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2281 ggaaagtaac ttctttcaca cggagcacaa tgcatttggt gatcctagca atgttgctag
2341 ggtgcgaagg attacattct actctgatga taatgccatg aatgagttct tccatttaaa
2401 tcctaagcct atgaagcttc gttcactttt ctgtttcaca aaagaccgtt gcatattttc
2461 tcaaatggct catcttaact tcaaattatt gcaagtgttg gttgtagtca tgtctcaaaa
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2641 agagaccctg gatatatttc atagctctag taaacttcct tttggtgttt gggagtctaa
2701 aatattgaga catctttgtt acacagaaga atgttactgt gtctcttttg caagtccatt
2761 ttgccgaatc atgcctccta ataatctaca aactttgatg tgggtggatg ataaattttg
2821 tgaaccaaga ttgttgcacc gattgataaa tttaagaaca ttgtgtataa tggatgtatc
2881 cggttctacc attaagatat tatcagcatt gagccctgtg cctagagcgt tggaggttct
2941 gaagctcaga tttttcaaga acacgagtga gcaaataaac ttgtcgtccc atccaaatat
3001 tgtcgagttg ggtttggttg gtttctcagc aatgctcttg aacattgaag cattccctcc
3061 aaatcttgtc aagcttaatc ttgtcggctt gatggtagac ggtcatctat tggcagtgct
3121 taagaaattg cccaaattaa ggatacttat attgctttgg tgcagacatg atgcagaaaa
3181 aatggatctc tctggtgata gctttccgca acttgaagtt ttgtatattg aggatgcaca
3241 agggttgtct gaagtaacgt gcatggatga tatgagtatg cctaaattga aaaagctatt
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3361 gttgagaata tcacaggtac tataaataat tatttacgtt taatatccat gatttttta
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3481 gaaaatgatt ctgtgttgga gagaatcttc tgatgcctgt tggtattata atactaataa
3541 taagagaaaa agtttgatta ctgtttcaag ttaattgctt gtgatttgta aaaacaaatt
3601 acttttatat ttctctttgt tttattttat gtttatttat ctttaattaa tggagtaata
3661 aaataaaaat cttattttca atagaaaaaa gtagacctta tttgtggtgc atgtatggta
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3781 ttgcataaat ataaaatatt atacaaatac ctatgggttg gaaaatatag aaatatgcca
3841 atcaaatgta tacaaaaatc attaatagat agaatcgtaa aagatataca aatgagaaat
3901 gcttgactaa gaagcttcgt gcaacctctc acactgagca caatgcattt ggtgatctcg
3961 gcactattgc tgttacttgt aagactacgt tccccaataa gtctttccaa acggcttgca
4021 aagctgagaa tatgaaaatc tcataggtta gtttgctgcg ttaattattt acatttaata
4081 tgctcgataa ggtgatttta aaaaaatttg tactagttaa ttcatgaact aaatatttca
4141 tttaatactc cataattctg aatatggaaa ataaataata tttaataaca agaataaaat
4201 gataaattat tcattgattt tataaattgg ataaatatta ttaaatattc ttaaataata
4261 taatgaacaa gtgaagatga acggagggag tatgaagcct cttttcaaag
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SEQ. ID. 2A = NUCLEIC ACID SEQUENCE OF *Rpi-mcq1.1*

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   1 atggctgaaa ttcttcttac agcagtcatc aataaatctg tagaaatagc tggaaatgta
  61 ctctttcaag aaggtacgcg tttatattgg ttgaaggagg atatagattg gctccaaaga
 121 gaaatgagac acattcgatc atatgtagac aatgcaaagg ccaaggaagt tggaggtgat
 181 tcaagggtga aaaacttatt aaaagatatt caacaactcg caggtgatgt ggaggatctc
 241 ctagatgagt ttcttccaaa aattcaacaa tccagtaagt tcaaaggcgc aatttgttgc
