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; Rouppe van der Voort *et al.*, 1997, 2000). The clustering of functional genes for qualitative and quantitative resistance to various pathogens suggests their evolution from common ancestors by local gene duplication followed by functional diversification (Gebhardt and Valkonen, 2001; Leister *et al.*, 1996; Leonards-Schippers *et al.*, 1994; Oberhagemann *et al.*, 1999). Molecular cloning of the *R1* gene should therefore facilitate study at the molecular level of several factors participating in the control of qualitative and quantitative resistance to various potato pathogens.

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

Results

High-resolution genetic mapping of the R1 locus

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

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

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

Chromosome walking towards the R1 locus and identification of candidate genes

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

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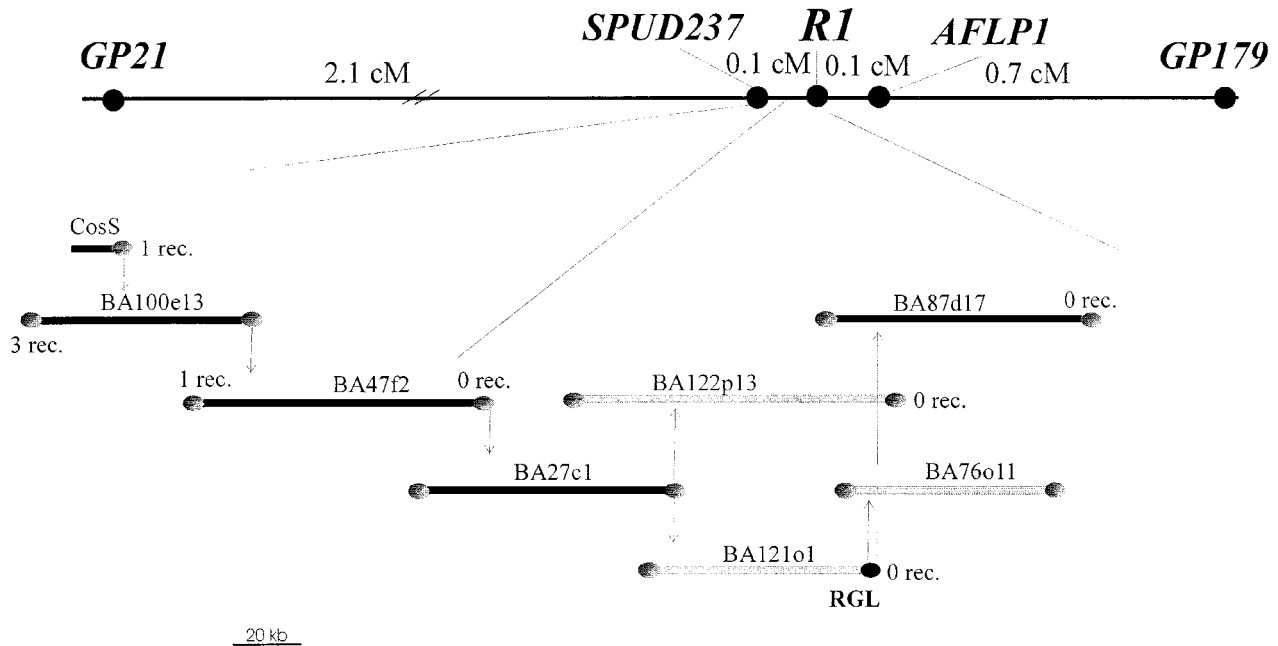
Figure 1. Genetic and physical map of the *R1* region.
GP21 and *GP179* are the markers used to construct the high-resolution map of the *R1* region. *SPUD237* and *AFLP1* are converted AFLP markers (De Jong *et al.*, 1997; Meksem *et al.*, 1995) flanking *R1*. Genetic distances are given in cM. CosS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

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

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

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

Complementation analysis

Complementation analysis of susceptible cultivar Desirée with whole BAC DNA confirmed that clone BA87d17 contained the *R1* gene (unpublished data). To identify the

