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Disclosed are nucleic acid molecules encoding polypeptides which are capable of conferring resistance against, and being triggered by, plant pathogens such as fungi (e.g. *Phytophthora infestans* and related isolates). Preferred nucleic acid molecules encode R1 from *Solanum tuberosum*, or a variety of homologs, naturally occurring or derivatives) thereof. Particular methods of activating resistance by using the R1 resistance gene are disclosed, which in certain cases lead to a hypersensitive response. Further aspects of the invention include specific primers, vectors, host cells, polypeptides, antibodies, aptamers, transgenic plants, methods of producing and employing these, and methods of influencing the resistance trait in a plant. Furthermore, screening methods for identifying and obtaining plant protective compounds are provided.

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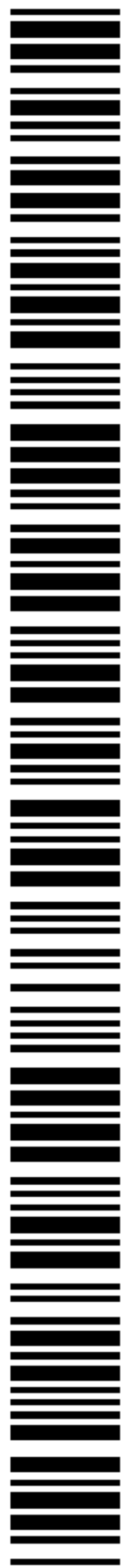
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(54) Title: PLANT-DERIVED RESISTANCE GENE

(57) Abstract: Disclosed are nucleic acid molecules encoding polypeptides which are capable of conferring resistance against, and being triggered by, plant pathogens such as fungi (e.g. Phytophthora infestans and related isolates). Preferred nucleic acid molecules encode R1 from Solanum tuberosum, or a variety of homologs, naturally occurring or derivatives) thereof. Particular methods of activating resistance by using the R1 resistance gene are disclosed, which in certain cases lead to a hypersensitive response. Further aspects of the invention include specific primers, vectors, host cells, polypeptides, antibodies, aptamers, transgenic plants, methods of producing and employing these, and methods of influencing the resistance trait in a plant. Furthermore, screening methods for identifying and obtaining plant protective compounds are provided.



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PLANT-DERIVED RESISTANCE GENE

The present invention relates to the *R1* resistance gene from potato. It further relates to methods and materials employing the gene, and processes for identifying or producing other related genes. It also relates generally to methods for identifying plant protective agents which are capable of inducing the *R1* gene or the activity of its encoded protein. Furthermore, the present invention relates to transgenic plants which became resistant to Late Blight because of the expression of an *R1* transgene.

Several documents are cited throughout the text of this specification by name. Full bibliographic citations may be found at the end of the specification immediately preceding the sequence listing or claims. There is no admission that any document cited is indeed prior art as to the present invention.

Late blight is worldwide the most destructive disease for potato cultivation causing billion-dollar losses every year (Kamoun et al. 1999). The causal pathogen is *Phytophthora infestans*, an oomycetous fungus infecting also 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. Introgression into potato cultivars of *R* genes conferring race specific resistance provided, however, only transient resistance to late blight, as new races rapidly overcame the *R* gene mediated resistance (Wastie 1991, Fry and Goodwin 1997). Quantitative or field resistance to late blight has also been identified in wild potato species (Ross 1986). This resistance is more durable than the one mediated by *R*

genes, but difficult to move into cultivated varieties by crossing and phenotypic selection. Late blight is still mostly controlled by the frequent application of fungicides which loose efficiency by selection of fungicide resistant isolates.

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

Thus, the technical problem underlying the present invention was to comply with the need for plant pathogen resistance genes and their regulatory sequences.

The solution to the technical problem is achieved by providing the embodiments characterized in the claims and described further below.

In accordance with the present invention *R1*, the first gene for resistance to Late blight has been cloned and characterized at the molecular level. The gene was identified by a particular combined positional cloning and candidate gene approach. The molecular structure of the gene allows to classify *R1* among plant resistance genes containing conserved NBS-LRR and leucine-zipper motifs (Ellis et al. 2000, Dangl and Jones, 2001).

R1 was cloned by using a positional cloning strategy in combination with searching for candidate genes having DNA sequence similarity to known plant resistance genes (Hammond-Kosack and Jones 1997, Ellis et al. 2000). A similar approach

was successful for 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). A chromosome walk towards *R1* was initiated from two marker loci *SPUD237* and *AFLP1*, flanking *R1* at short genetic distances of 0.1 cM. Walking from marker *AFLP1* proved unproductive, however, due to scarcity of BAC and YAC clones (Leister et al. 1997) having the *AFLP1* marker. The identification of potato genomic clones with overlapping inserts was facilitated by BAC technology in combination with the use of macroarrays of BAC clones. Application of this approach was successful in rice (Nakamura et al. 1997, Yang et al. 1997, Yang et al. 1998) and tomato (Folkertsma et al. 1998). The physical map (Figure 1) covers at least 250 kb of the potato genome. Around 200 kb were a candidate region for containing the *R1* gene based on linkage without recombination to BAC end markers. The candidate region was open-ended towards the *AFLP1* locus because the single recombination event separating *R1* and *AFLP1* was not included in the physical map. Partial sequence information from the candidate region identified an RGL resistance-gene like gene fragment that detected a gene family with members present on both chromosomes carrying the susceptibility allele *r1* or the resistance allele *R1*. In fact, the RGL probe used to identify cDNA and BAC clones for *R1* was part of an *r1* susceptibility allele. Based on an allele specific PCR assay derived from a cDNA clone encoding part of *R1*, a functional member of the candidate gene family was shown to be present in plants having the *R1* resistance allele and to be absent in susceptible plants. This candidate gene was subcloned from BAC BA87d17 and stably transformed into the susceptible cultivar Desirée. Complementation of the *R1* phenotype in several transgenic plants showed that the candidate gene was, indeed, the *R1* gene.

Accordingly, the present invention relates to a nucleic acid molecule encoding a polypeptide which is capable of conferring resistance against a pathogen in a plant in which said polypeptide is expressed, said nucleic acid molecule comprising or consisting of a nucleotide sequence selected from the group consisting of:

- (a) a nucleotide sequence encoding at least the mature form of a protein (*R1*) comprising the amino acid sequence as given in SEQ ID NO: 2;

- (b) a nucleotide sequence comprising at least one or more coding regions of the DNA sequence as given in SEQ ID NO: 1;
- (c) a nucleotide sequence hybridizing with the complementary strand of a nucleotide sequence as defined in (a) or (b) under stringent hybridization conditions;
- (d) a nucleotide sequence encoding a protein derived from the protein encoded by a nucleotide sequence of (a) or (b) by way of substitution, deletion and/or addition of one or several amino acids of the amino acid sequence encoded by the nucleotide sequence of (a) or (b);
- (e) a nucleotide sequence encoding a protein having an amino acid sequence at least 60 % identical to the amino acid sequence encoded by the nucleotide sequence of (a) or (b);
- (f) a nucleotide sequence encoding at least a Leucine zipper (LZ) domain corresponding to amino acid position 308-329 of SEQ ID NO: 2, a nucleic binding site (NBS) domain corresponding to amino acid position 572-682 of SEQ ID NO: 2 and/or a Leucine rich repeat (LRR) domain corresponding to amino acid position 780-1280 of SEQ ID NO: 2;
- (g) a nucleotide sequence encoding an epitope-bearing portion of a R1 protein encoded by a nucleotide sequence of (a) or (b);
- (h) a nucleotide sequence comprising at least 15 consecutive nucleotides of a nucleotide sequence of any one of (a) to (g);
- (i) a nucleotide sequence encoding a polypeptide comprising a one or more motifs as given in the SEQ ID NOS: 10 and 12, in Figure 4 or the amino acid sequence LHD;
- (j) DNA sequences obtainable by screening an appropriate library under stringent conditions with a probe having at least 17 consecutive nucleotides of a nucleotide sequence of any one of SEQ ID NOS: 1 or 5 to 8;
- (k) a nucleotide sequence encoding a fragment of at least 6 consecutive amino acids of a protein encoded by a nucleotide sequence of (a) or (b); and

- (l) the nucleotide sequence of which is degenerate as a result of the genetic code to a nucleotide sequence of any one of (a) to (i).

According to a first aspect of the present invention there is provided a nucleic acid molecule encoding a polypeptide which is capable of conferring resistance against a pathogen, such as fungi, in a plant into which said polypeptide is expressed.

Nucleic acid molecules according to the present invention may be provided in recombinant form or free or substantially free of nucleic acid or genes of the species of interest or origin other than the sequence encoding a polypeptide with the required function. The nucleic acid molecules (and their encoded polypeptide products) may also be (i) isolated and/or purified from their natural environment (although not necessarily in pure form per se), or (ii) in substantially pure or homogeneous form.

Nucleic acid according to the present invention may include cDNA, RNA, genomic DNA, preferably the intact gene, and may be wholly or partially synthetic (constructs'). Where a DNA sequence is specified, e. g. with reference to a figure or SEQ ID NO, unless context requires otherwise the RNA equivalent, with U substituted for T where it occurs, is encompassed. Also encompassed is the complement of the various disclosed sequences, which may be used in probing experiments, or in down-regulation of the sequence.

A particular aspect of the invention is a nucleic acid molecule having the sequence all or part of the sequence shown in SEQ ID NO: 1 including (where appropriate) both coding and/or non-coding regions. Within SEQ ID NO: 1 there is apparently a large open reading frame (ORF). Subsequent comparison of the genomic DNA sequence with the sequence of cDNAs revealed that the gene contains three exons and three introns; see Example 5 and Figure 4. The putative *R1* polypeptide sequence is shown in Figure 4 designated SEQ ID NO: 2. *R1* appears to contain 1293 amino acid residues and has a molecular weight of 149.4 kDa. Particular nucleic acid molecules of this aspect of the invention include those encoding the *R1* protein product and cDNA, believed to be base 2223-6321 excluding the introns marked as shown (4878-4970 and 6130-6229 inclusive). Surprisingly the primary structure of *R1* is similar to that of the L. Zipl NBS-LRR (Hammond-Kosack and Jones 1997) class of R proteins. Based on the deduced protein sequence, *R1*

belongs to the L.Zip/NBS/LRR class of plant resistance genes (Hammond-Kosack and Jones 1997). The leucine zipper motif (L.Zip) in the amino-terminal region is thought to feature in dimerization or interaction with other proteins. The downstream putative nucleotide-binding site (NBS) domain may be involved in the signal transduction pathway leading to the onset of the resistance response. The C-terminal leucine-rich repeat (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).

R1 is located on the short arm of chromosome V (Leonards-Schippers et al. 1992, Dong et al. 2000) and is sequence related to the tomato *Prf* gene for resistance to *Pseudomonas 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 colinear with each other except a paracentric inversion of the short arm (Tanksley et al. 1992). The potato locus *StPto* corresponding to *Pto/Fen* maps more than 10 cM proximal to *R1* (Leister et al. 1996), excluding, therefore, the possibility that *R1* and *Prf* are located in a colinear genomic region. This is the case, however, when considering the tomato *Bs4* gene conferring resistance to the bacterial pathogen *Xanthomonas campestris*. The position of the potato locus corresponding to *Bs4* can be inferred from the tight linkage (1 cM) between *Bs4* and the marker TG432 (Ballvora et al. 2001) which maps 3.8 cM distal to *GP21* on the tomato molecular map (Tanksley et al. 1992). This region of the tomato chromosome 5 that extends distal from the *GP21* marker should be colinear with the potato interval *GP21* – *GP179* including *R1*, when taking into account the paracentric inversion between the two genomes.

Two potato genes for resistance to *Potato Virus X*, *Rx2* and *Nb*, also map to similar positions as *R1* (Ritter et al. 1991, Leonards-Schippers et al. 1992, De Jong et al.

1997). The *Rx2* gene has been cloned and is, like *R1*, a member of the L.Zip/NBS/LRR class of resistance genes (Bendahmane et al. 2000). The two resistance genes share only 32 % sequence identity and are, therefore, rather different members of the same superfamily of genes. *Nb* is located in the interval *GP21* – *SPUD237* (De Jong et al. 1997) not containing *R1* and is genetically separated, therefore, from *R1*.

In a further aspect of the invention there are disclosed active, homologous, variants of the *R1* sequences, which may for instance be mutants or other derivatives, or naturally occurring *R1* homologues such as allelic variants, paralogues (from the same species, but at a different location e. g. pseudoalleles at linked loci), or orthologues (related genes from different species). Examples of these are shown below. In each case the variant encodes a product which is homologous (similar) to *R1*, which may be isolated or produced on the basis of that sequence, and is capable of conferring pathogen resistance against one or more pathogens.

Resistance gene activity can be tested by conventional methods known in the art, as appropriate to the nature of the resistance being investigated. Example methods can be found in the following publications: bacterial (Grant, (1995) Science 269, 843-846); fungal (Dixon, (1996) Cell 84, 451-459; Jones, (1994) Science 266, 789-793; Thomas, (1997) The Plant Cell 9, 2209-2224; nematode and viral (Whitham, (1994) Cell 78, 1101-1115). Typically, activity is tested by complementation of trait in a plant; see example 4. This can be achieved by using the isolated gene or for example by coupling the putative active variant to a promoter and terminator for expression in plants and transforming it into a susceptible plant that lacks a given resistance trait. The activity of the *R1* variant is then confirmed by challenge with the appropriate pathogen. Alternatively a transient expression assay can be used to test for activation of the *R1* variant analogous to the assay used by Mindrinos, (1994) Cell 78, 1089-1099. Briefly, the putative active *R1* variant is coexpressed from a plasmid with a pathogen-derived gene which is an elicitor of the resistance specified by the putative

R1 homologue and a reporter gene (e.g. GUS). If the variant is activated by the continuous expression of the pathogen derived gene, then an HR would result and

the reporter gene activity would be abolished. If no activity was initiated, then the reporter gene would be detectable.