 301 cttaagaccg tttcttttgc ggatgagttt gctatggaga ttgagaagat aaaaagaagg
 361 gttgtggaca ttgatcgtgt aaggacaact tacaacatca tggatacaaa taacaacaat
 421 gattgcattc cattggacca gagaagattg ttccttcatg ttgatgaaac agaggtcatc
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 661 agggcgggag aaatcttaat cgacatagcc aaacaagttg gactgacgga agacgaaagg
 721 aaagaaaact tggagaacaa cctacggtca ctcttgaaaa gaaaaaggta tgttattctc
 781 ttagatgaca tttgggatgt tgaaatttgg gatgatctaa aacttgtcct tcctgaatgt
 841 gattcaaaaa ttggcagtag gataattata acctctcgaa atagtaatgt aggcagatac
 901 ataggagggg atttctcaat tcacgtgttg caacctctaa attcggagaa cagttttgaa
 961 ctctttacca agaaaatctt tatttttgat aacaataata attggaccaa tgcttcacca
1021 aacttggtag atattggtag aagtatagtt ggtagatgtg gtggtatacc actagccatt
1081 gtggtgactg caggcatgtt aagggcaaga gaaagaacag aacgtgcatg gaacaggtta
1141 cttgagagta tgagccataa agttcaagat ggatgtgcta aggtattggc tctgagttac
1201 aatgatttgc caattgcatt aaggccatgt ttcttgtatt ttggccttta ccccgaggat
1261 catgaaattc gtgcttttga tttgacaaat atgtggattg ctgagaagtt gatagttgta
1321 aatagtggca atgggcgaga ggctgaaagt ttggcggatg atgtcctaaa tgatttggtt
1381 tcaagaaaca tgattcaagt tgccaaaagg acatatgatg gaagaatttc aagttgtcgc
1441 atacatgact tgttacatag tttgtgtgtt gacttggcta aggaaagcaa cttctttcac
1501 accgagcaca atgcattggg tgatcccgga aatgttgcta ggctgcgaag gattacattc
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1981 ttgtactcat tgcctcataa taatgtacaa actttgatgt ggctggatga taaatttttt
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2341 aagaaattgc ccaaattaag gatacttaaa atgtttgggt gcagtcataa tgaagaaaag
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2461 ccagatgggt tgtctgaagt aacgtgtagg gatgatgtca gtatgcctaa attgaaaaag
2521 ttgttacttg tacaacgccg cccttctcca attagtctct cagaacgtct tgcaaagctc
2581 agaatatga
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SEQ. ID. 2B = NUCLEIC ACID SEQUENCE OF *Rpi-mcq1.2*

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   1 atggctgaaa ttcttcttac aacagtcatc aataaatctg taggaatagc tgcaaatgta
  61 ctctttcaag aaggaacgcg tttatattgg ttgaaagagg acatagattg gctccacaga
 121 gaaatgagac acattcgatc atatgtagac gatgcaaagg ccaaggaagt tggaggcgat
 181 tcaagggtca gaaacttatt aaaagatatt caacaactgg caggtgatgt ggaggatcta
 241 ttagatgagt ttcttccaaa aattcaacaa tccaataagt tcatttgttg ccttaagaca
 301 gtttcttttg ccgatgagtt tgccatggag attgagaaga taaaaagaag agttgctgat
 361 attacccgtg taaggacaac ttacaacatc acagatacaa gtaacaataa tgatgattgc
 421 attccattgg accggagaag attgttcctt catgctgatg aaacagaggt catcggtctg
 481 gaagatgact tcaatacact aaaagccaaa ttacttgatc aagatttgcc ttatggagtt
 541 gtttcaatag ttggcatgcc cggtctagga aaaacaactc ttgccaagaa actttatagg
 601 catgtccgtg atcaatttga gagctcggga ctggtctacg tgtcccaaca gccaagagcg
 661 ggagaaatct tacgtgacat agccaaacaa gttggactgc caaaagagga aaggaaagaa
 721 aacttggagg gcaacctacg atcactcttg aaaacaaaaa ggtatgttat cctcctagat
