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

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Summary

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

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

Introduction

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

Several *R* genes originating from introgressions of *S. demissum*, *S. bulbocastanum* and *S. berthaultii* have been mapped to potato chromosomes using DNA markers (El-Kharbotly *et al*., 1994; El-Kharbotly *et al*., 1996; Ewing *et al*., 2000; Leonards-Schippers *et al*., 1992; Li *et al*., 1998; Naess *et al*., 2000). *R1* is located on potato chromosome V.
(Leonards-Schippers et al., 1992) in a hot spot for resistance to various pathogens. Single genes for resistance to potato virus X have been mapped to the same region (De Jong et al., 1997; Ritter et al., 1991). This region also contains major quantitative trait loci (QTL) for resistance to late blight (Collins et al., 1999; Leonards-Schippers et al., 1994; Oberhagemann et al., 1999) and the parasitic root cyst nematode Globodera pallida (Kreike et al., 1994; Roupe van der Voort et al., 1997, 2000). The clustering of functional genes for qualitative and quantitative resistance to various pathogens suggests their evolution from common ancestors by local gene duplication followed by functional diversification (Gebhardt and Valkonen, 2001; Leister et al., 1996; Leonards-Schippers et al., 1994; Oberhagemann et al., 1999). Molecular cloning of the R1 gene should therefore facilitate study at the molecular level of several factors participating in the control of qualitative and quantitative resistance to various potato pathogens.

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

Results

High-resolution genetic mapping of the R1 locus

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

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

<table>
<thead>
<tr>
<th></th>
<th>GP21-</th>
<th>GP179-</th>
<th>GP21-GP179</th>
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<tr>
<td></td>
<td>R1</td>
<td>R1</td>
<td></td>
</tr>
<tr>
<td>Number of recombinants</td>
<td>23</td>
<td>8</td>
<td>31</td>
</tr>
<tr>
<td>Recombinants with genotype R1r1</td>
<td>12</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>Recombinants with genotype r1R1</td>
<td>11</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>Recombination frequency (%)</td>
<td>2.2</td>
<td>0.8</td>
<td>3.0</td>
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Chromosome walking towards the R1 locus and identification of candidate genes

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

The partially overlapping BACs in the contig shown in Figure 1 had insertions of between 70 and 100 kb, as...
estimated from BAC DNA digested with NotI and size-separated by pulsed-field gel electrophoresis. Based on the length of the individual BACs, the length of the physical map was estimated at 250–300 kb. The region co-segregating with the \( R_1 \) locus covered around 200 kb of the contig.

Line P6/210 from which the BAC library originated is heterozygous for the \( R_1 \) resistance allele (\( R_1r_1 \)). BACs in the contig therefore corresponded to either the chromosome carrying the \( R_1 \) or the chromosome carrying the \( r_1 \) allele. The polymorphisms of BAC end markers that were instrumental in mapping and contig construction (data not shown) were used to assign BAC clones to either the \( R_1 \) or the \( r_1 \) allele. BAC clones BA100e13, BA47f2, BA27c1 and BA87d17 (Figure 1) were in cis with the \( R_1 \) allele, whereas clones BA121o1, BA122p13 and BA76o11 were derived from the homologue with the \( r_1 \) allele (Figure 1).

Of all BACs in the contig, clone BA87d17 was the best candidate for hosting \( R_1 \) because (i) it co-segregates with the \( R_1 \) locus, (ii) it contains at least one RGL, and (iii) it is derived from the \( R_1 \)-containing chromosome.