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

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

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

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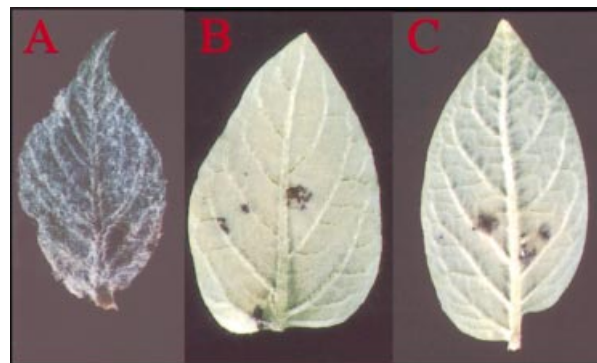[c]Plants were scored as resistant if HR was visible 8–10 days after inoculation with *P. infestans* race 4; R, resistant; S, sensitive; nd, resistance status not determined as lines showed inconsistent results.

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

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

they were susceptible (not shown). The candidate gene present in clone g10 was therefore necessary and sufficient to transfer the HR response upon infection with *P. infestans* to the susceptible cultivar Desirée. The observed response was as expected for the *R1* gene according to the nomenclature of Black *et al.* (1953). As line P41 from which the transgene originated carried only the *R1* gene (Leonards-Schippers *et al.*, 1992), we concluded that the candidate gene present in g10 corresponded to *R1*.
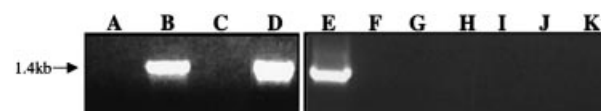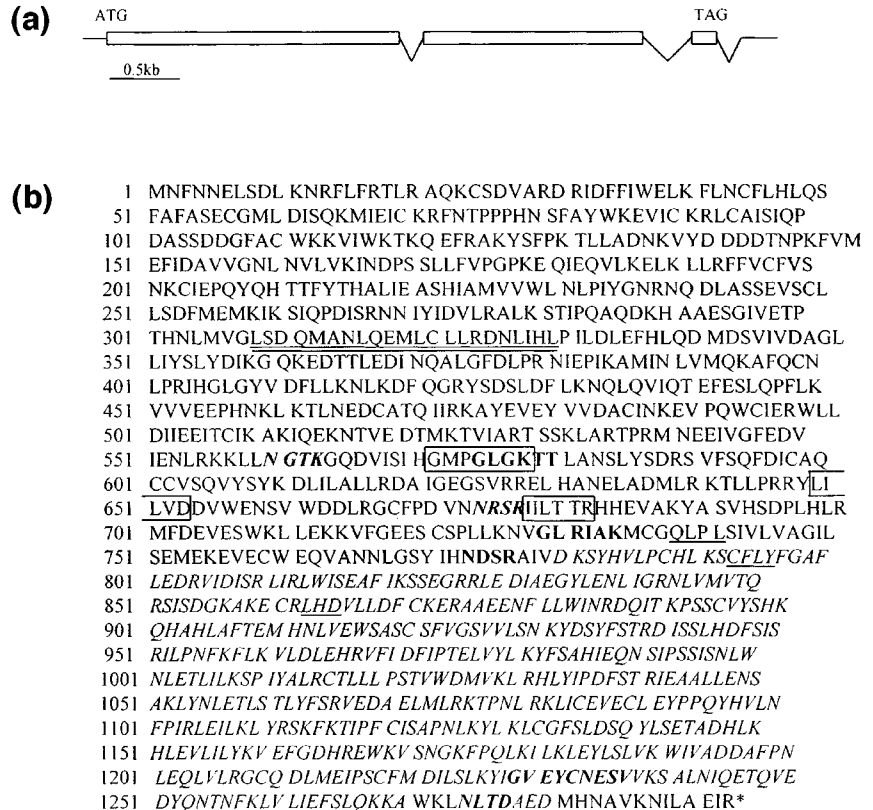
All transgenic lines that showed the HR response contained the gene corresponding to cDNA 76-2 (see below), as demonstrated by the presence of a 1.4 kb PCR product amplified by the sequence-specific primers 76-2sf2 and 76-2SR. This product was absent in untransformed Desirée (Figure 3).