 781 gacatttggg atgttgaaat ttgggatgat ctaaaactcg tccttcctga atgtgattca
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 901 ggggatttct caattcacat gttgcaacct ctagattcgg agaacagttt tgaactcttt
 961 accaagaaaa tctttacttt tgataacaat aataattggg ccaatgcttc accagacttg
1021 gtagatattg gtagaagtat agttggtaga tgcggaggta tacctctagc cattgtggtc
1081 actgcaggca tgttaagggc aagagaaaga acagaacatg catggaacag agtacttgag
1141 agtatgggcc ataaagttca agatggatgt gctaaggtat tggctttgag ttacaatgat
1201 ttgcccattg cattaaggcc atgtttcttg taccttggcc ttttccccga ggaccatgaa
1261 attcgtgcct ttgatttgac aaatatgtgg attgctgaga agctgatagt tgtaaatagt
1321 ggcaatgggc gagaggctga aagtttggcg gaggatgttc taaatgattt tgtttctaga
1381 aacttgattc aagtttccca aagaaaatgt aatggaagaa tttcaagtta tcgcatacat
1441 gacttgttac atagtttgtg cgtcgaattg ggcaaggaaa gtaacttttt tcacactgaa
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1621 cttttctgtt tcacaaattt agactcttgc atattttctc atttggctca tcatgacttc
1681 aaattattac aagtgttggt tgtagttatc tcttataatt ggttgagtgt cagtatctca
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2101 atattatcag cattgagccc tgtgccaaca gcgttggagg ttctgaagct ctacttttg
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2401 gatggtgatg gtgatagctt tccgcaactt gaagttttgc atattgagag accattcttc
2461 ttgtttgaaa taacgtgcac agatgatgac agtatgccta aattgaaaaa gctattactt
2521 accacttcga acgttaggct ctcggaaaga cttgcaaaac tgagagtatg a
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SEQ. ID. 2C = NUCLEIC ACID SEQUENCE OF A PORTION OF *Rpi-mcq1.1* TRANSGENE FROM PSLJ21153 (INCLUDES OWN PROMOTER AND TERMINATOR)

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   1  ggatctgggt tttacccggt cttttattaa atgggtggta gaaaataaat
  51  tatatatata tatttttttgg agtgaacaca cgcgcaggtg ctgagattac
 101  cattgttgtc caaatggtgt atttataatg gttgaaaatt gtttcgtggt
 151  gtaataggac tcccacaaac tttaagtgtc tgcttcaaaa aatggtttaa
```

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 201  gtttaatggg gtaactatgt atttcctcta actaaaaatc aaaaaccata
 251  gcaaaaaaat aaggtaaaga accataatat aatcaaataa gcataaaccc
 301  atctcaaaaa actcattttt tttaacaata aaccaaacat aaaaccaata
 351  taccccaaag acttaacaaa gtttcatatt aactaaaaat caaaaaccat
 401  agtaaagcaa taacgtaaag aaccataata taatcaaata agcataaacc
 451  catctcaaaa actcattttt ttcatcaaac atcaaaaaac aatgagtaaa
 501  agttctacaa caagaaccaa acataaaacc aagagacccc aaagacttaa
 551  caaagttcca tattaacaaa aaatcaacaa ccataacaaa acaataaggc
 601  aaagaacaat agcataacca aataagcata aacccatctt aaaaaactca
 651  tttttatcac caaacattaa aaaactcatt tttttcacca aacatcaaaa
 701  aacaatgagt aaaagttcta caacatgaac caaacataaa accaacatac
 751  cccaaacact taacaaagct ccatataaac aacaaaacaa caaggcaaag
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1201  gtttttcact ttatttcaaa agcagtttga ttgtaaaaat cgtcaatttt