**Complementation analysis**

Complementation analysis of susceptible cultivar Desirée with whole BAC DNA confirmed that clone BA87d17 contained the \( R_1 \) gene (unpublished data). To identify the \( R_1 \) gene within the BAC insertion, a random genomic sub-library with, on average, 10 kb insertions was constructed from BA87d17. The library was screened by colony hybridization for clones containing the candidate gene. Positive clones were evaluated for the presence of a complete copy of the candidate gene (including the putative promoter region) by the size of the amplification products obtained by PCR with forward primers from the vector borders and reverse primers from the candidate gene (data not shown). Subclone g10 was selected, which had an insertion of about 10 kb and contained at least 2 kb sequence upstream and 4 kb downstream of the candidate gene. Subsequent sequence analysis confirmed the presence of a single open reading frame in g10. Clone g10 was transformed into *Agrobacterium tumefaciens* and three different bacterial colonies were used to transform the susceptible cultivar Desirée. From three transformation experiments, 15 independent transgenic lines were regenerated and tested in four independent experiments for expression of hypersensitive resistance to *P. infestans* race 4 (\( Avr1 \)) (Table 2). Nine transgenic lines consistently showed a typical HR response, similar to the resistant line P41 hosting \( R_1 \) (Figure 2); three lines gave inconsistent results and the remaining three were susceptible, like the untransformed Desirée control. When transgenic lines that expressed HR upon inoculation with *P. infestans* race 4 (\( Avr1 \)) were infected with race 1,4 (\( avr1 \))...
they were susceptible (not shown). The candidate gene present in clone g10 was therefore necessary and sufficient to transfer the HR response upon infection with *P. infestans* to the susceptible cultivar Desiree. The observed response was as expected for the *R1* gene according to the nomenclature of Black et al. (1953). As line P41 from which the transgene originated carried only the *R1* gene (Leonards-Schippers et al., 1992), we concluded that the candidate gene present in g10 corresponded to *R1*.

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

**Candidate cDNA clones**

The whole insertions of BACs BA121o1 and BA76o11 both containing copies of the candidate RGL family were used to screen a cDNA library prepared from infected leaves of genotype P41 (*R1r1*). Fourteen cDNA clones were isolated, eight of which were, based on DNA sequence analysis, similar to known plant resistance genes. The highest similarity was obtained with the tomato *Prf* gene for resistance to *Pseudomonas syringae* (Salmeron et al., 1996). The sequences of the eight candidate cDNAs shared approximately 80–90% identity among each other. The sequence of one cDNA clone, c76-2, was 2292 nucleotides long and was identical, with the exception of the introns, to the sequence of the genomic clone g10 used for complementation analysis, thus indicating that the gene present in clone g10 was functional and expressed in leaves infected with an incompatible race of *P. infestans*.

Sequence comparison to known resistance genes in the database indicated that c76-2 was not full-length. Using RACE analysis, the cDNA was extended at the 5′ end by 1943 nucleotides, resulting in a full-length cDNA sequence of 4235 nucleotides including a 5′ untranslated region of 59 nucleotides and 297 nucleotides of 3′ untranslated sequence between the stop codon and the poly(A) tail. The cDNA included a start codon at position 2223 of the genomic sequence corresponding to the first methionine in the amino acid sequence deduced from the genomic clone g10 (Figure 4b). Two adenines were identified at positions -3 and +4 (with the A of the ATG initiation codon at position +1), referred to as the ribosome recognition sequence in plants, insects, yeast and mammals (Kozak, 1991).
PCR primers specific for cDNA c76-2 were designed based on sequence alignment with the other seven candidate cDNAs (not shown). Primers 76-2sf2 and 76-2SR (see Experimental procedures) generated a 1.4 kb PCR product that was present in the diploid line P41 (R1R1), the source of the R1 gene (Leonards-Schippers et al., 1992), in BAC clone BA87d17 hosting R1, and in all g10 transgenic plants showing hypersensitive resistance. The 1.4 kb fragment was not amplified in the susceptible genotypes P40 (r1r1) and cultivar Desireé (r1r1r1r1). The product was also absent in the other BACs in the contig (Figure 3).