Similarity or homology between the variant and *R1* may be as defined and determined by the TBLASTN program, of Altschul, (1990) J. Mol. Biol. 215, 403-10, which is in standard use in the art, or, and this may be preferred, the standard program BestFit, which is part of the Wisconsin Package, Version 10, January 1999, (Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA, Wisconsin 53711), which has been used to calculate sequence homologies in the present application. DNASTAR software using the CLUSTAL method with PAM250 residue weight table (gap penalty 10, gap length 10) may also be used. Homology (or similarity, or identity) may be at the nucleotide sequence and/or the expressed amino acid sequence level. Preferably, the nucleic acid and/or amino acid sequence shares homology with the coding sequence or the sequence encoded by the nucleotide sequence of SEQ ID NO:1 or other sequences set out herein, preferably at least about 50%, or 60%, or 70%, or 80% homology, most preferably at least about 90%, 95%, 96%, 97%, 98% or 99% homology. Homology may be over the full-length of the relevant sequence shown herein, or may more preferably be over a contiguous sequence of about or greater than about e. g. 20, 100, 200, 300, 500, 600 or more amino acids or codons, compared with the relevant amino acid sequence or nucleotide sequence as the case may be.

There are believed to be more than two homologues of *R1* in the potato genome. It is likely that one or more of these homologues are R genes against viruses, fungi, bacteria or nematodes.

Naturally occurring *R1* variants may be isolated, in the light of the present disclosure, without burden from any suitable plant. Naturally occurring *R1* variants may be isolated, from e.g. genomic or cDNA. The putative resistance genes can be obtained using materials (e. g. primers or probes) based on regions peculiar to *R1*, for instance designated in Figure 4. As discussed in the appended examples, the *R1* gene identified according to the present invention in potato is expected to define a novel class of plant resistance genes. Corresponding genes encoding proteins displaying similar properties should therefore be present in other plants as well.

Nucleic acid molecules of the invention can be obtained, e.g., by hybridization of the above-described nucleic acid molecules with a (sample of) nucleic acid molecule(s) of any source. Nucleic acid molecules hybridizing with the above-described nucleic acid molecules can in general be derived from any plant possessing such molecules, preferably from dicotyledonous plants, in particular from any plant of interest in agriculture, horticulture or wood culture, such as crop plants, namely those of the family Solanaceae, such as potato and tomato but also from plants such as manioc, leguminous plants, oil producing plants, such as oilseed rape, linseed, etc., plants using polypeptide as storage substances, such as soybean, plants using sucrose as storage substance, such as sugar beet or sugar cane, trees, ornamental plants as well as plants that can be used for the production of biomass, regenerative energy, or building materials such as cambric grass etc.

Thus a further aspect of the present invention provides a method of identifying and/or cloning homologous *R1* genes from a plant, which method employs all or part of a nucleotide sequences as described above. Thus in one embodiment, nucleotide sequence information provided herein may be used in a data-base (e.g. of ESTs, or STSs, or other genomic sequence information) search to find homologous sequences, expression products of which can be tested for pathogen resistance activity e.g. using methods based on the transient assays of the present invention, or conventional phenotype assays in transgenic plants.

Alternatively, probes based on the sequence may be used e.g. in southern blotting. For instance DNA may be extracted from cells taken from plants displaying the appropriate resistance trait and digested with different restriction enzymes. Restriction fragments may then be separated (e.g. by electrophoresis on an agarose gel) before denaturation and transferred to a nitrocellulose filter. Labelled probe may be hybridised to the DNA fragments on the filter and binding determined.

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 example, hybridizations may be performed using a hybridization solution comprising: 5X SSC (wherein SSC= 0.15

M sodium chloride; 0.15 M sodium citrate; pH 7), 5X Denhardt's reagent, 0.5-1.0% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes-1 hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-65°C in 1X SSC and 1% SDS, changing the solution every 30 minutes.

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): $T_m = 81.5^{\circ}\text{C} + 16.6\text{Log} [\text{Na}^+] + 0.41 (\% \text{ G+C}) - 0.63 (\% \text{ formamide}) - 600/\text{\#bp in duplex}$. As an illustration of the above formula, using $[\text{Na}^+] = [0.368]$ 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. It is well known in the art to increase stringency of hybridisation gradually until only a few positive clones remain. Other suitable conditions include, e. g. for detection of sequences that are about 80-90% identical, hybridization overnight at 42°C in 0.25M Na₂HPO₄, 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 Na₂HPO₄, pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 60°C in 0.1X SSC, 0.1% SDS.

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 (including, where appropriate, RACE PCR), RNase protection and allele specific oligonucleotide probing.

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 by cloning in a vector that replicates in a suitable host.

In each case, if need be clones (e.g. lambda, cosmid, plasmid, BACs, biBACS) or fragments identified in the search can be extended or supplemented. For instance if it is suspected that they are incomplete, the original DNA source (e.g. a clone library, mRNA preparation etc.) can be revisited to isolate missing portions e.g. using sequences, probes or primers based on that portion which has already been obtained to identify other clones containing overlapping sequence (see e.g. "Principles of Genome Analysis" by S B Primrose (1995) Pub. Blackwell Science Ltd, Oxford, UK).

The nucleic acid molecules or corresponding genes can then be tested for functionality, for example, as described in the examples. One scheme for isolating R1 homologues is as follows:

- I) produce a population in which a resistance trait is segregating.
- II) PCR amplify DNA from individual members of the population with primers based on the sequence of R1 (but not from the R gene conserved motifs).
- III) test the PCR products (either by direct sequence analysis or restriction enzyme digestion) for sequence polymorphism that cosegregates with the R trait. Identify an appropriate polymorphic marker sequence.
- IV) Isolate the complete coding sequence of the polymorphic gene. This could be done from an appropriate cloned library or by amplifying it using primers from the 5' and 3' extremes of R1. In each case the identified polymorphic PCR product, or sequence information provided by it, is used to identify the gene.

Resistance gene coding activity can then be tested as described above or in the examples.

A more specific approach is based on the understanding that homologous *R1*-genes may be linked in clusters. Clustering of R-genes in potato has already been reported (Leister et al. 1996; De Jong et al. 1997). One of the large R-gene clusters is on the short arm of potato chromosome V.

The resistance hot spot on potato chromosome V which includes *R1*, also contains major QTL (Quantitative Trait Loci) for resistance to *Phytophthora infestans* (Leonards-Schippers et al. 1994, Oberhagemann et al. 1999, Collins et al. 1999) and the root cyst nematode *Globodera pallida* (Kreike et al. 1994, Rouppe van der Voort et al. 1997, 2000). Linkage disequilibrium mapping revealed strong association between markers in the 0.8 cM interval *SPUD237* – *GP179* containing *R1* and resistance of foliage and tubers to late blight supporting tight linkage between *R1* and the 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 first molecular analysis of the *R1* locus now revealed that the latter option is more favourable as *R1* is a member of a gene family and is present as an extra copy in a DNA insertion in the *R1* bearing 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). A second highly homologous member of the *R1* gene family, having two alleles *r1.1* and *r1.2*, is located physically close to *R1*. Further studies on the functionality of this gene are required. With the *R1* sequence being available, other members of the *R1* family can now be identified that might be present in those parts of the *GP21* – *GP179* interval not yet covered by the physical map and/or in other parts of the potato genome. Allelic variants in *S. tuberosum* and homologs in other Solanaceae species may be isolated which are involved in quantitative resistance to *P. infestans*.

It is thus a preferred embodiment of the present invention that said pathogen which a plant expressing a nucleic acid molecule of the invention is resistant against is *Phytophthora infestans*.

The interaction between *R1* and the late blight pathogen is in concordance with the gene-for-gene concept (Person et al. 1962, Flor 1971). 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* (all races except those with race 1 specificity). *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). No avirulence factor of *P. infestans* has been cloned so far. Further characterisation of *R1* at the molecular level and cloning of the *Avr1* gene should contribute to clarify how the resistance protein recognises the avirulence effector molecule. Cloning of late blight resistance genes that recognise avirulence factors different from *Avr1* might allow identification of the molecular motifs that determine the specificity of effector recognition and may help to engineer R-proteins with broader and more durable resistance to late blight. Other, linked, *R1* variants (providing different R traits) may be isolated essentially as set out above, but wherein the DNA used for the initial amplification step is taken from members of the population in which the required R trait co-segregates with *R1* itself (or an *R1* variant).

It has been noted by the present inventors that the sequence of *R1* is similar to the sequence of the otherwise unrelated *Prf* gene that confers resistance in tomato against a bacterial pathogen, i.e. *P. syringae* (Salmeron et al. 1996). In the light of this information it appears that the sequence of *R1* could be modified e.g. by site-directed or random mutation, to produce *R1* mutants or other derivatives which can confer resistance against (i.e. is switched on by) pathogens that are quite different from *P. infestans*. This can be achieved as described below, with *R1* mutants being tested with the transient expression assay methods described above.

Preferably the nucleic acid molecule which is the mutant or other derivative is generated either directly or indirectly (e.g. via one or amplification or replication steps) from an original nucleic acid corresponding to all or part of the sequence shown in SEQ ID NO:1 or other sequences disclosed herein.

Thus a further aspect of the present invention is a method of producing a nucleic acid encoding an *R1* derivative comprising the step of modifying a nucleic acid molecule encoding *R1*. The derivative may include changes to the nucleic acid molecule which make no difference to the encoded amino acid sequence (i. e. degeneratively equivalent). Changes to a sequence, to produce a mutant or

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. In addition to one or more changes within the *R1* sequence, a variant nucleic acid may encode an amino acid sequence including additional amino acids at the C-terminus and/or N-terminus.

Specifically included are parts or fragments (however produced) corresponding to portions of the sequences provided, and which encode polypeptides having biological activity, for instance pathogen resistance or the ability to raise or bind *R1*-binding antibodies.

Generally speaking, 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 for glycosylation, lipoylation etc. Leader or other targeting sequences may be added to 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). Preferred modifications include those which decreases the net negative charge of the region in or around QLPL, CFLY or LHD motifs. Means and methods how to modify resistant genes are known to the person skilled in the art and described, for example in WO 01/29239 for the Rx gene of *Solanum tuberosum*. 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.

As is well-understood, homology at the amino acid level is determined in terms of amino acid similarity or identity. Similarity allows for 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 homologues 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 alter the properties of the polypeptide. Indeed, changes such as those described above may confer slightly advantageous properties on the peptide e. g. altered stability or specificity, in particular broader specificity.

Mutants having these properties can then be selected as described above.

Other methods may include mixing or incorporating sequences from related resistance genes into the *R1* sequence. For example restriction enzyme fragments of *R1* could be ligated together with fragments of an *R1* homologue or even of an unrelated gene to generate recombinant versions of *R1*. An alternative strategy for modifying *R1* would employ PCR as described above (Ho et al., 1989 Gene 77, 51-59) or DNA shuffling (Cramer et al., 1998 Nature 391).

Thus the methods of the invention, described above, may include hybridisation of one or more (e.g. two) probes or primers based on the *R1* sequence either to screen for *R1* homologues or to produce *R1* derivatives. Such, oligonucleotides, probes or primers form a further part of the present invention. An oligonucleotide for use in probing or PCR may be about 30 or fewer nucleotides in length (e.g. 18, 21 or 24). Generally specific primers are upwards of 14 or 15 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.

In one aspect of the present invention, the nucleic acid molecule 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, mammalian, 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 the coding region of the *R1* gene, or a variant (e. g

mutant, derivative or allele) 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, 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis (see above), 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.

In one embodiment of this aspect of the present invention provides 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. The preferable situation is where the level of expression increases upon application of the relevant stimulus by an amount effective to alter a phenotypic characteristic. Thus an inducible (or "switchable") promoter may be used which causes a basic level of expression in the absence of the stimulus which level is too low to bring about a desired phenotype (and may in fact be zero). Upon application of the stimulus, expression is increased (or switched on) to a level which brings about the desired phenotype.

Possible regulatory elements permitting expression in prokaryotic host cells comprise, e.g., the P_L , *lac*, *trp* or *tac* promoter in *E. coli*, and examples for regulatory elements permitting expression in eukaryotic host cells are the *AOX1* or *GAL1* promoter in yeast or the CMV-, SV40-, RSV-promoter (Rous sarcoma virus), CMV-enhancer, SV40-enhancer or a globin intron in mammalian and other animal cells. In this context, suitable expression vectors are known in the art such as Okayama-Berg cDNA expression vector pcDV1 (Pharmacia), pCDM8, pRc/CMV, pcDNA1, pcDNA3 (In-vitrogene), pSPORT1 (GIBCO BRL).

Particularly of interest in the present context are plant vectors. Specific procedures and vectors previously used with wide success upon plants are described by Bevan (Nucl. Acids Res. 12,8711-8721 (1984)) and 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) gene promoter that is expressed at a high level in virtually all plant tissues (Benfey et al, 1990a and 1990b); the cauliflower meri 5 promoter that is expressed in the vegetative apical meristem as well as several well localised positions in the plant body, e.g. inner phloem, flower primordia, branching points in root and shoot (Medford, 1992; Medford et al, 1991) and the Arabidopsis thaliana LEAFY promoter that is expressed very early in flower development (Weigel et al, 1992). Other promoters include the rice actin promoter.