1251  tagttgtttt attctttcat ttgcaagaaa aaaaaattaa gcataaatct
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1351  atcaaatgcc catttagttt ttttttttt aaactttaat acgagacttt
1401  tttcatattt tatattttcc tcaaattaga tccttttttt tcctttcctt
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1951  ttatcaatca tcagccacaa aatattaaaa gtcacagttt gtttcttaaa
2001  ttccatatcg aattaaattg aatgacagtt aaattggaat gaatggtgta
2051  atttcctttg actattgtac tagtatctta tccacagcat gtgttgttcc
2101  ttccttcttt cgtttttcat ttacttgaca ttagtaggag acttggcagt
2151  ggactccaac tattctaagc tgacctttct tttcctttac caattatctt
2201  ctcttttcta atttctcatt ctgatcggtt tttgtagcta ctgaaaaaga
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2351  ggttgaagga ggatatagat tggctccaaa gagaaatgag acacattcga
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4651  aatgaagaaa agatggatct ctctggtgat ggtgatagct ttccgcaact
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5201  atttttttat cggttttggg tccttagcgg ttcgatattt gatttaacca
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5601  caatattaaa aatcaaaatc gaaccggtaa cccaatattt ttttctttct
5651  ataaaaccat taaaacctca ttgacccaat aacccaataa caataaatca
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5751  actaatataa attactacct gttatagcaa gtgcaagtag agaattgata
5801  tatagctcac attttacaaa ttctttctag tgttaatcgt caaaaacatt
5851  agcttctcaa taatatatgg c
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SEQ. ID. 2D = NUCLEIC ACID SEQUENCE OF A PORTION OF *Rpi-mcq1.2* TRANSGENE FROM PSLJ21148 (INCLUDES OWN PROMOTER AND TERMINATOR)

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 151  ccagcaatat gaacaagaaa aataatacaa acagtaaatg aaaataaaat
 201  tatctgataa tatatttagc ttcagaacca aagattgctt gttacaggtg
 251  aaaaaaatac tactccaatg caacgcttaa aactcttcga taatcatata
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 451  ggttgaaaga ggacaatgac tggagaagtg aggcacattc gatattatct
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 551  caatatgact acgttccact agaccgtgga agattattcc ttcatgttga
 601  tgaaacagag gtcattggtt tggggaaaat actatcaaat attcaagttt
 651  actggtaatt aaaactactg atagtttagt ggatttgaat aaaatgtgtt
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1101  tgataaaata tgcaaggctc ttttgtgaaa caaaaagcct tttaggttta
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1201  ccttcgcagc gtagcaacgt tgtcaggatc accaaatgca tagtgatcag
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SEQ. ID. 3 = NUCLEIC ACID SEQUENCE OF *Rpi-nrs1*

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2581 tctgaagtaa cgtgcatgga tgatatgagt atgcctaaat tgaaaaagct atttcttgta
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SEQ. ID. 4A = AMINO ACID SEQUENCE OF *Rpi-oka1*

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 121 IQQSNKFICC LKTVSFADEF AMEIEKIKRR VADIDRVRTT YSITDTSNNN DDCIPLDRRR
 181 LFLHADETEV IGLEDDFNTL QAKLLDHDLP YGVVSIVGMP GLGKTTLAKK LYRHVCHQFE