Structure of the R1 gene

The sequence of the complementing genomic clone g10 (GenBank accession no. AF447489) was 10388 nucleotides long. It contained one open reading frame (ORF) with sequence similarity to other plant resistance genes, and included 2222 base pairs of the putative promoter region (Figures 4 and 5). No other ORFs or sequence homology were identified in the public databases. Sequence alignment with cDNA c76-2 and the 5’ RACE product revealed the presence of three exons and three introns. Two introns of 92 bp (positions 4878–4970) and 126 bp (positions 6103–6229) interrupt the coding region. The third intron of 81 bp (positions 6323–6404) is located in the 3’ untranslated region immediately downstream of the stop codon (Figure 4a). The deduced amino acid sequence suggests a polypeptide of 1293 amino acids with a molecular mass of 149.4 kDa (Figure 4b). The predicted R1 protein has a putative nucleotide binding site (NBS) domain consisting of P-loop (amino acids 572–578), kinase 2 (amino acids 649–653) and kinase 3a (amino acids 677–682) motifs (Figure 4b). Downstream of the kinase motifs were other sequences with similarity to domains of unknown function conserved among resistance genes: GLPL (QLPL in R1), CKLY (CFLY in R1) and MHD (LHD in R1). Searching for conserved motifs by using the ExPASY algorithm, four myristylation, nine glycosylation, 43 phosphorylation and one amidation putative sites were found in the deduced R1 amino acid sequence. The putative leucine-rich repeat (LRR) domain of R1 has 15/16 imperfect repeats located in the C-terminal part of the gene. Like some other plant R proteins with cytoplasmic LRRs, the deduced R1 protein contains a leucine zipper motif at amino acid positions 308–329, which may fold into a coiled-coil structure (Hammond-Kosack and Jones, 1997; Lupas, 1996). The deduced amino acid sequence of the R1 gene is most similar (36% identity) to the Prf gene for resistance to P. syringae of tomato (Salmeron et al., 1996). The sequence similarity extends throughout the deduced polypeptide sequences with the exception of the first 240 and 785 characters.
to position effects, co-suppression or rearrangements after transfer of a typical HR response to the susceptible cultivar Desireé in 60% of all transgenic plants tested. Failure of the candidate genes present in BAC clone BA87d17 was able (gene and the second one being allelic with a single member present in BA122p13. The functional R1 gene was part of a 15 kb insertion present in the R1-bearing chromosome in the region covered by BA87d17, but absent in the chromosome bearing r1 (Figure 5).

Discussion

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

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

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

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

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

Figure 5. Schematic representation of the potato chromosome V region around the R1 locus. Boxes filled with vertical lines represent regions homologous between the chromosomes bearing the R1 and r1 alleles. The functional R1 allele and the neighbouring locus with the r1.1 and r1.2 alleles are shown as open boxes. The boxes with diagonal lines including the R1 gene show the 15 kb insertion present on the R1 chromosome when compared to the r1 chromosome (deletion marked with dashed line). The genomic clone g10 (10 kb) used for functional complementation is shown relative to the R1 gene as straight line.
and plasma membranes (Freytag et al., 1994). The putative cytoplasmic localization of the R1 protein implies that the avirulence factor that interacts with R1 is translocated from the haustorium into the cytoplasm. In the comparable pathosystem of rice and the hemibiotrophic rice blast fungus Magnaporthe grisea, it has been shown that only the processed form of the extracellular avirulence protein AVR-Pita (Orbach et al., 2000) interacts inside the host plant cell with the corresponding cytoplasmic resistance protein Pi-ta (Jia et al., 2000). Alternatively, the R1 protein may be anchored intracellularly to the plasma membrane via a myristylation site and interacts directly or indirectly with the so far unknown product of the Avr1 gene of Phytophthora infestans.

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

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

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

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

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

**Experimental procedures**

**Plant material**

F1 offspring of a cross between the heterozygous diploid clones H79.1506/1 (R1r1) and H80.696/4 (r1r1), referred to as P41 and P40,
respectively (Gebhardt et al., 1989; Leonards-Schippers et al., 1992), was used for high-resolution genetic mapping of R1. Recombinants in the marker interval GP21–GP179 originating from the P41 (R1R1) parent were selected as described previously (Meksem et al., 1995). Hybrid P6210 derived from the cross P41 × P40 (Leister et al., 1996) which carries R1 in the heterozygous state was used for constructing genomic cosmid and BAC libraries. Parent P41 (R1R1) was used for cDNA library construction.

Test for resistance to Phytophthora infestans

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

Potato genomic libraries

The BAC library was supplied by LION Bioscience AG (Heidelberg, Germany). The library was constructed from HindIII partially digested high-molecular-weight genomic DNA of the potato genotype P6/210 in the binary vector pCLD04541 (Jones et al., 1992) as described previously (Meksem et al., 2000). The BAC library consists of approximately 100 000 clones with an average insert size of 70 kb. With the size of a haploid potato genome being approximately 10^9 base pairs, the genome coverage was 6–7 times per haploid genome. The colonies were stored in 264 384-microtitre plates (Genetix, Oxford, UK) in 2YT medium (Sambrook et al., 1989) with freezing buffer (5.5% w/v glycine, 7 mM MgSO4, 1.5 mM sodium citrate, 0.3 mM MgSO4, 13 mM KH2PO4, 27 mM K2HPO4).