The promoter may include one or more sequence motifs or elements conferring developmental and/or tissue-specific regulatory control of expression.

Thus the vectors of the present invention may include the *R1* gene or a variant thereof, in addition to various sequences required to give them replicative, integrative and/or expression functionality. Such vectors can be used, for instance, to make plants into which they are introduced resistant to *P. infestans* or other fungi.

If it is desired to induce broader-spectrum resistance, various further options are available in the light of the present disclosure:

(a) Modify the *R1* sequence, to produce mutants or other derivatives as discussed above, such that its effect can be initiated by elicitors or pathogens other than *P. infestans* alone or the other natural elicitors discussed herein.

(b) Co-express *R1* directly with an appropriate elicitor (e.g. Avr 1 from an avirulent strain).

(c) Co-express *R1* and an elicitor gene, the transcription or translation of which is suppressed by the activation of *R1*.

This would recouple *R1* to its elicitor, and better mimic the natural response to *P. infestans* infection which results in broad specificity silencing.

(d) Co-express *R1* with an elicitor gene, the translation of which is only switched on in the presence of pathogen (s).

(e) Co-express *R1* with an elicitor gene, whereby one or both are inactivated, and reactivate the gene (s) in a variegated manner, such that the HR is limited only to certain sectors of the plant (e.g. somatically defined sectors) but whereas the defensive response extends beyond these sectors. This could be achieved, for instance, by analogy with the methods disclosed in WO95/31564, wherein, following a backcross between a plant carrying a transposon tagged resistance gene (in that case *cf-9*) plus intact elicitor (Avr-9) and a plant carrying an activator transposase, the progeny exhibited a somatic reactivation of the *cf-9*, leading to a localised necrotic response but widespread resistance.

In addition to the vectors and constructs above, the present invention also provides methods comprising introduction of the *R1* constructs discussed above (such as vectors) into a host cell and/or induction of expression of a construct within a plant cell, by application of a suitable stimulus, an effective exogenous inducer. The vectors described above may be introduced into hosts by any appropriate method e.g. conjugation, mobilisation, transformation, transfection, transduction or electroporation, as described in further detail below.

In a further aspect of the invention, there is disclosed a host cell containing nucleic acid or a vector according to the present invention, especially a plant or a microbial cell. The host cell can be any prokaryotic or eukaryotic cell, such as bacterial,

insect, fungal, plant or animal cells. Preferred fungal cells are, for example, those of the genus *Saccharomyces*, in particular those of the species *S. cerevisiae*.

For the expression of the nucleic acid molecules according to the invention in sense or antisense orientation in plant cells, the molecules are placed under the control of regulatory elements which ensure the expression in plant cells. These regulatory elements may be heterologous or homologous with respect to the nucleic acid molecule to be expressed as well with respect to the plant species to be transformed. In general, such regulatory elements comprise a promoter active in plant cells. To obtain expression in all tissues of a transgenic plant, preferably constitutive promoters are used, such as the 35 S promoter of CaMV (Odell, *Nature* 313 (1985), 810-812) or promoters of the polyubiquitin genes of maize (Christensen, *Plant Mol. Biol.* 18 (1982), 675-689). In order to achieve expression in specific tissues of a transgenic plant it is possible to use tissue specific promoters (see, e.g., Stockhaus, *EMBO J.* 8 (1989), 2245-2251). Known are also promoters which are specifically active in tubers of potatoes or in seeds of different plants species, such as maize, *Vicia*, wheat, barley etc. Inducible promoters may be used in order to be able to exactly control expression. An example for inducible promoters are the promoters of genes encoding heat shock proteins. Also microspore-specific regulatory elements and their uses have been described (WO96/16182). Furthermore, the chemically inducible Tet-system may be employed (Gatz, *Mol. Gen. Genet.* 227 (1991); 229-237). Further suitable promoters are known to the person skilled in the art and are described, e.g., in Ward (*Plant Mol. Biol.* 22 (1993), 361-366). The regulatory elements may further comprise transcriptional and/or translational enhancers functional in plants cells. Furthermore, the regulatory elements may include transcription termination signals, such as a poly-A signal, which lead to the addition of a poly A tail to the transcript which may improve its stability; for literature see also supra.

In the case that a nucleic acid molecule according to the invention is expressed in sense orientation it is in principle possible to modify the coding sequence in such a way that the protein is located in any desired compartment of the plant cell. These include the endoplasmatic reticulum, the vacuole, the mitochondria, the plastids, the apoplast, the cytoplasm etc. Methods how to carry out this modifications and signal sequences ensuring localization in a desired compartment are well known to the person skilled in the art.

Methods for the introduction of foreign DNA into plants are also well known in the art. These include, for example, the transformation of plant cells or tissues with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*, the fusion of protoplasts, direct gene transfer (see, e.g., EP-A 164 575), injection, electroporation, biolistic methods like particle bombardment and other methods known in the art. The vectors used in the method of the invention may contain further functional elements, for example "left border"- and "right border"-sequences of the T-DNA of *Agrobacterium* which allow for stably integration into the plant genome. Furthermore, methods and vectors are known to the person skilled in the art which permit the generation of marker free transgenic plants, i.e. the selectable or scorable marker gene is lost at a certain stage of plant development or plant breeding. This can be achieved by, for example cotransformation (Lyznik, Plant Mol. Biol. 13 (1989), 151-161; Peng, Plant Mol. Biol. 27 (1995), 91-104) and/or by using systems which utilize enzymes capable of promoting homologous recombination in plants (see, e.g., WO97/08331; Bayley, Plant Mol. Biol. 18 (1992), 353-361); Lloyd, Mol. Gen. Genet. 242 (1994), 653-657; Maeser, Mol. Gen. Genet. 230 (1991), 170-176; Onouchi, Nucl. Acids Res. 19 (1991), 6373-6378). Methods for the preparation of appropriate vectors are described by, e.g., Sambrook (Molecular Cloning; A Laboratory Manual, 2nd Edition (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Suitable strains of *Agrobacterium tumefaciens* and vectors as well as transformation of *Agrobacteria* and appropriate growth and selection media are well known to those skilled in the art and are described in the prior art (GV3101 (pMK90RK), Koncz, Mol. Gen. Genet. 204 (1986), 383-396; C58C1 (pGV 3850kan), Deblaere, Nucl. Acid Res. 13 (1985), 4777; Bevan, Nucleic. Acid Res. 12(1984), 8711; Koncz, Proc. Natl. Acad. Sci. USA 86 (1989), 8467-8471; Koncz, Plant Mol. Biol. 20 (1992), 963-976; Koncz, Specialized vectors for gene tagging and expression studies. In: Plant Molecular Biology Manual Vol 2, Gelvin and Schilperoort (Eds.), Dordrecht, The Netherlands: Kluwer Academic Publ. (1994), 1-22; EP-A-120 516; Hoekema: The Binary Plant Vector System, Offsetdrukkerij Kanters B.V., Alblasterdam (1985), Chapter V, Fraley, Crit. Rev. Plant. Sci., 4, 1-46; An, EMBO J. 4 (1985), 277-287). Although the use of *Agrobacterium tumefaciens* is preferred in the method of the

invention, other *Agrobacterium* strains, such as *Agrobacterium rhizogenes*, may be used, for example if a phenotype conferred by said strain is desired.

Methods for the transformation using biolistic methods are well known to the person skilled in the art; see, e.g., Wan, *Plant Physiol.* 104 (1994), 37-48; Vasil, *Bio/Technology* 11 (1993), 1553-1558 and Christou (1996) *Trends in Plant Science* 1, 423-431. Microinjection can be performed as described in Potrykus and Spangenberg (eds.), *Gene Transfer To Plants*. Springer Verlag, Berlin, NY (1995).

The transformation of most dicotyledonous plants is possible with the methods described above. But also for the transformation of monocotyledonous plants several successful transformation techniques have been developed. These include the transformation using biolistic methods as, e.g., described above as well as protoplast transformation, electroporation of partially permeabilized cells, introduction of DNA using glass fibers, etc. The resulting transformed plant cell can then be used to regenerate a transformed plant in a manner known by a skilled person. This can be found, for example, in Hood, *Molecular Breeding* 3 (1997), 291-306; Coleman, *Proc. Natl. Acad. Sci. USA* 94 (1997), 7094-7097; Shilito, *Biotechnology* 7 (1989), 581-587.

In general, the plants which can be modified according to the invention and which either show overexpression of a protein according to the invention or a reduction of the synthesis of such a protein can be derived from any desired plant species. They can be monocotyledonous plants or dicotyledonous plants, preferably they belong to plant species of interest in agriculture, wood culture or horticulture interest, such as crop plants (e.g. maize, rice, barley, wheat, rye, oats etc.), potatoes, oil producing plants (e.g. oilseed rape, sunflower, pea nut, soy bean, etc.), cotton, sugar beet, sugar cane, leguminous plants (e.g. beans, peas etc.), wood producing plants, preferably trees, etc.

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. If desired, selectable genetic markers may be used consisting of chimaeric genes that confer selectable phenotypes such as resistance to antibiotics such as kanamycin, hygromycin, phosphinotricin, chlorsulfuron, methotrexate, gentamycin, spectinomycin, imidazolinones and glyphosate.

Thus a further aspect of the present invention provides a method of transforming a plant cell involving introduction of a vector comprising a nucleic acid of the present invention (e. g. *R1* or *R1* variant) into a plant cell and causing or allowing recombination between the vector and the plant cell genome to introduce the sequence of nucleotides into the genome.

The invention further encompasses a host cell transformed with nucleic acid molecule 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.

The term "heterologous" is used broadly in this aspect to indicate that the gene/sequence of nucleotides in question 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 be additional to a corresponding endogenous gene. Nucleic acid heterologous, or exogenous or foreign, 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.

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.

The generation of fertile transgenic plants has been achieved in the cereals rice, maize, wheat, oat, and barley (reviewed in Shimamoto, K. (1994) Current Opinion in Biotechnology 5,158-162.; Vasil, et al. (1992) Bio/Technology 10,667-674; Vain et al., 1995, Biotechnology Advances 13 (4): 653-671; Vasil, 1996, Nature Biotechnology 14 page 702).

Plants which include a plant cell according to the invention are also provided, along with any part or propagule thereof, seed, selfed or hybrid progeny and descendants. A plant according to the present invention may be one which does not breed true in one or more properties. Plant varieties may be excluded, particularly registrable plant varieties according to Plant Breeders' Rights. It is noted that a plant need not be considered a "plant variety" simply because it contains stably within its genome a transgene, introduced into a cell of the plant or an ancestor thereof.

In a preferred embodiment of the invention, the transgenic plant of the invention upon the presence of the *R1* gene of the invention attained resistance or improved resistance against a pathogen the corresponding wild-type plant was susceptible to.

The term "resistance" covers the range of protection from a delay to complete inhibition of disease development. Examples for pathogens of importance comprise *Phytophthora infestans*, the causal agent of potato late blight disease, *Phytophthora sojae*, root rot pathogen of soybean, *Peronospora parasitica* (downy mildew), *Magnaporthe grisea*, causal agent of rice blast disease, *Erysiphe* spp (powdery mildew), *Pseudomonas syringae* (agent of bacterial blight), *Erwinia amylovora* (fire blight disease), *Erwinia carotovora* (soft rot), *Botrytis cinerea* (downy mildew of grape), *Rhizoctonia solani* and *Pythium debaryanum* (agents of seedling blight or damping off disease). Preferably, the transgenic plant of the invention attains resistance to *P. infestans*.

In addition to the regenerated plant, the present invention embraces all of the following: a clone of such a plant, seed, selfed or hybrid progeny and descendants (e. g. F1 and F2 descendants) and any part of any of these, such as cuttings, seed. The invention also provides a plant propagule from such a plant, that is any part

which may be used in reproduction or propagation, sexual or asexual, including cuttings, seed and so on.

As an alternative to the molecular-biology based methods of introducing *R1* (or variants thereof) into plants, the sequences disclosed herein may be used to facilitate selection of plants into which it is desired to introduce the resistance trait using conventional plant breeding methods. Progeny from crosses which carry the gene may be readily identified by screening on the basis of the *R1* sequence, particularly the *R1* signature sequence.

The methods disclosed herein for identifying proximal markers to the *R1* locus may be generally applicable to other genes found in clusters (e.g. plant derived resistance genes). Such methods are characterised in that they employ a step using low stringency PCR with non-degenerate primers which avoid conserved sequence motifs. The general approach may be summarised as follows: (a) Prepare a population in which the gene of interest is segregating, (b) Identify resistance gene homologue (s) linked to the locus of interest on the basis of highly conserved (resistance gene) motifs and highly degenerate primers (Leister et al., 1996) Nature Genet. 14,421-428, (c) Identify further markers corresponding to homologous genes, which are within the (resistance) locus and that are closer to the gene, using low stringency PCR with nondegenerate primers which avoid conserved sequence motifs, (d) Use said further markers to identify a clone carrying the (resistance) gene of interest genomic library from a resistant plant, optionally in conjunction with transient assays for activity (Mindrinos et al (1994) or as described herein), (e) Optionally, confirm the identity of the cloned gene on the basis of phenotype in transgenic plants.

The present invention also encompasses the expression product of any of the *R1* or variant nucleic acid sequences disclosed above, and methods of making the expression product by expression from encoding nucleic acid molecules therefore under suitable conditions, which may be in suitable host cells in vitro, or chemically synthesized, in particular if antigens for raising antibodies are desired.

Antibodies may be raised to a purified *R1*/variant polypeptide or peptide by any method known in the art (for an overview see e.g. "Immunology-5th Edition" by Roitt, Brostoff, Male: Pub 1998-Mosby Press, London). Such antibodies, or fragments or derivatives thereof, can be used to bind *R1* or in the identification and/or isolation of proteins homologous to *R1* (i.e. which share epitopes therewith), which in turn can provide the basis of an alternative method to those described above to isolate their encoding genes.