Candidate cDNA clones

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

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

(a)

(b)

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   1 MNFNNELSDL KNRFLFRTLR AQKCSDVARD RIDFFIWELK FLNCFLHLQS
  51 FAFASECGML DISQKMIEIC KRFNTPPPHN SFAYWKEVIC KRLCAISIQP
 101 DASSDDGFAC WKKVIWKTKQ EFRAKYSFPK TLLADNKVYD DDDTNPKFVM
 151 EFIDAVVGNL NVLVKINDPS SLLFVPGPKE QIEQVLKELK LLRFFVCFVS
 201 NKCIEPQYQH TTFYTHALIE ASHIAMVVWL NLPIYGNRNQ DLASSEVSCL
 251 LSDFMEMKIK SIQPDISRNN IYIDVLRALK STIPQAQDKH AAESGIVETP
 301 THNLMVGLSD QMANLQEMLC LLRDNLIHLP ILDLEFHLQD MDSVIVDAGL
 351 LIYSLYDIKG QKEDTTLEDI NQALGFDLPR NIEPIKAMIN LVMQKAFQCN
 401 LPRIHGLGYV DFLLKNLKDF QGRYSDSLDF LKNQLQVIQT EFESLQPFLK
 451 VVVEEPHNKL KTLNEDCATQ IIRKAYEVEY VVDACINKEV PQWCIERWLL
 501 DIIEEITCIK AKIQEKNTVE DTMKTVIART SSKLARTPRM NEEIVGFEDV
 551 IENLRKKLLN GTKGQDVISI HGMPGLGKTT LANSLYSDRS VFSQFDICAQ
 601 CCVSQVYSYK DLILALLRDA IGEGSVRREL HANELADMLR KTLLPRRYLI
 651 LVDDVWENSV WDDLRGCFPD VNNRSHIILT TRHHEVAKYA SVHSDPLHLR
 701 MFDEVESWKL LEKKVFGEES CSPLLKNVGL RIAKMCGQLP LSIVLVAGIL
 751 SEMEKEVECW EQVANNLGSY IHNDSRAIVD KSYHVLPCHL KSCFLYFGAF
 801 LEDRVIDISR LIRLWISEAF IKSSEGRRLE DIAEGYLENL IGRNLVMVTQ
 851 RSISDGKAKE CRLHDVLLDF CKERAAEENF LLWINRDQIT KPSSCVYSHK
 901 QHAHLAFTEM HNLVEWSASC SFVGSVVLSN KYDSYFSTRD ISSLHDFSIS
 951 RILPNFKFLK VLDLEHRVFI DFIPTELVYL KYFSAHIEQN SIPSSISNLW
1001 NLETLILKSP IYALRCTLLL PSTVWDMVKL RHLYIPDFST RIEAALLENS
1051 AKLYNLETLS TLYFSRVEDA ELMLRKTPNL RKLICEVECL EYPPQYHVLN
1101 FPIRLEILKL YRSKFKTIPF CISAPNLKYL KLCGFSLDSQ YLSETADHLK
1151 HLEVLILYKV EFGDHREWKV SNGKFPQLKI LKLEYLSLVK WIVADDAFPN
1201 LEQLVLRGCQ DLMEIPSCFM DILSLKYIGV EYCNESVVKS ALNIQETQVE
1251 DYQNTNFKLV LIEFSLQKKA WKLNLTDAED MHNAVKNILA EIR*
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PCR primers specific for cDNA c76-2 were designed based on sequence alignment with the other seven candidate cDNAs (not shown). Primers 76-2sf2 and 76-2SR (see Experimental procedures) generated a 1.4 kb PCR product that was present in the diploid line P41 (*R1r1*), the source of the *R1* gene (Leonards-Schippers *et al.*, 1992), in BAC clone BA87d17 hosting *R1*, and in all g10 transgenic plants showing hypersensitive resistance. The 1.4 kb fragment was not amplified in the susceptible genotypes P40 (*r1r1*) and cultivar Desirée (*r1r1r1r1*). The product was also absent in the other BACs in the contig (Figure 3).