A cosmid library of approximately 150 000 clones was constructed using standard procedures (Sambrook et al., 1989) from Sau3AI partially digested genomic DNA (17–23 kb fragments) of P6/210 in the same vector (BamHI cloning site) as the BAC library. Cosmids were packaged using Gigapack II Gold Packaging extract (Stratagene, California, USA) and transfected into E. coli strain SURE (Stratagene). Plasmid DNA was extracted from pools of about 1500 bacterial colonies (Sambrook et al., 1989). One hundred and three cosmid pools were generated and screened by PCR using S/PUD237-specific primers (De Jong et al., 1997). Positive pools were plated and screened by colony hybridization using standard protocols (Sambrook et al., 1989).

BAC DNA isolation

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

Preparation of probes from BAC insertions

A 1.5 μg aliquot of BAC DNA was digested to completion with HindIII plus EcoRI and separated from the vector on 0.8% low-melting-temperature agarose gel (Sea Plaque GTG Agarose, Bioproducts, Rockland, Maine, USA). Inserted DNA was dissolved from the gel using the GE Lase system (Epigen Technologies or Biozym) following the manufacturer’s instructions. The DNA was ethanol-precipitated, dissolved in water and labelled with 32p-dCTP by random primed labelling (Feinberg and Vogelstein, 1984).

Subcloning of BAC BA87d17

A 10 μg aliquot of BAC DNA was partially digested with 1 U Taq509I for 15 min at 65°C and size-separated on a 0.8% low-melting-temperature agarose gel (Sea Plaque GTG Agarose). Fragments of about 10 kb in size were eluted using the GE Lase system (Epigen Technologies, Madison, Wisconsin, USA), following the manufacturer’s instructions. The purified fragments were cloned into the pCLD04541 binary vector linearized with EcoRI, dephosphorylated using SHRIMP phosphatase (Roche, Mannheim, Germany) and transformed into E. coli strain DH10B (Life Technologies, Rockville, MD, USA). Two hundred recombinant colonies were picked into microtitre plates.

CDNA library construction and screening

Cut shoots of approximately 8-week-old plants of parent P41 (R1R1) and of the susceptible cultivar Desireè were infected with P.
infestans race 4 and maintained under a glass cylinder (to increase humidity) in water in a growth chamber at 17°C with 18 h light. Under these conditions, leaves of the susceptible control were overgrown by P. infestans mycelium after 8 days. Equal amounts of uninfected leaves of parent P41 and infected leaves 2 and 19 h and 3, 7 and 9 days after inoculation were collected. Poly(A)+ RNA was isolated using the RNeasy Plant Mini Kit and the Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. A λZAP II cDNA library (Stratagene) was constructed from the poly(A)+ RNA according to the manufacturer’s instructions. The different cDNA preparations were pooled prior to ligation into the λZAP vector. Approximately 5 x 10⁶ plaque-forming units were plated and screened by plaque hybridization (Sambrook et al., 1989) using as probe the insertions of BACs BA121o1 and BA76o11.

**RACE analysis**

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

**DNA sequence analysis**

Custom DNA sequencing was performed by the ADIS unit at the Max Planck Institute for Breeding Research. The dideoxy chain-termination sequencing method was employed using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit and an ABI377 automated DNA Sequencer (PE Biosystems, Foster City, California, USA). DNA sequence analysis was performed using the Wisconsin Package, version 10.0 (Genetics Computer Group (GCG), Madison, Wisconsin, USA). DNA sequence analysis was performed using the Wisconsin Package, version 10.0 (Genetics Computer Group (GCG), Madison, Wisconsin, USA). Sequence databases were searched with BlastX and other algorithms available through the National Center for Biotechnology Information (Bethesda, Maryland, USA) and the ExPASY www server (Appel et al., 1994).

**Transformation of Agrobacterium tumefaciens**

Subclone g10 of BAC BA87d17 was electroporated into A. tumefaciens strain LB4404 according to the method described by Wen-jun and Forde (1989). Three Agrobacterium strains, LBAg10-2, LBAg10-5 and LBAg10-23, were used for potato transformation.

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

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

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