Likewise, aptamers that bind to the *R1* polypeptide of the invention may be employed. The preparation of aptamers is known to the person skilled in the art; see, e.g., Thomas, and Dinshaw (2000) Adaptive recognition by nucleic aptamers. Science 287:820-825.

The invention further provides a method of influencing or affecting a resistance trait in a plant, whereby the method includes the step of causing or allowing expression of a heterologous nucleic acid sequence as discussed above (e.g. *R1* or *R1* variant, in each case plus an optional elicitor) within cells of the plant.

As an alternative, it may be desirable to down-regulate *R1* activity. This may be achieved, for instance used antisense technology (which is reviewed in Bourque, (1995), Plant Science 105,125-149, and Flavell, (1994) PNAS USA 91, 3490-3496). An alternative to anti-sense is to use a copy of all or part of the target gene inserted in sense, that is the same orientation as the target gene, to achieve reduction in expression of the target gene by cosuppression; see, for example, van der Krol et al., (1990) The Plant Cell 2, 291-299; Napoli et al., (1990) The Plant Cell 2, 279-289; Zhang et al., (1992) The Plant Cell 4, 1575-1588, and US-A-5,231,020.

Thus, in the invention also relates to a transgenic plant cell - and to transgenic plants comprising such plants cells - which contain, preferably stably integrated into the genome, a nucleic acid molecule according to the invention or part thereof, wherein the transcription and/or expression of the nucleic acid molecule or part thereof leads to reduction of the synthesis of an *R1* protein. In a preferred embodiment, the reduction is achieved by an anti-sense, sense, ribozyme, co-suppression, dominant mutant effect, or knock out mutant in the *R1* gene.

Preferably, though, the invention provides a method which includes expressing SEQ ID NO:1 or a variant thereof within the cells of a plant (thereby producing the encoded polypeptide), following an earlier step of introduction of the nucleic acid into a cell of the plant or an ancestor thereof. Generally such a method may be used to introduce fungal resistance into the plant whereby an *R1*-mediated resistance is triggered by contact with an appropriate fungal elicitor or other initiator or inducer. Broadly speaking the elicitor or other trigger may be encoded directly by the invading fungi (such as the virulence protein of *P. infestans* or certain other fungi). Alternatively it may be expressed by a separate construct or transgene which is itself triggered or upregulated by the fungal infection. Additionally, in both of these cases, modification of the *R1* (variant) sequence may allow triggering by a non-natural elicitor, if this is preferred.

The formats described above, to assess *R1* or *R1*-derivative function with respect to a putative or known elicitor, themselves form a further aspect of the present invention. In particular the methods, for establishing gene for gene compatibility between elicitor and resistance gene, are characterised in that they include the steps of: (a) causing or permitting the co-expression in cell of *R1* or an *R1* derivative with the elicitor, (b) observing said cell for an HR, (c) correlating the result of the observation made in (b) with the specificity of the *R1* or the *R1* derivative for the elicitor

In accordance with the above, the present invention also relates to such transgenic plants which are more sensitive to Late Blight infection compared to a corresponding wild type plant. Likewise, the present invention relates to harvestable parts and propagation material of such plants.

As described in the examples, an *R1* gene has been isolated which upon transformation into a susceptible potato cultivar Desireé conferred resistance to *P. infestans*. Since the genomic clone the corresponding DNA sequence of which is depicted in SEQ ID NO:1 was able to give rise to this effect, it is apparent that the regulatory sequences of the *R1* gene necessary and sufficient to mediate the expression of the *R1* polypeptide upon pathogen infection are contained in the

isolated DNA sequence. It is immediately evident to the person skilled in the art that such regulatory sequences have important applications on their own, for example for the expression of heterologous DNA sequences specifically upon pathogen infection, e.g., for the induction of a hypersensitive response to a given pathogen.

Accordingly, the present invention also relates to a regulatory sequence of a promoter naturally regulating the expression of a nucleic acid molecule of the invention described above or of a nucleic acid molecule homologous to a nucleic acid molecule of the invention, said regulatory sequence being capable of conferring or modulating the expression of a heterologous DNA sequence upon pathogen infection.

In context with the present invention, the term "regulatory sequence" refers to sequences which influence the specificity and/or level of expression, for example in the sense that they confer cell and/or tissue specificity. Such regions can be located upstream of the transcription initiation site, but can also be located downstream of it, e.g., in transcribed but not translated leader sequences, or in introns.

The term "promoter", within the meaning of the present invention refers to nucleotide sequences necessary for transcription initiation, i.e. RNA polymerase binding and successful start of processive transcript formation, and may also include, for example, the TATA box.

The term "nucleic acid molecule homologous to a nucleic acid molecule of the invention", as used herein includes promoter regions and regulatory sequences of other *R1* genes, such as genes from other species, for example, tomato which are homologous to potato *R1* genes and which display substantially the same expression pattern. Such promoters are characterized by their capability of conferring preferably exclusively expression of a heterologous DNA sequence in a plant upon pathogen infection.

The term "capable of conferring or modulating the expression of a heterologous DNA sequence upon pathogen infection" as used herein means that said promoter is capable of controlling the expression of a heterologous DNA sequence in plants at infection sites, analogous or closely related to the controlled expression of pathogen related genes which are involved in the natural resistance in most incompatible

host/pathogen interactions, such as the hypersensitive cell death at infection sites of a part of a plant. Thus, the regulatory sequence of the invention is characterized by its capability of mediating localized transcriptional activation selectively in response to pathogen attack or in response to stimuli that mimic pathogen attack such as elicitors prepared from, e.g., pathogens such as fungi or bacteria or derivatives thereof. The transcriptional activation by the regulatory sequence of the invention may also occur in cells surrounding the actual infection site due to cell-cell interactions. The regulatory sequence of the invention and chimeric promoters comprising such sequences may advantageously not or only to a small extent be inducible upon other stimuli such as abiotic stress. Preferably, the induction from the chimeric promoter upon pathogen attack or elicitor treatment is at least about 10-fold higher, preferably 20-fold higher and particularly 30-fold higher than its activation, if any, by abiotic stress.

However, the expression specificity conferred by the regulatory sequences of the invention may not be limited to local gene expression due to pathogens, for example, they may be combined with further regulatory sequences that provide for tissue specific gene expression. The particular expression pattern may also depend on the plant/vector system employed. However, expression of heterologous DNA sequences driven by the regulatory sequences of the invention predominantly occurs upon pathogen infection or treatment with a corresponding elicitor unless certain elements of the invention were taken and designed by the person skilled in the art to control the expression of a heterologous DNA sequence certain cell types.

Thus, according to the present invention, regulatory sequences from other species can be used that are functionally homologous to the regulatory sequences of the promoter of the above defined *R1* specific nucleic acid molecules, or promoters of genes that display an identical or similar pattern of expression. The particular expression pattern may also depend on the plant/vector system employed. However, expression of heterologous DNA sequences driven by the regulatory sequences of the invention predominantly occurs in any cell infected by a particular pathogen unless certain elements of the regulatory sequences of the invention, were taken and designed by the person skilled in the art to control the expression of a heterologous DNA sequence in a particular tissue or otherwise controlled manner.

In accordance with the present invention, novel regulatory sequences of *R1* genes, can be isolated and have been exemplified for the regulatory sequence of the *R1* gene of potato. For example, genomic DNA can be digested with appropriate restriction enzymes, denatured and allowed to anneal to a reverse primer derived from the cDNA sequence of the invention. After primer extension, a blunt-ended adaptor can be ligated and PCR can be performed using a nested reverse primer derived from the mentioned cDNA, and a forward primer derived from the adaptor sequence. In another strategy for the cloning of the regulatory sequences of the invention a physical map of the genomic sequences upstream the coding region can be constructed by mean of genomic southern analysis. With this information, genomic DNA can be digested with selected restriction enzymes, genomic fragments containing a piece of the upstream sequences and the coding sequence can be gel purified and self-ligated in a large volume to favour the formation of circular molecules, that can subsequently be amplified by PCR with forward and reverse primers, derived from the coding sequence of the gene. Within the cloned genomic sequence, the transcription start site can be determined by standard procedures well known to everyone skilled in the art, such as 5'-RACE, primer extension or S1 mapping. To define cis-regulatory elements upstream of the transcription start site (i.e. within the putative promoter region), the respective region is fused to marker genes such as genes encoding GUS or GFP, and 5' deletion derivatives of these construct are generated. They are transformed into suitable plant material, and the expression of the marker gene depending on the remaining upstream sequence (putative promoter) is determined. These techniques are well known to a person skilled in the art.

In one embodiment the regulatory sequence of the invention comprises a DNA sequence selected from the group consisting of

- (a) DNA sequences comprising the nucleotide sequence as depicted in SEQ ID NO. 1 from nucleotides 1 to 2222 or (a) part(s) thereof;
- (b) DNA sequences comprising at least 14 consecutive nucleotides of the nucleotide sequence as depicted in SEQ ID NO: 1 from nucleotides 1 to 2222;

- (c) DNA sequences hybridizing with a nucleotide sequence as defined in (a) or (b) under stringent conditions;
- (d) DNA sequences of a gene or fragment thereof obtainable by screening an appropriate genomic DNA library with a probe having a nucleotide sequence as defined in claim 1; and
- (e) DNA sequences comprising nucleotide sequences which are conserved in (a), (b) and (c).

Homologous regulatory sequences differ at one or more positions from the regulatory sequence of (a) or (b) but still have the same specificity, namely they comprise the same or similar sequence motifs, preferably 6 to 10 nucleotides in length, responsible for the above described expression pattern. Preferably such regulatory sequences hybridize to one of the above-mentioned regulatory sequences, most preferably under stringent conditions. Particularly preferred are regulatory sequences which share at least 85%, more preferably 90-95%, and most preferably 96-99% sequence identity with one of the above-mentioned regulatory sequences and have the same or substantially the same specificity. Such regulatory sequences also comprise those which are altered, for example by one or more nucleotide deletion(s), insertion(s), substitution(s), addition(s), and/or recombination(s) and/or any other modification(s) known in the art either alone or in combination in comparison to the above-described nucleotide sequence. Methods for introducing such modifications in the nucleotide sequence of the regulatory sequences of the invention are well known to the person skilled in the art. It is also immediately evident to the person skilled in the art that further regulatory elements may be added to the regulatory sequences of the invention. For example, transcriptional enhancers and/or sequences which allow for induced expression of the regulatory sequences of the invention may be employed. A suitable inducible system is for example tetracycline-regulated gene expression as described, e.g., by Gatz, *supra*.

The possibility exists to modify the regulatory sequences as described above or sequence motifs thereof by, e.g., nucleotide replacements which do not affect the overall structure or binding motif of the regulatory sequence so that it remains capable of conferring gene expression upon pathogen infection. The regulatory sequence of the invention may be derived from the R1 genes of potato (see

Examples) although other plants may be suitable sources for such regulatory sequences as well. Furthermore, the nucleotide sequences of the invention can be compared as appropriate computer programs known in the art such as BLAST, which stands for Basic Local Alignment Search Tool (Altschul, 1997; Altschul, J. Mol. Evol. 36 (1993), 290-390; Altschul, J. Mol. Biol. 215 (1990); 403-410), can be used to search for local sequence alignments. BLAST produces alignments of nucleotide sequences to determine sequence similarity. Because of the local nature of the alignments, BLAST is especially useful in determining exact matches or in identifying homologues. With such means it is possible to identify conserved nucleotide sequences that may play a role in pathogen specific expression.

Usually, said regulatory sequence is part of a recombinant DNA molecule. In a preferred embodiment of the present invention, the regulatory sequence in the recombinant DNA molecule is operatively linked to a heterologous DNA sequence. The term heterologous with respect to the DNA sequence being operatively linked to the regulatory sequence of the invention means that said DNA sequence is not naturally linked to the regulatory sequence of the invention. Expression of said heterologous DNA sequence comprises transcription of the DNA sequence, preferably into a translatable mRNA. Regulatory elements ensuring expression in eukaryotic cells, preferably plant cells, are well known to those skilled in the art. They usually comprise poly-A signals ensuring termination of transcription and stabilization of the transcript, see also supra. Additional regulatory elements may include transcriptional as well as translational enhancers; see supra.

In a preferred embodiment, the heterologous DNA sequence of the above-described recombinant DNA molecules encodes a peptide, protein, antisense RNA, sense RNA and/or ribozyme. The recombinant DNA molecule of the invention can be used alone or as part of a vector to express heterologous DNA sequences, which, e.g., encode proteins for, e.g., seed storage proteins, toxins, antibodies ("plantibodies") or diagnostics of R1 related gene expression. The recombinant DNA molecule or vector containing the DNA sequence encoding a protein of interest is introduced into the cells which in turn produce the protein of interest. For example, the regulatory sequences of the invention can be operatively linked to sequences encoding Barstar

and Barnase, respectively, for use in the production of HR response in plants. Applications of the regulatory sequences of the invention are evident to the person skilled in the art and can be derived from the literature, e.g., Strittmatter and Wegener, *Zeitschrift für Naturforschung* 48c (1993), 673-688; Kahl, J. *Microbiol. Biotechnol.* 11 (1995), 449-460 and references cited therein.