Structure of the R1 *gene*

The sequence of the complementing genomic clone g10 (GenBank accession no. AF447489) was 10 388 nucleotides long. It contained one open reading frame (ORF) with sequence similarity to other plant resistance genes, and included 2222 base pairs of the putative promoter region (Figures 4 and 5). No other ORFs or sequence homology were identified in the public databases. Sequence alignment with cDNA c76-2 and the 5′ RACE product revealed the presence of three exons and three introns. Two introns of 92 bp (positions 4878–4970) and 126 bp (positions 6103–6229) interrupt the coding region. The third intron of 81 bp (positions 6323–6404) is located in the 3′ untranslated

region immediately downstream of the stop codon (Figure 4a). The deduced amino acid sequence suggests a polypeptide of 1293 amino acids with a molecular mass of 149.4 kDa (Figure 4b). The predicted R1 protein has a putative nucleotide binding site (NBS) domain consisting of P-loop (amino acids 572–578), kinase 2 (amino acids 649–653) and kinase 3a (amino acids 677–682) motifs (Figure 4b). Downstream of the kinase motifs were other sequences with similarity to domains of unknown function conserved among resistance genes: GLPL (QLPL in *R1*), CKLY (CFLY in *R1*) and MHD (LHD in *R1*). Searching for conserved motifs by using the ExPASY algorithm, four myristylation, nine glycosylation, 43 phosphorylation and one amidation putative sites were found in the deduced R1 amino acid sequence. The putative leucine-rich repeat (LRR) domain of R1 has 15/16 imperfect repeats located in the C-terminal part of the gene. Like some other plant R proteins with cytoplasmic LRRs, the deduced R1 protein contains a leucine zipper motif at amino acid positions 308–329, which may fold into a coiled-coil structure (Hammond-Kosack and Jones, 1997; Lupas, 1996). The deduced amino acid sequence of the *R1* gene is most similar (36% identity) to the *Prf* gene for resistance to *P. syringae* of tomato (Salmeron *et al.*, 1996). The sequence similarity extends throughout the deduced polypeptide sequences with the exception of the first 240 and 785
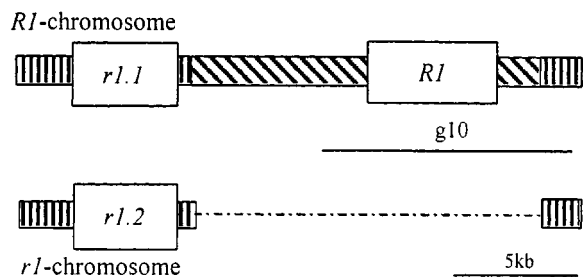
Figure 5. Schematic representation of the potato chromosome V region around the *R1* locus.

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

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

Genomic organization of the R1 *locus*

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

Discussion

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

Complementation analysis showed that one of two candidate genes present in BAC clone BA87d17 was able to transfer a typical HR response to the susceptible cultivar Desirée in 60% of all transgenic plants tested. Failure of the transgene to express the HR response may have been due to position effects, co-suppression or rearrangements after

transformation, and has also been observed in other complementation experiments with resistance genes (Grant *et al.*, 1995; Whitham *et al.*, 1994).

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

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

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

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

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

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

The resistance hot spot on potato chromosome V that includes *R1* also contains major QTL (quantitative trait loci) for resistance to *Phytophthora infestans* (Collins *et al.*, 1999; Leonards-Schippers *et al.*, 1994; Oberhagemann *et al.*, 1999) and the root cyst nematode *Globodera pallida* (Kreike *et al.*, 1994; Rouppe van der Voort *et al.*, 1997; Rouppe van der Voort *et al.*, 2000). Linkage disequilibrium mapping revealed strong association of markers in the 0.8 cM interval *SPUD237–GP179* containing *R1*, with the