 241 CSGLVYVSQQ PRAGEILHDI AKQVGLTEEE RKENLENNLR SLLKIKRYVI LLDDIWDVEI
 301 WDDLKLVLPE CDSKIGSRII ITSRNSNVGR YIGGDFSIHV LQPLDSEKSF ELFTKKIFNF
 361 VNDNWANASP DLVNIGRCIV ERCGGIPLAI VVTAGMLRAR GRTEHAWNRV LESMAHKIQD
 421 GCGKVLALSY NDLPIALRPC FLYFGLYPED HEIRAFDLTN MWIAEKLIVV NTGNGREAES
 481 LADDVLNDLV SRNLIQVAKR TYDGRISSCR IHDLLHSLCV DLAKESNFFH TEHNAFGDPS
 541 NVARVRRITF YSDDNAMNEF FHLNPKPMKL RSLFCFTKDR CIFSQMAHLN FKLLQVLVVV
 601 MSQKGYQHVT FPKKIGNMSC LRYVRLEGAI RVKLPNSIVK LKCLETLDIF HSSSKLPFGV
 661 WESKILRHLC YTEECYCVSF ASPFCRIMPP NNLQTLMWVD DKFCEPRLLH RLINLRTLCI
 721 MDVSGSTIKI LSALSPVPRA LEVLKLRFFK NTSEQINLSS HPNIVELGLV GFSAMLLNIE
 781 AFPPNLVKLN LVGLMVDGHL LAVLKKLPKL RILILLWCRH DAEKMDLSGD SFPQLEVLYI
 841 EDAQGLSEVT CMDDMSMPKL KKLFLVQGPN ISPISLRVSE RLAKLRISQV L*
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SEQ. ID. 4B = AMINO ACID SEQUENCE OF *Rpi-oka2*

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   1 MNYCVYKTWA VDSNTKANST SFLSFFSYFP FLILTFRKKK FNEKLKEMAE ILLTAVINKS
  61 IEIAGNVLFQ EGTRLYWLKE DIDWLQREMR HIRSYVDNAK AKEVGGDSRV KNLLKDIQQL
 121 AGDVEDLLDE FLPKIQQSNK FICCLKTVSF ADEFAMEIEK IKRRVADIDR VRTTYSITDT
 181 SNNNDDCIPL DRRRLFLHAD ETEVIGLEDD FNTLQAKLLD HDLPYGVVSI VGMPGLGKTT
 241 LAKKLYRHVC HQFECSGLVY VSQQPRAGEI LHDIAKQVGL TEEERKENLE NNLRSLLKIK
 301 RYVILLDDIW DVEIWDDLKL VLPECDSKIG SRIIITSRNS NVGRYIGGDF SIHVLQPLDS
 361 EKSFELFTKK IFNFVNDNWA NASPDLVNIG RCIVERCGGI PLAIVVTAGM LRARGRTEHA
 421 WNRVLESMAH KIQDGCGKVL ALSYNDLPIA LRPCFLYFGL YPEDHEIRAF DLTNMWIAEK
 481 LIVVNTGNGR EAESLADDVL NDLVSRNLIQ VAKRTYDGRI SSCRIHDLLH SLCVDLAKES
 541 NFFHTEHYAF GDPSNVARVR RITFYSDDNA MNEFFHLNPK PMKLRSLFCF TKDRCIFSQM
 601 AHLNFKLLQV LVVVMSQKGY QHVTFPKKIG NMSCLRYVRL EGAIRVKLPN SIVKLKCLET
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661 LDIFHSSSKL PFGVWESKIL RHLCYTEECY CVSFASPFCR IMPPNNLQTL MWVDDKFCEP
721 RLLHRLINLR TLCIMDVSGS TIKILSALSP VPKALEVLKL RFFKNTSEQI NLSSHPNIVE
781 LGLVGFSAML LNIEAFPPNL VKLNLVGLMV DGHLLAVLKK LPKLRILILL WCRHDAEKMD
841 LSGDSFPQLE VLYIEDAQGL SEVTCMDDMS MPKLKKLFLV QGPNISPISL RVSERLAKLR
901 ISQVL*
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SEQ. ID. 5A = AMINO ACID SEQUENCE OF *Rpi-mcq1.1*

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  1 MAEILLTAVI NKSVEIAGNV LFQEGTRLYW LKEDIDWLQR EMRHIRSYVD NAKAKEVGGD
 61 SRVKNLLKDI QQLAGDVEDL LDEFLPKIQQ SSKFKGAICC LKTVSFADEF AMEIEKIKRR
121 VVDIDRVRTT YNIMDTNNNN DCIPLDQRRL FLHVDETEVI GLDDDFNTLQ AKLLDQDLPY
181 GVVSIVGMPG LGKTTLAKKL YRHVRHKFEC SGLVYVSQQP RAGEILIDIA KQVGLTEDER
241 KENLENNLRS LLKRKRYVIL LDDIWDVEIW DDLKLVLPEC DSKIGSRIII TSRNSNVGRY
301 IGGDFSIHVL QPLNSENSFE LFTKKIFIFD NNNNWTNASP NLVDIGRSIV GRCGGIPLAI
361 VVTAGMLRAR ERTERAWNRL LESMSHKVQD GCAKVLALSY NDLPIALRPC FLYFGLYPED
421 HEIRAFDLTN MWIAEKLIVV NSGNGREAES LADDVLNDLV SRNMIQVAKR TYDGRISSCR
481 IHDLLHSLCV DLAKESNFFH TEHNALGDPG NVARLRRITF YSDNNAMNEF FRSNPKLEKL