On the other hand, said protein can be a scorable marker, e.g., luciferase, green fluorescent protein or β -galactosidase. This embodiment is particularly useful for simple and rapid screening methods for compounds and substances described herein below capable of modulating R1 gene expression. For example, a transgenic plant can be cultured in the presence and absence of a candidate compound in order to determine whether the compound affects the expression of genes which are under the control of regulatory sequences of the invention, which can be measured, e.g., by monitoring the expression of the above-mentioned marker. It is also immediately evident to those skilled in the art that other marker genes may be employed as well, encoding, for example, a selectable marker which provides for the direct selection of compounds which induce or inhibit the expression of said marker.

The regulatory sequences of the invention may also be used in methods of antisense approaches. The antisense RNA may be a short (generally at least 10, preferably at least 14 nucleotides, and optionally up to 100 or more nucleotides) nucleotide sequence formulated to be complementary to a portion of a specific mRNA sequence and/or DNA sequence of the gene of interest. Standard methods relating to antisense technology have been described; see, e.g., Klann, *Plant Physiol.* 112 (1996), 1321-1330 and *supra*. Following transcription of the DNA sequence into antisense RNA, the antisense RNA binds to its target sequence within a cell, thereby inhibiting translation of the mRNA and down-regulating expression of the protein encoded by the mRNA.

In a further embodiment, the invention relates to nucleic acid molecules of at least 15 nucleotides in length hybridizing specifically with a regulatory sequence as described above or with a complementary strand thereof. Specific hybridization

occurs preferably under stringent conditions and implies no or very little cross-hybridization with nucleotide sequences having no or substantially different regulatory properties. Such nucleic acid molecules may be used as probes and/or for the control of gene expression. Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary in length. Preferred are nucleic acid probes of 17 to 35 nucleotides in length. Of course, it may also be appropriate to use nucleic acids of up to 100 and more nucleotides in length. The nucleic acid probes of the invention are useful for various applications. On the one hand, they may be used as PCR primers for amplification of regulatory sequences according to the invention. Another application is the use as a hybridization probe to identify regulatory sequences hybridizing to the regulatory sequences of the invention by homology screening of genomic DNA libraries. Nucleic acid molecules according to this preferred embodiment of the invention which are complementary to a regulatory sequence as described above may also be used for repression of expression of a gene comprising such regulatory sequences, for example due to an antisense, cosuppression or triple helix effect or for the construction of appropriate ribozymes (see, e.g., EP-B1 0 291 533, EP-A1 0 321 201, EP-A2 0 360 257) which specifically cleave the (pre)-mRNA of a gene comprising a regulatory sequence of the invention. Selection of appropriate target sites and corresponding ribozymes can be done as described for example in Steinecke, Ribozymes, Methods in Cell Biology 50, Galbraith et al. eds Academic Press, Inc. (1995), 449-460. Furthermore, the person skilled in the art is well aware that it is also possible to label such a nucleic acid probe with an appropriate marker for specific applications, such as for the detection of the presence of a nucleic acid molecule of the invention in a sample derived from an organism.

The above described nucleic acid molecules may either be DNA or RNA or a hybrid thereof. Furthermore, said nucleic acid molecule may contain, for example, thioester bonds and/or nucleotide analogues, commonly used in oligonucleotide anti-sense approaches; see supra.

The present invention also relates to vectors, particularly plasmids, cosmids, viruses and bacteriophages used conventionally in genetic engineering that comprise a regulatory sequence or corresponding recombinant DNA molecule of the invention.

Preferably, said vector is an expression vector and/or a vector further comprising a selection marker for plants. For example of suitable selector markers, see supra. Methods which are well known to those skilled in the art can be used to construct recombinant vectors; see, for example, the techniques described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989). Alternatively, the recombinant DNA molecules and vectors of the invention can be reconstituted into liposomes for delivery to target cells.

The present invention furthermore relates to host cells transformed with a regulatory sequence, a DNA molecule or vector of the invention. Said host cell may be a prokaryotic or eukaryotic cell. The regulatory sequence, vector or recombinant DNA molecule of the invention which is present in the host cell may either be integrated into the genome of the host cell or it may be maintained extrachromosomally. The host cell can be any prokaryotic or eukaryotic cell, such as a bacterial, insect, fungal, plant, animal or human cell. Preferred cells are plant cells.

In a further preferred embodiment, the present invention provides for a method for the production of transgenic plants, plant cells or plant tissue comprising the introduction of a nucleic acid molecule, recombinant DNA molecule or vector of the invention into the genome of said plant, plant cell or plant tissue. For the expression of the heterologous DNA sequence under the control of the regulatory sequence according to the invention in plant cells, further regulatory sequences such as poly A tail may be fused, preferably 3' to the heterologous DNA sequence, see also supra. Further possibilities might be to add transcriptional or translational enhancers that increase gene expression, or sequences that increase mRNA stability. Methods for the introduction of foreign DNA into plants, plant cells and plant tissue are described above.

Thus, the present invention relates also to transgenic plant cells which contain, preferably stably integrated into the genome, a regulatory sequence, a recombinant DNA molecule or vector according to the invention. Furthermore, the present

invention also relates to transgenic plants and plant tissue comprising the above-described transgenic plant cells.

Furthermore, the present invention relates to a method for the identification of a plant protective agent comprising the steps of:

- (a) culturing a plant cell or tissue or maintaining a plant comprising a recombinant DNA molecule comprising a readout system operatively linked to a regulatory sequence of the present invention in the presence of a compound or a sample comprising a plurality of compounds under conditions which permit expression of said readout system;
- (b) identifying or verifying a sample and compound, respectively, which leads to suppression or activation and/or enhancement of expression of said readout system in said plant, plant cell, or plant tissue.

The term "read out system" in context with the present invention means a DNA sequence which upon transcription and/or expression in a cell, tissue or organism provides for a scorable and/or selectable phenotype. Such read out systems are well known to those skilled in the art and comprise, for example, recombinant DNA molecules and marker genes as described above.

The term "plurality of compounds" in a method of the invention is to be understood as a plurality of substances which may or may not be identical.

Said compound or plurality of compounds may be inorganic or organic, naturally occurring or man made compounds and may be comprised in, for example, samples, e.g., cell extracts from, e.g., plants, animals or microorganisms. Furthermore, said compound(s) may be known in the art but hitherto not known to be capable of suppressing or activating and/or enhancing the transcription of an R1 gene. The plurality of compounds may be, e.g., added to the culture medium or injected into the plant, plant cells or tissue or sprayed onto the plant or supplied in the soil.

If a sample containing a compound or a plurality of compounds is identified in the method of the invention, then it is either possible to isolate the compound from the original sample identified as containing the compound capable of suppressing or activating and/or enhancing the transcription of a R1 gene, or one can further

subdivide the original sample, for example, if it consists of a plurality of different compounds, so as to reduce the number of different substances per sample and repeat the method with the subdivisions of the original sample. Depending on the complexity of the samples, the steps described above can be performed several times, preferably until the sample identified according to the method of the invention only comprises a limited number of or only one substance(s). Preferably said sample comprises substances of similar chemical and/or physical properties, and most preferably said substances are identical. Preferably, the compound identified according to the above described method is further formulated in a form suitable for the application in plant breeding or plant cell and tissue culture.

The compounds which can be tested and identified according to a method of the invention may be expression libraries, e.g., cDNA expression libraries, peptides, proteins, nucleic acids, antibodies, small organic compounds, hormones, peptidomimetics, PNAs or the like (Milner, *Nature Medicine* 1 (1995), 879-880; Hupp, *Cell* 83 (1995), 237-245; Gibbs, *Cell* 79 (1994), 193-198 and references cited supra). Furthermore, genes encoding a putative regulator of an R1 gene may be identified using, for example, insertion mutagenesis using, for example, gene targeting vectors known in the art (see, e.g., Hayashi, *Science* 258 (1992), 1350-1353; Fritze and Walden, Gene activation by T-DNA tagging. In *Methods in Molecular biology* 44 (Gartland, K.M.A. and Davey, M.R., eds). Totowa: Human Press (1995), 281-294) or transposon tagging (Chandlee, *Physiologia Plantarum* 78 (1990), 105-115). Said compounds can also be functional derivatives or analogues of known inhibitors or activators. Methods for the preparation of chemical derivatives and analogues are well known to those skilled in the art and are described in, for example, Beilstein, *Handbook of Organic Chemistry*, Springer edition New York Inc., 175 Fifth Avenue, New York, N.Y. 10010 U.S.A. and *Organic Synthesis*, Wiley, New York, USA. Furthermore, said derivatives and analogues can be tested for their effects according to methods known in the art. Furthermore, peptidomimetics and/or computer aided design of appropriate derivatives and analogues can be used, for example, according to the methods described above.

Determining whether a compound is capable of suppressing or activating and/or enhancing the transcription of an *R1* gene can be done, for example, in plants by monitoring the reporter gene. It can further be done by monitoring the phenotypic characteristics of the transgenic plant of the invention contacted with the compounds and compare it to that of wild-type plants. In an additional embodiment, said characteristics may be compared to that of a transgenic plant contacted with a compound which is either known to be capable or incapable of suppressing or activating and/or enhancing *R1* gene expression or the activity of the protein. The compounds identified according to the method of the invention are expected to be very beneficial since promoters that have been known so far are only of limited use due to the non or not tightly regulated pathogen specificity of their regulatory sequences.

The inhibitor or activator identified by the above-described method may prove useful as a herbicide, pesticide and/or as a plant growth regulator. Thus, in a further embodiment the invention relates to a compound obtained or identified according to the method of the invention. Such useful compounds can be for example transacting factors which bind to the regulatory sequence of the invention. Identification of transacting factors can be carried out using standard methods in the art (see, e.g., Sambrook, supra, and Ausubel, supra). To determine whether a protein binds to the regulatory sequences of the invention, standard DNA footprinting and/or native gel-shift analyses can be carried out. In order to identify a transacting factor which binds to the regulatory sequence of the invention, the regulatory sequence can be used as an affinity reagent in standard protein purification methods, or as a probe for screening an expression library. Once the transacting factor is identified, modulation of its binding to the regulatory sequences of the invention can be pursued, beginning with, for example, screening for inhibitors against the binding of the transacting factor to the regulatory sequences of the present invention. Activation or repression of *R1* genes could then be achieved in plants by applying of the transacting factor (or its inhibitor) or the gene encoding it, e.g. in a vector for transgenic plants. In addition, if the active form of the transacting factor is a dimer, dominant-negative mutants of the transacting factor could be made in order to inhibit its activity. Furthermore, upon identification of the transacting factor, further

components in the pathway leading to activation (e.g. signal transduction) or repression of a gene under the control of the regulatory sequences of the present invention can then be identified. Modulation of the activities of these components can then be pursued, in order to develop additional drugs and methods for modulating the expression of a gene under the control of the regulatory sequences of the present invention.

Preferably, the compound identified according to the above described method or its analog or derivative is further formulated in a form suitable for the application in plant breeding or plant cell and tissue culture. For example, it can be combined with a agriculturally acceptable carrier known in the art. The plant protection composition can be prepared by employing the above-described method of the invention and synthesizing the compound identified as inhibitor or activator in an amount sufficient for use in agriculture. Thus, the present invention also relates to a method for the preparation of an agricultural plant protection composition comprising the above-described steps of the method of the invention and synthesizing the compound so identified or an analog or derivative thereof.

In the plant protection composition of the invention, the compound identified by the above-described method may be preferentially formulated by conventional means commonly used for the application of, for example, herbicides and pesticides or agents capable of inducing systemic acquired resistance (SAR). For example, certain additives known to those skilled in the art comprising stabilizers or substances which facilitate the uptake by the plant cell, plant tissue or plant may be used, for example, carborundum, or 0.01% SDS (sodium dodecylsulfate) solution.

In a still further embodiment the present invention relates to a method for identifying and obtaining an avirulence or a virulence factor of a pathogen comprising the steps of:

- (a) screening the R1 protein of the present invention or a fragment thereof against a peptide or protein expression library derived from a pathogen in a readout system under suitable conditions which permit interaction of the protein and peptide in said readout system;
- (b) identifying or verifying a cDNA which leads to suppression or activation of the readout system.

Beside the above described possibilities of using the nucleic acid molecules according to the invention for the genetic engineering of plants with modified characteristics and their use to identify homologous molecules, the described nucleic acid molecules may also be used for several other applications, for example, for the identification of nucleic acid molecules which encode proteins which interact with the *R1* proteins described above. This can be achieved by assays well known in the art, for example, as described in Scofield (Science 274 (1996), 2063-2065) by use of the so-called yeast "two-hybrid system". In this system the protein encoded by the nucleic acid molecules according to the invention or a smaller part thereof is linked to the DNA-binding domain of the GAL4 transcription factor. A yeast strain expressing this fusion protein and comprising a lacZ reporter gene driven by an appropriate promoter, which is recognized by the GAL4 transcription factor, is transformed with a library of cDNAs which will express plant proteins or peptides thereof fused to an activation domain. Thus, if a peptide encoded by one of the cDNAs is able to interact with the fusion peptide comprising a peptide of a protein of the invention, the complex is able to direct expression of the reporter gene. In this way the nucleic acid molecules according to the invention and the encoded peptide can be used to identify peptides and proteins interacting with *R1* proteins. This method can also be employed for identifying inhibitors and activators as described above.

Other methods for identifying compounds which interact with the proteins according to the invention or nucleic acid molecules encoding such molecules are, for example, the in vitro screening with the phage display system as well as filter binding assays or "real time" measuring of interaction using, for example, the BIAcore apparatus (Pharmacia); see references cited supra.

A similar strategy can be pursued with the so called three hybrid system.