resistance of foliage and tubers to late blight (unpublished data), supporting the tight linkage between *R1* and factors controlling quantitative resistance to late blight. It has been suggested, based on the observed genetic linkage, that *R1* and the factors controlling quantitative resistance to late blight may be alleles of the same gene or members of a clustered gene family (Leonards-Schippers *et al.*, 1994; Oberhagemann *et al.*, 1999). The molecular analysis of the *R1* locus supports the latter option as *R1* is a member of a gene family with at least one additional member physically closely linked to *R1*. Further members might be present in those parts of the *SPUD237–GP179* interval not yet covered by the physical map and/or in other parts of the potato genome. Now that the *R1* sequence is available, those members can now be identified and characterized for structure and function. Allelic variants of *R1* in *S. tuberosum* and homologues in other Solanaceae species, that are involved in quantitative resistance to *P. infestans*, may be isolated.

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

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

Experimental procedures

Plant material

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

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

Test for resistance to Phytophthora infestans

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

Potato genomic libraries

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

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

BAC library screening and contig construction

High-density colony filters for hybridization-based screening of the BAC library were prepared using a BioGRID robot (Oxford, UK). Clones were gridded in double spots using a 5 × 5 array with 6 × 384 arrays per 22.5 × 22.5 cm nylon membrane (PALL, Biodyne, Portsmouth, UK). Each 5 × 5 array contained 2 × 12 colonies with the control position of the array occupied by the clone pSW1 (PE Biosystems, Foster City, California, USA). This gridding pattern allowed 27 648 colonies to be represented twice on each filter. Library screening was performed using a set of four filters carrying 101 376 clones. Colony filters were incubated on LB medium for 15 h at 37°C and processed for colony hybridization using standard techniques (Sambrook *et al.*, 1989). Filter hybridization was performed as described previously (Gebhardt

et al., 1989), except that 300 pg of pSW1 control insert were labelled and hybridized together with the probe to facilitate the determination of addresses of positive clones. Plasmid DNA was purified from positive clones and insertions were sequenced from both ends using T3 and T7 oligonucleotides as sequencing primers. DNA sequence information for BAC insertion ends was used to design specific PCR primer pairs. PCR products amplified with these primers and the respective BACs as template were used as probes for a new filter hybridization to identify overlapping BAC clones, for orientation of overlapping BAC clones relative to each other, and for mapping in the recombinant plants. Overlaps were confirmed by sequencing the PCR products. Direction of contig extension was verified by genetic mapping using the recombinant plants and RFLP- or PCR-based marker analysis. To determine the size of BAC insertions, the BAC DNA was digested with *Not*I and the fragments were separated by pulsed-field gel electrophoresis on a CHEF DRIII (Bio-Rad, Hercules, California, USA) for 12 h at 11°C with an initial pulse time of 5 sec and a final pulse time of 10 sec, at a 120° angle and 6 V cm^{-1}.

BAC DNA isolation

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

Preparation of probes from BAC insertions

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

Subcloning of BAC BA87d17

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

CDNA library construction and screening

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

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

RACE analysis

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

DNA sequence analysis

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

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

Transformation of *Agrobacterium tumefaciens*

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

Agrobacterium tumefaciens-mediated potato transformation and analysis of transgenic plants

The susceptible potato cultivar Desirée was used in all transformation experiments. *Agrobacterium tumefaciens*-mediated transformation was performed as described by Rocha-Sosa *et al.* (1989), except that the MS medium contained 250 mg l⁻¹ claforan. Kanamycin-resistant transgenic plants were tested by PCR for the presence of the g10 insert using the insert-specific primers 87e (5'-

ATTACAATGGGTTGAACTCAG-3') and 87s (5'-ACCTCTTTC-AATTGTTCTGGTG–3'). PCR conditions were: annealing at 55°C for 45 sec and polymerization at 72°C for 60 sec. Transgenic plants were screened with the *R1*-specific primers 76-2sf2 (5'-CACTCGTGACATATCCTCACTA-3') and 76-2SR (5'-CAACCC-TGGCATGCCACG-3') derived from cDNA c76-2. PCR conditions were: annealing at 55°C for 45 sec and polymerization at 72°C for 90 sec. Tests for resistance to *P. infestans* race 4 were performed using three leaflets per plant in each test.

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