541 RALFCFTEDP CIFSQLAHLD FKLLQVLVVV IFVDDICGVS IPNTFGNMRC LRYLRFQGHF
601 YGKLPNCMVK LKRLETLDIG YSLIKFPTGV WKSTQLKHLR YGGFNQASNS CFSISPFFPN
661 LYSLPHNNVQ TLMWLDDKFF EAGLLHRLIN LRKLGIAGVS DSTVKILSAL SPVPTALEVL
721 KLKIYRDMSE QINLSSYPNI VKLRLNVCGR MRLNCEAFPP NLVKLTLVGD EVDGHVVAEL
781 KKLPKLRILK MFGCSHNEEK MDLSGDGDSF PQLEVLHIDE PDGLSEVTCR DDVSMPKLKK
841 LLLVQRRPSP ISLSERLAKL RI*
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SEQ. ID. 5B = AMINO ACID SEQUENCE OF *Rpi-mcq1.2*

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  1 MAEILLTTVI NKSVGIAANV LFQEGTRLYW LKEDIDWLHR EMRHIRSYVD DAKAKEVGGD
 61 SRVRNLLKDI QQLAGDVEDL LDEFLPKIQQ SNKFICCLKT VSFADEFAME IEKIKRRVAD
121 ITRVRTTYNI TDTSNNNDDC IPLDRRRLFL HADETEVIGL EDDFNTLKAK LLDQDLPYGV
181 VSIVGMPGLG KTTLAKKLYR HVRDQFESSG LVYVSQQPRA GEILRDIAKQ VGLPKEERKE
241 NLEGNLRSLL KTKRYVILLD DIWDVEIWDD LKLVLPECDS EIGSRIIITS RNSNVGRYIG
301 GDFSIHMLQP LDSENSFELF TKKIFTFDNN NNWANASPDL VDIGRSIVGR CGGIPLAIVV
361 TAGMLRARER TEHAWNRVLE SMGHKVQDGC AKVLALSYND LPIALRPCFL YLGLFPEDHE
421 IRAFDLTNMW IAEKLIVVNS GNGREAESLA EDVLNDFVSR NLIQVSQRKC NGRISSYRIH
481 DLLHSLCVEL GKESNFFHTE HNAFGDPDNV ARVRRITFYS DNNAMSKFFR SNPKPKKLRA
541 LFCFTNLDSC IFSHLAHHDF KLLQVLVVVI SYNWLSVSIS NKFGKMSCLR YLRLEGPIVG
601 ELSNSIVKLK RVETIDIAGD NIKIPCGVWE SKQLRHLRNR EERRYFFSVS PFCLNMYPLP
661 PNNLQTLVWM DDKFFEPRLL HRLINLRKLG IWGTSDSTIK ILSALSPVPT ALEVLKLYFL
721 RDLSEQINLS TYPNIVKLNL QGFVRVRLNS EAFPPNLVKL ILDKIEVEGH VVAVLKKLPT
781 LRILKMYGCK HNEEKMDLSG DGDGDSFPQL EVLHIERPFF LFEITCTDDD SMPKLKKLLL
841 TTSNVRLSER LAKLRV*
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SEQ. ID. 6 = AMINO ACID SEQUENCE OF *Rpi-nrs1*

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  1 MNYCVYKTWA VDSNTKANST SFLSFFSYFP FLILTFRKKK FNEKLKEMAE ILLTAVINKS
 61 IEIAGNVLFQ EGTRLYWLKE DIDWLQREMR HIRSYVDNAK AKEVGGDSRV KNLLKDIQQL
121 AGDVEDLLDE FLPKIQQSNK FICCLKTVSF ADEFAMEIEK IKRRVADIDR VRTTYSITDT
181 SNNNDDCIPL DRRRLFLHAD ETEVIGLEDD FNTLQAKLLD HDLPYGVVSI VGMPGLGKTT
241 LAKKLYRHVC HQFECSGLVY VSQQPRAGEI LHDIAKQVGL TEEERKENLE NNLRSLLKIK
301 RYVILLDDIW DVEIWDDLKL VLPECDSKIG SRIIITSRNS NVGRYIGGDF SIHVLQPLDS
361 EKSFELFTKK IFNFVNDNWA NASPDLVNIG RCIVERCGGI PLAIVVTAGM LRARGRTEHA
421 WNRVLESMAH KIQDGCGKVL ALSYNDLPIA LRPCFLYFGL YPEDHEIRAF DLTNMWIAEK
481 LIVVNTGNGR EAESLADDVL NDLVSRNLIQ VAKRTYDGRI SSCRIHDLLH SLCVDLAKES
541 NFFHTEHYAF GDPSNVARVR RITFYSDDNA MNEFFHLNPK PMKLRSLFCF TKDRCIFSQM
601 AHLNFKLLQV LVVVMSQKGY QHVTFPKKIG NMSCLRYVRL EGAIRVKLPN SIVKLKCLET
661 LDIFHSSSKL PFGVWESKIL RHLCYTEECY CVSFASPFCR IMPPNNLQTL MWVDDKFCEP
721 RLLHRLINLR TLCIMDVSGS TIKILSALSP VPKALEVLKL RFFKNTSEQI NLSSHPNIVE
781 LGLVGFSAML LNIEAFPPNL VKLNLVGLMV DGHLLAVLKK LPKLRILILL WCRHDAEKMD
841 LSGDSFPQLE VLYIEDAQGL SEVTCMDDMS MPKLKKLFLV QGPNISPISL RVSERLAKLR
901 ISQVL*
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