The yeast two-hybrid system originally has been described by Fields and Song (Nature 340 (1989), 245-246; see also for review Vidal, M, in Bartel, P.L. and Fields, S. (eds.), The yeast two-hybrid system. Oxford University Press, New York, NY, (1997), 109-147). A modified version of the yeast two-hybrid system has been described by (Gyuris, Cell 75 (1993), 223-232; Zervos, Cell 72 (1993), 223-232). Briefly, a domain of the polypeptide is used as bait for binding compounds. Positives are then selected by their ability to grow on plates lacking leucine, and then further

tested for their ability to turn blue on plates with X-gal, as previously described in great detail; see also WO 95/31544. A modified version is the "reverse yeast two-hybrid system" which allows to select for interaction defective alleles using a negative selection strategy as, for example, described in (Vidal, Proc. Natl Acad Sci. USA 93 (1996), 10321-10326; Vidal, Proc. Natl Acad Sci. USA 93 (1996), 10315-10320). This system uses the counter selectable reporter gene *URA3*. Yeast cells expressing Ura3p convert the compound 5-fluoroorotic acid (FOA) into the toxic derivative 5-flourouracil. A two-hybrid interaction which leads to activation of the *URA3* reporter gene can, thus, be counterselected in the presence of FOA and loss of function mutants can be specifically selected out of a large pool of wild type alleles.

Another convenient method, for example, could be the yeast three-hybrid system as described (SenGupta, Proc. Natl. Acad. Sci. USA 93 (1996), 8496-8501). The yeast three-hybrid selection system was developed for isolating the genes of the proteins that interact with RNA, and to study RNA-protein interactions. This system, based on the yeast two-hybrid system, consists of a DNA-binding domain fused to a known RNA-binding protein, an activation domain fused to a prospective RNA-binding protein, and a hybrid RNA. Transcription of reporter genes only occurs when both hybrid proteins interact with the hybrid RNA. In the reverse three-hybrid system, interaction of the proteins with the hybrid RNA results in expression of a reporter gene whose product is toxic to yeast cells. All these methods can be employed in accordance with the above described method of the present invention with the R1 protein or peptide fragments thereof as a bait for identifying and obtaining an avirulence or virulence factor and their encoding cDNAs or parts thereof. Methods for obtaining the DNA sequence of those clones tested positive in the screening assay are known to the person skilled in the art and are described in the above referenced publications.

The present invention also relates to the cDNA and its encoded product obtained or identified by the above described method.

The invention also relates to compositions comprising at least one of the aforementioned nucleic acid molecules and/or comprising a nucleic acid molecule

which is complementary for such a nucleic acid molecule, a vector of the invention, a *R1* protein of the invention or an immunologically or biologically active fragment thereof or an antibody or aptamer specifically recognizing such a protein or fragment; a regulatory sequence or recombinant DNA, or a corresponding vector of the invention, a compound designed orientated according to the protein of the invention and/or identified according to the method described above and/or an antibody specifically recognizing such a compound or a regulatory sequence of the invention, and optionally suitable means for detection or suitable means for plant cell and tissue culture.

Diagnostic compositions may be used for methods for detecting expression of *R1* gene by detecting the presence of corresponding mRNA which comprises isolation of mRNA from a cell and contacting the mRNA so obtained with a probe comprising a nucleic acid probe as described above under hybridizing conditions, detecting the presence of mRNA hybridized to the probe, and thereby detecting the expression of the gene by the cell. Further methods of detecting the presence of a protein according to the present invention comprises immunotechniques well known in the art, for example enzyme linked immunosorbent assay.

Moreover, the present invention relates to a kit comprising at least one of the aforementioned nucleic acid molecules, vectors, proteins, compounds, antibodies, or aptamers of the invention. The kit of the invention may contain further ingredients such as selection markers and components for selective media suitable for the generation of transgenic plant cells, plant tissue or plants. Furthermore, the kit may include buffers and substrates for reporter genes that may be present in the recombinant gene or vector of the invention. The kit of the invention may advantageously be used for carrying out the method of the invention and could be, inter alia, employed in a variety of applications referred to herein, e.g., in the diagnostic field or as research tool. The parts of the kit of the invention can be packaged individually in vials or in combination in containers or multicontainer units. Manufacture of the kit follows preferably standard procedures which are known to the person skilled in the art. The kit or its ingredients according to the invention can be used in plant cell and plant tissue cultures, for example, for any of the above described methods for detecting inhibitors and activators of *R1* genes. The kit of the

invention and its ingredients are expected to be very useful in breeding new varieties of, for example, plants which display improved properties such as nutritial value or disease resistance.

It is also immediately evident to the person skilled in the art that the regulatory sequences, recombinant DNA molecules, vectors and compounds of the present invention can be employed to produce transgenic plants with a desired trait; see for review TIPTEC Plant Product & Crop Biotechnology 13 (1995), 312-397.

Furthermore, it is possible to use the nucleic acid molecules according to the invention as molecular markers in plant breeding. Moreover, the overexpression of nucleic acid molecules according to the invention may be useful for the alteration or modification of plant/pathogene interaction. The term "pathogene" includes, for example, bacteria, viruses and fungi as well as protozoa. Preferably, said pathogene is *P. infestans*.

Furthermore, the present invention relates to the use of a nucleic acid molecule, vector, host cell, protein, a regulatory sequence, an aptamer recombinant DNA molecule, a vector, a compound an aptamer and/or the antibody of the invention for use in a screening method for the identification of virulence and avirulence genes of pathogens, for screening plant protective compounds, for inducing pathogen resistance in plants, as a marker in marker-assisted plant breeding. The regulatory sequence or a recombinant DNA molecule of the present invention is preferably used for the expression of a heterologous DNA sequence.

These and other embodiments are disclosed and encompassed by the description and examples of the present invention. Further literature concerning any one of the methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries, using for example electronic devices. For example the public database "Medline" may be utilized which is available on the Internet. Databases are maintained, for example, by the National Center for Biotechnology Information, National Library of Medicine, Building 38A, Bethesda, Maryland 20894 United States of America, the Swiss Institute of Bioinformatics, Biozentrum - University of Basel, Klingelbergstrasse 50-70, 4056

Basel, Switzerland, and The J. Craig Venter Institute, 9704 Medical Center Drive, Rockville, Maryland 20850 United States of America. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

The present invention is further described by reference to the following non-limiting figures and examples.

The figures show:

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 (Meksem et al. 1995, De Jong et al. 1997) 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 90 kb. Solid black bars: BACs from the chromosome carrying *R1*. Grey bars: BACs from the chromosome carrying *r1*. White bar: BAC origin not determined. 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.

Figure 2. 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 10-2_5; (E) BAC clone BA87d17 carrying the *R1* allele; (F), (G), (H), (I), (J) BAC clones BA122p13, BA12101, BA76011, BA47F2 and BA27c1, respectively, (K) negative control.

Figure 3. *R1* complementation test. Disease symptoms are shown 9 days post-inoculation on leaflets from (A) susceptible Desirée; (B) transgenic Desirée line no 10-2_5 transformed with clone g10-2 and (C) the resistant parent P41 (*R1r1*).

Figure 4. The *R1* gene. (A) Structural organisation. 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 region and N-glycosylation sites are indicated in bold. The conserved motifs QLPL, CFLY and LHD specific for plant resistance proteins, are underlined. Single letter codes for the amino acids are standard.

Figure 5. Schematic representation of the chromosome 5 region around the *R1* locus. Boxes filled with vertical lines represent homologous regions between the chromosomes bearing the *R1* and *r1* alleles. The functional allele *R1.1* and the *R1.2/r1.2* locus are marked with opened boxes. The angled line indicates the deletion present on the *r1*- chromosome when compared to the *R1*-chromosome.

EXAMPLES

Material and Methods:

Plant material :

F1 offspring of a cross between the heterozygous diploid clones H79.1506/1 (*R1 r1*) and H80.696/4 (*r1 r1*), 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 (Meksem et al. 1995). The hybrid clone P6/210 derived from the cross P41 x P40 (Leister et al. 1997) 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 a *P. infestans* isolate having the corresponding avirulence factor *Avr1* (race 4) was determined as described (Leonards-Schippers et al. 1992), except that whole leaflets instead of leaf disks were used for inoculation. Presence or absence of 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 has been constructed from *Hind*III partially digested high molecular weight genomic DNA of clone P6/210 in the binary vector pCLD04541 (Jones et al. 1992) as described (Meksem et al. 2000). The BAC library consists of 101,376 clones with an insert average size of 70 kb. The colonies were stored in 264 384-microtiter plates (Genetix, Oxford, UK) in 2YT medium (Sambrook et al. 1989) with freezing buffer (5.5 % w/v glycin, 7 mM (NH₄)SO₄, 1.5 mM Na-Citrate, 0.3 mM MgSO₄, 13 mM KH₂PO₄, 27 mM K₂HPO₄).

A cosmid library of ca. 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 and in the same vector (*Bam*H1 cloning site) as the BAC library. Cosmids were packaged using Gigapack II Gold Packaging extract (Stratagene, CA, USA) and transfected into *E. coli* strain SURE™ (Stratagene, CA, USA). 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 hybridisation using standard protocols (Sambrook et al. 1989).

BAC library screening and contig construction:

High-density colony filters for hybridisation-based screening of the BAC library were prepared using a BioGRID robot (Oxford, UK). Clones were gridded in double spots using a 5 x 5 array with 6 x 384 arrays per 22.5 x 22.5 cm nylon membrane (PALL, Biotek, Portsmouth, UK). Each 5 x 5 array contained 2 x 12 colonies with the control position of the array occupied by the clone pSW1 (PE Biosystems, Foster City, CA 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 hybridisation using standard techniques (Sambrook et al. 1989). Filter hybridisation was performed as described (Gebhardt et al. 1989), except that 300 pg pSW1 control insert were labelled and hybridised 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 employing T3 and T7 oligonucleotides as sequencing primers. DNA sequence information of 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 new filter hybridisation 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 *NotI* and the fragments were separated by pulsed field gel electrophoresis on a CHEF DRIII (BioRad, Hercules, CA, USA) for 12 h at 11°C with an initial pulse time of 5 s and a final pulse time of 10 s, at 120° angle and 6 V/cm.

BAC DNA isolation:

BAC DNA was extracted using QIAfilter Plasmid Purification Kit 100 (Qiagen, Hilden, Germany) according to manufactures instructions with minor modifications. A single colony was precultured in liquid LB medium for 8 h at 37°C. 75 µl preculture were 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:

1.5 µg BAC DNA were digested to completion with *HindIII* plus *EcoR1* 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 supplier'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 BA87dI7:

10 µg BAC DNA were partially digested with 1U *Tsp5091* for 15 min at

65°C and size separated on a 0.8 % low melting temperature agarose gel (Sea Plaque GTG Agarose, Bioproducts, Rockland, Maine, USA). Fragments of about 10 kb in size were eluted using the GELase system (Epicentre Technologies, Madison, USA), following the supplier's instructions. The purified fragments were cloned into the pCLD04541 binary vector linearized with *EcoRI*, dephosphorylated using SHRIMP phosphatase (Roche, Germany) and transformed into *E. coli* strain DH10B (Life Technologies, USA). Two hundred recombinant colonies were picked into microtiter plates.

cDNA library construction and screening:

Cut shoots of ca. 8 weeks old plants of parent P41 (*R1r1*) and of the susceptible cv 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 16h 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 2h, 19h, 3d, 7d and 9d after inoculation were collected. Poly A⁺ RNA was isolated using the RNeasy Plant Mini Kit or the Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany) according to the supplier's instructions. A Lambda ZAP II cDNA library (Stratagene, CA, USA) was constructed from the poly-A⁺ RNA, following the manufacturer's instructions. The different cDNA preparations were pooled prior to ligation into the Lambda ZAP vector. 5 x 10⁵ pfu's were plated and screened by plaque hybridisation (Sambrook et al. 1989) using as probe the insertions of BACs BA121o1 and BA76o11.

5'Rapid amplification of cDNA ends (RACE) analysis:

Total RNA was isolated from uninfected leaf tissue of P41 (*R1r1*) using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to supplier's instructions. RACE analysis was performed with 1µg total RNA using the SMARTTM Race cDNA Amplification Kit (Clontech, CA, USA) following the manufacturer's instructions. The nested gene-specific primers used for the PCR amplification were first RT1-1: 5'-AAACCCGGTGTTCCAAATCTAACACT-3' (SEQ ID NO: 3) and second RT2-1: 5'-CATGTAGTGAGGATATGTCACGAGTG-3'. (SEQ ID NO: 4) The final PCR products

of the RACE reaction were cloned into pGEM-T vector (Promega, CA, USA). Two independent clones were sequenced.

DNA Sequence Analysis:

Custom DNA sequencing was done 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, CA USA).

DNA sequence analysis was done using the Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison, Wisc, USA. Sequence databases were searched with BlastX and other algorithms available through the National Center for Biotechnology Information, Bethesda, MD, 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 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 Claforan. Kanamycin resistant transgenic plants were tested by the polymerase chain reaction (PCR) for presence of the g10 insert using the insert specific primers 87e (5'-ATTACAATGGGTTGAACTCAG-3' (SEQ ID NO: 5)) and 87s (5'-ACCTCTTTCAATTGTTCTGGTG-3' (SEQ ID NO: 6)). PCR conditions were: Ta at 55°C for 45 sec and polymerisation at 72°C for 60 seconds. Transgenic plants were screened with the *R1* specific primers 76-2sf2 (5'-CACTCGTGACATATCCTCACTA-3' (SEQ ID NO: 7)) and 76-2SR (5'-CAACCCTGGCATGCCACG-3' (SEQ ID NO: 8)) derived from cDNA c76-2. PCR

conditions were: Ta at 55°C for 45 sec and polymerisation at 72°C for 90 sec. Tests for resistance to *P. infestans* race 4 were done using three leaflets per plant in each test.

Example 1: High-resolution genetic mapping of the *R1* locus

To facilitate physical mapping of the *R1* locus, 16 recombinants between the markers *GP21* and *GP179* flanking *R1* (Leonards-Schippers et al. 1992) were selected from 588 plants and tested for resistance to a *P. infestans* having the corresponding avirulence factor *Avr1*. Together with 15 recombinants previously selected in the same interval (Meksem et al. 1995), 31 recombinants in total were available in the interval *GP21* – *GP179* from 1049 plants, corresponding to 3.0 % recombination frequency (3 cM). Recombination frequencies between *GP21* and *R1* and between *R1* and *GP179* were 2.2 % and 0.8 %, respectively (Table 1).

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

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

The markers *SPUD237* and *AFLP1*, both mapping in the interval *GP21* – *GP179* (De Jong et al. 1997, Meksem et al. 1995) flank the *R1* locus. Both markers were separated from *R1* by one recombination event in 1049 plants (0.1 cM, Figure 1).

Example 2: Chromosome walking towards the *R1* locus and identification of an *R1* candidate gene

Marker *SPUD237* was used as probe for screening the cosmid library. One positive clone CosS (Figure 1) was identified. End sequencing of the CosS insert generated

a new marker separated by one recombination event (0.1 cM) from the *R1* locus. Screening the BAC library with this marker identified BAC clone BA100e13. Three recombination events separated the distal end of BA100e13 from *R1*. The BA100e13 end proximal to *R1* identified BA47f2. The BA47f2 end distal to *R1* overlapped with BA100e13 and was separated from *R1* by one recombination event. The proximal end co-segregated with *R1*, like all subsequent BAC ends analysed (right part of Figure 1). The BA47f2 end that co-segregated with *R1* identified 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). This resistance-gene-like (RGL) fragment was used as probe to rescreen the BAC library. The RGL probe identified, in addition to BA122p13, several new positive clones of which two, BA87d17 and BA76o11, were further analysed. They contained full length copies of the RGL gene which was envisaged as a possible *R1* candidate. The non-overlapping ends of BA76o11 and BA87d17 co-segregated with *R1*.

BAC end markers instrumental for physical map construction were also used to assign BAC clones to the P6/210 (*R1r1*) chromosome carrying either an *r1* or the *R1* allele. Clones BA100e13, BA47f2 and BA87d17 (Figure 1) were in *cis* with the *R1* allele, whereas clones BA121o1, BA122p13 and BA76o11 were derived from the homologue having *r1* (Figure 1). Clone BA27c1 could not be assigned to an *r1* or *R1* chromosome, based on the markers used.

Example 3: *R1* candidate cDNA clones

Using the whole insertions of BACs BA121o1 and BA76o11 as probes, six and eight cDNA clones, respectively, were isolated from a cDNA library prepared from infected leaves of genotype P41 (*R1r1*). Eight of the 14 cDNA clones were 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 ca. 80-90 % identity among each other. The cDNA clone c76-2, 2292 nucleotides long, was identical, with

exception of the introns, to the genomic sequence of clone g10, a subclone representing part of BA87d17 (see later). Sequence comparison to known resistance genes in the database indicated that c76-2 was not full length. Using RACE analysis, the cDNA was extended to 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 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 clone g10 (Figure 4B). Two adenines were identified at positions -3 and + 4 (where the a of the ATG is +1) referred to as 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. Primers 76-2sf2 and 76-2SR (see Material and Methods) generated a 1.4 kb PCR product only in parent P41 (*R1r1*) but not in parent P40 (*r1r1*) (Figure 2). This polymorphism suggested the possibility that BAC BA87d17 (derived from the *R1* hosting chromosome) contained the *R1* gene, even if the mapping data still indicated absence of recombination between the distal end of this BAC clone and *R1*.

Example 4: Complementation of the *R1* phenotype

A genomic sub-library in the pCLD04541 binary vector (see Material and Methods) with, on average, 10 kb insertions was constructed from BA87d17 (76 kb). The library was screened by colony hybridisation with the RGL probe of BA121o1. Positive clones were evaluated for the presence of the complete copy of the candidate RGL gene by the size of amplification products obtained by PCR with forward primers from the vector borders (T3 and T7) and reverse primers from the RGL. Clones were also tested by using the c76-2 cDNA specific primers 76-2sf2 and 76-2SR. Subclone g10-2 was selected and transformed into *A. tumefaciens*. Three different bacterial colonies were used to transform the susceptible cultivar Desirée. From three transformation experiments, fifteen independent transgenic lines were regenerated and tested in four independent experiments for expression of resistance to *P. infestans* race 4 (Table 2).

Table 2. Test for resistance to *P. infestans* race 4 of transgenic potato lines transformed with clone g10. Transgenic lines were tested in four independent experiments with three leaflets from each line for expression of hypersensitive resistance to *P. infestans* race 4.

Transgenic line no		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	n.d.
10-5	4	n.d.
10-5	5	R
10-23	1	n.d.
10-23	2	R
10-23	3	R
10-23	4	R
10-23	5	R
10-23	6	S

Nine transgenic lines showed consistently a typical HR response, similar to the resistant line P41 hosting *R1* (Figure 3); three gave inconsistent results and the remaining three were susceptible, like the untransformed Desirée control. Based on these results, it was concluded that the subclone g10 contained a functional *R1* gene.

All transgenic lines with the *R1* phenotype contained the gene corresponding to cDNA 76-2, as demonstrated by presence of the 1.4 kb PCR product amplified by primers 76-2sf2 and 76-2SR. This product was absent in untransformed Desirée (control) and in all BAC clones reported in Figure 1 as members of the contig around *R1*, except for BA87d17 (Figure 2).

Example 5: Structure of the *R1* gene

Subclone g10 containing the *R1* gene was sequenced; see SEQ ID NO: 1. The sequence was 10,388 nucleotides long and contained one gene with sequence similarity to other plant resistance genes. No other open reading frame or sequence homology was identified in the GenEMBL database. Sequence alignment with the cDNA c76-2 and the 5' RACE product revealed the presence of three exons and three introns. Two introns of 92 bp (position 4878 to 4970) and 126 bp (position 6103 to 6229) interrupt the coding region. The third intron of 81 bp (position 6323 to 6404) is located in the 3'untranslated region immediately downstream of the stop codon (Fig. 4A). The deduced amino acid sequence predicts a polypeptide of 1293 amino acids with a molecular mass of 149.4 kDa (Figure 4B). The deduced amino acid sequence of the *R1* gene is most similar (40 % identity) to the *Prf* gene for resistance to *P. syringae* of tomato (Salmeron et al. 1996). 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 (SEQ ID NO: 10) in *R1*), CKLY (CFLY (SEQ ID NO: 12) in *R1*) and MHD (LHD in *R1*). Searching for conserved motifs by using the ExPASy algorithm, 4 myristylation, 9 glycosylation, 43 phosphorylation and 1 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 carboxy-terminal part of the gene. Like some plant R-proteins with cytoplasmic LRRs, the R1 protein contains a leucine zipper from amino acid position 308 to 329 (Hammond-Kosack and Jones, 1997).

Example 6: Genomic organization of the *R1* locus

Southern gel blot analysis showed that *R1* is a member of a gene family.

The *R1* specific primers 76-2sf2 and 76-2SR amplified the 1.4 kb fragment in BA87d17 (*R1*) but not in the overlapping clones BA121o1, BA122p13 and BA76o11 (*r1*) (Figure 2). DNA sequence analysis of BACs BA87d17 (*R1*) and BA122p13 (*r1*) revealed that BA87d17 contained two highly homologous members of the *R1* gene

family, *R1* corresponding to the functional *R1* gene and *r1.1* being orthologous with the *r1.2* allele in BA122p13. The functional *R1* gene was part of a 15 kb insertion present in the *R1* bearing chromosome in the region represented by BA87d17, but absent in the chromosome hosting *r1* (Figure 5).

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

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Gln His Ala His Leu Ala Phe Thr Glu Met His Asn Leu Val Glu Trp	
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Ser Ala Ser Cys Ser Phe Val Gly Ser Val Val Leu Ser Asn Lys Tyr	
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Ile Ser Arg Ile Leu Pro Asn Phe Lys Phe Leu Lys Val Leu Asp Leu	
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Glu His Arg Val Phe Ile Asp Phe Ile Pro Thr Glu Leu Val Tyr Leu	
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Lys Tyr Phe Ser Ala His Ile Glu Gln Asn Ser Ile Pro Ser Ser Ile	
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Ser Asn Leu Trp Asn Leu Glu Thr Leu Ile Leu Lys Ser Pro Ile	
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cca ata cgg ctt Pro Ile Arg Leu 1105	gaa ata cta aag ctt Glu Ile Leu Lys Leu 1110	tat cga tca aaa ttt aaa Tyr Arg Ser Lys Phe Lys 1115	5663
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Lys Met Ile Glu Ile Cys Lys Arg Phe Asn Thr Pro Pro Pro His Asn
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Ser Phe Ala Tyr Trp Lys Glu Val Ile Cys Lys Arg Leu Cys Ala Ile
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Gly Pro Lys Glu Gln Ile Glu Gln Val Leu Lys Glu Leu Lys Leu Leu
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Lys His Ala Ala Glu Ser Gly Ile Val Glu Thr Pro Thr His Asn Leu
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1

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A nucleic acid molecule encoding a polypeptide which is capable of conferring resistance against a pathogen in a plant in which said polypeptide is expressed, said nucleic acid molecule comprising or consisting of a nucleotide sequence, wherein the nucleotide sequence is:

- (a) a nucleotide sequence encoding at least the mature form of a protein (R1) comprising the amino acid sequence as given in SEQ ID NO:2;
- (b) a nucleotide sequence comprising the coding regions of the DNA sequence as given in SEQ ID NO:1;
- (c) a nucleotide sequence hybridizing with the complementary strand of the nucleotide sequence as defined in (a) or (b) under stringent hybridization conditions, wherein said nucleotide sequence is at least 60% identical to the nucleotide sequences of (a) or (b);
- (d) a nucleotide sequence encoding a protein derived from the protein encoded by the nucleotide sequence of (a) or (b) by way of substitution, deletion and/or addition of one or several amino acids of the amino acid sequence encoded by the nucleotide sequence of (a) or (b), wherein said nucleotide sequence is at least 60% identical to the nucleotide sequences of (a) or (b);
- (e) a nucleotide sequence encoding a protein having an amino acid sequence at least 60% identical to the amino acid sequence encoded by the nucleotide sequence of (a) or (b);
- (f) a nucleotide sequence encoding at least a Leucine zipper (LZ) domain corresponding to amino acid position 308-329 of SEQ ID NO:2, a nucleic binding site (NBS) domain corresponding to amino acid position 572-682 of SEQ ID NO:2 and a Leucine rich repeat (LRR) domain corresponding to amino acid position 780-1280 of SEQ ID NO:2;
- (g) a nucleotide sequence encoding an epitope-bearing portion of a R1 protein encoded by the nucleotide sequence of (a) or (b), wherein said nucleotide sequence is at least 60% identical to the nucleotide sequences of (a) or (b);

- (h) DNA sequences obtained by screening an appropriate library under stringent conditions with a probe having at least 17 consecutive nucleotides of a nucleotide sequence of any one of SEQ ID NOs: 1 and 5 to 8, wherein said DNA sequence is at least 60% identical to the nucleotide sequence of SEQ ID NO:1 or a nucleotide sequence encoding the protein with the sequence of SEQ ID NO:2; or
 - (i) a nucleotide sequence of which is degenerate as a result of the genetic code to the nucleotide sequence of any one of (a) to (h).
2. The nucleic acid molecule of claim 1 wherein said pathogen is *Phytophthora infestans*.
 3. A vector comprising the nucleic acid molecule of claim 1 or 2.
 4. The vector of claim 3 which is an expression vector wherein the nucleic acid molecule is operatively linked to one or more control sequences allowing the transcription in prokaryotic and/or eukaryotic host cells.
 5. The vector of claim 4 which is an expression vector wherein the nucleic acid molecule is operatively linked to one or more control sequences allowing the transcription and expression in prokaryotic and/or eukaryotic host cells.
 6. A host cell transformed with the vector of any one of claims 3 to 5 or the nucleic acid molecule of any one of claims 1 or 2.
 7. A polypeptide which is capable of conferring resistance against a pathogen in a plant which is encoded by the nucleic acid molecule of claim 1 or 2.
 8. A transgenic plant cell transformed with the nucleic acid molecule of claim 1 or 2 which is operably linked to regulatory elements allowing transcription and/or expression of the DNA sequence in plant cells.
 9. A regulatory sequence of a promoter regulating the expression of a gene comprising the nucleic acid molecule of claim 1 or 2, said regulatory sequence being capable of conferring or modulating the expression of a heterologous DNA sequence

upon pathogen infection, wherein said regulatory sequence comprises a DNA sequence, wherein the DNA sequence is:

- (a) a DNA sequence comprising the nucleotide sequence as depicted in SEQ ID NO:1 from nucleotides 1 to 2222;
- (b) a DNA sequence hybridizing with the nucleotide sequence as defined in (a) under stringent conditions, wherein said DNA sequence is at least 60% identical to the nucleotide sequence of SEQ ID NO:1 from nucleotides 1 to 2222; or
- (c) a DNA sequence having at least 85% identity to the sequence defined in (a).

10. A recombinant DNA molecule comprising the regulatory sequence of claim 9.

11. The recombinant DNA molecule of claim 10, wherein said regulatory sequence is operatively linked to a heterologous DNA sequence.

12. A host cell transformed with the regulatory sequence of claim 9 or with the recombinant DNA molecule of claim 10 or 11.

13. A transgenic plant cell comprising the regulatory sequence of claim 9 or the recombinant DNA molecule of claim 10 or 11 as a transgene.

14. A method for the identification of a plant protective agent comprising the steps of:

- (a) culturing a plant cell or tissue or maintaining a plant comprising a recombinant DNA molecule comprising a read out system operatively linked to the regulatory sequence of claim 9 in the presence of a compound or a sample comprising a plurality of compounds under conditions which permit expression of said read out system; and
- (b) identifying or verifying a sample and compound, respectively, which leads to activation and/or enhancement of expression of said read out system in said plant, plant cell, or plant tissue as said plant protective agent.

15. A method for identifying and obtaining an avirulence or a virulence factor of a pathogen comprising the steps of:

- (a) screening the polypeptide of claim 7 or a fragment thereof against a peptide or protein expression library derived from a pathogen in a read out system under suitable conditions which permit interaction of the polypeptide of claim 7 and peptide in said read out system; and
- (b) identifying or verifying a cDNA which leads to suppression or activation of the read out system as a virulence or avirulence factor.

16. A composition comprising the nucleic acid molecule of claim 1 or 2, the vector of any one of claims 3 to 5, the polypeptide of claim 7, the regulatory sequence of claim 9 or the recombinant DNA molecule of claim 10 or 11 and suitable means for detection or suitable means for plant cell and tissue culture.

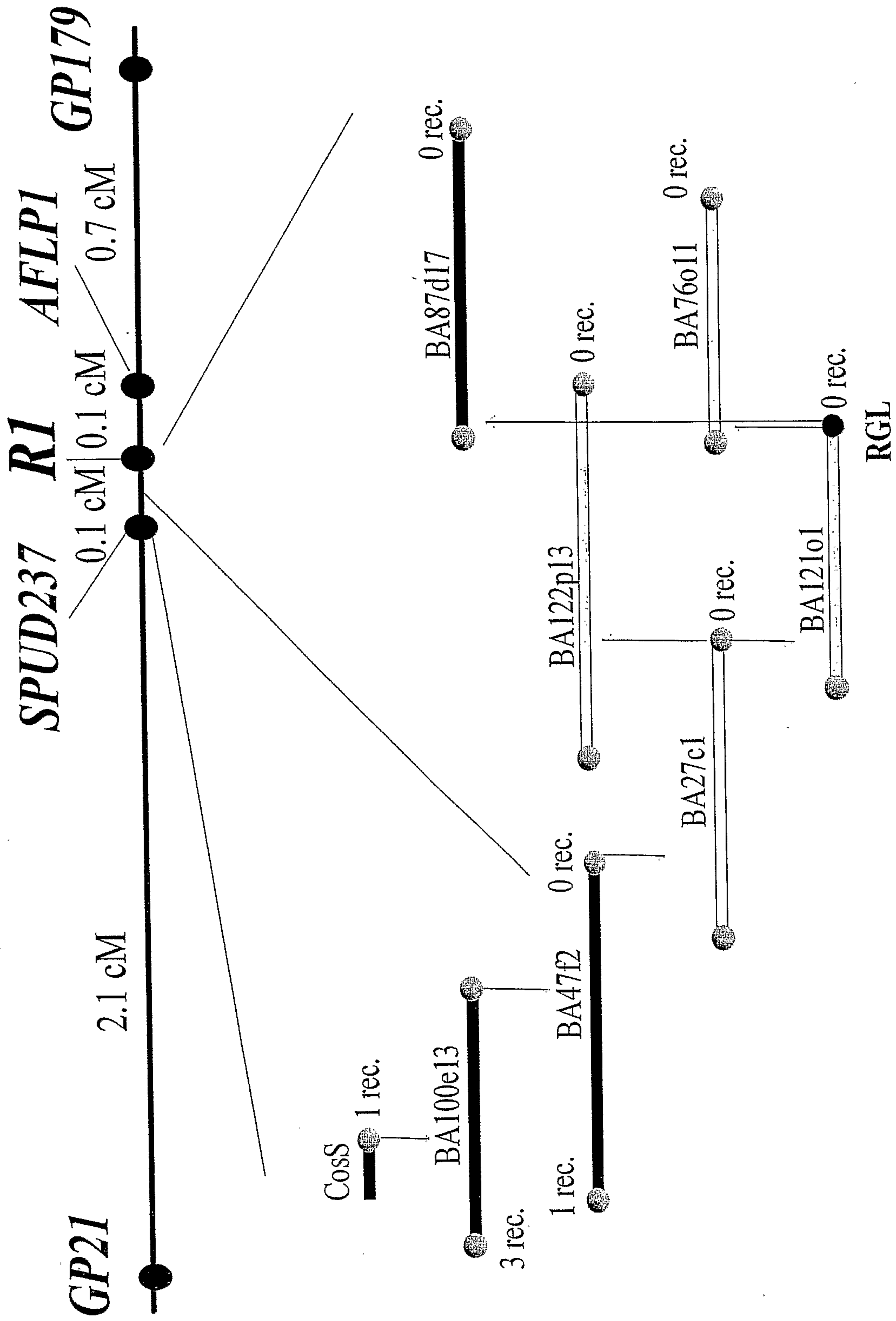


Figure 1

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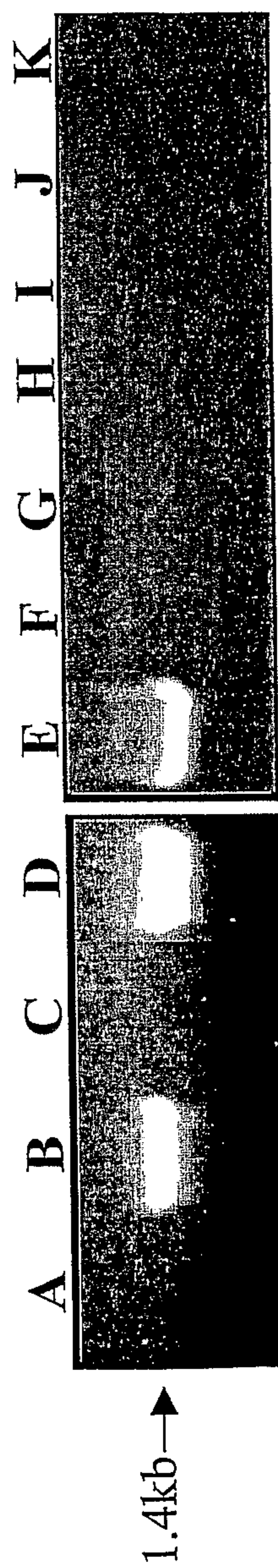


Figure 2

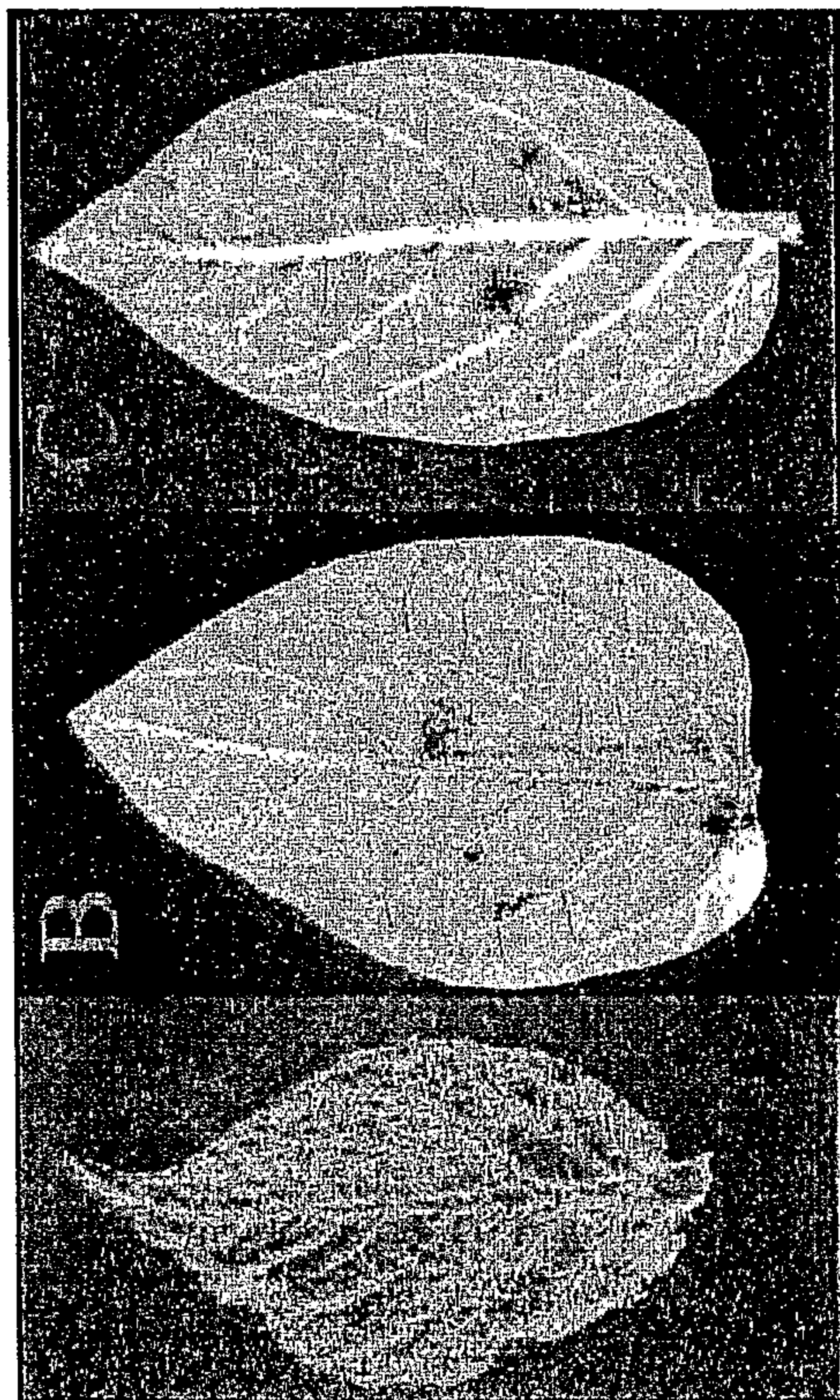
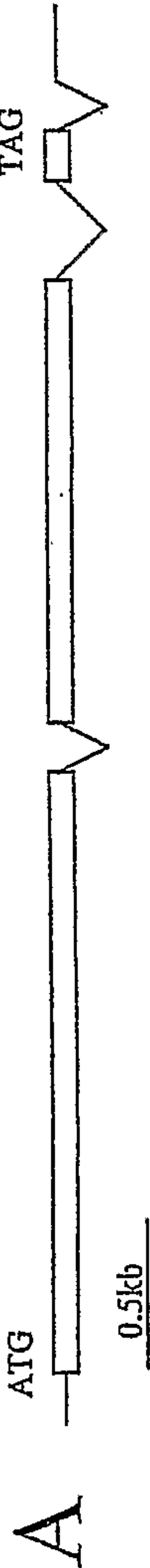


Figure 3



B

1 MNFNNELSDL KNRFLFRTL A QKCDVARD RIDFFIWELK FLNCFHLQS
51 FAFASECGML DISQKMEIC KRFTPPPHN SFAYWKEVIC KRLCAISIQP
101 DASSDDGFAC WKKVIWKTQ EFRAYSEPK TLLADNKVYD DDDTNPKFVM
151 EFIDAVVGNL NVLVKINDPS SLLFVGPKE QIEQVLKELK LLRFFVCFVS
201 NKCIEPQYQH TTFYTHALIE ASHAMVVWL NLPYGNRNQ DLASSEVSL
251 LSDFMEMKIK SIQPDISRNN IYIDVLALK STIPQAQDKH AAESGIVETP
301 THNLMVGLSD QMANLOEMLC LLRDNLHLP ILDFEHLQD MDSVIVDAGL
351 LIYSLYDIKG QKEDTTLEDI NQALGFDLPR NIEPIKAMIN LVMQKAFQCN
401 LPRIHGLGYV DFLKKNLKDF QGRYSDSLDF LKNQLQVIQT EFESLQPFK
451 VVVEEPHNKL KTLNEDCATQ IIRKAYEVEY VVDACINKEV PQWCIERWLL
501 DIIIEITCIK AKIQEKNTVE DTMKTVIART SSKLARTPRM NEEIVGFEDV
551 IENLRKKLLN GTKGQDVISI HGMPGLGKITT LANSLYSDRS VFSQFDICAQ
601 CCVSQVVSYP DLILALLRDA IGEQSVRREL HANELADMLR KTLPPRRYLI
651 LYDDVWENSV WDDLRCFPD VNNRSHILTT TRHHEVAKYA SVHSDPLHLR
701 MFDEVESWKL LEKKVFGEES CSPLLNKVG LRIAKMCGQLP LSIVLVAGIL
751 SEMEKEVECW EQVANNLGSY IHNDSRAVD KSYHVLPCHL KSCFLYFGAF
801 LEDRVIDIS LIRLWISSEAF IKSSSEGRRL E DIAEGYLEN IGRNLVMTQ
851 RSISDGKAKE CRLHDVLLDF CKERAAEENF LLWINRDQIT KPSSCVYSHK
901 QHAHLAFTEM HNLVEWSASC SFVGSVVLN KYDSYFSTRD ISSLHDFSSIS
951 RILPNFKFLK VLDLEHRVFI DFIPTLVYL KYFSAHIEQN SIPSSISNLW
1001 NLETILKSP IYALRCTLL PSTVWDMVKL RHLYPDFTST RIEAALLENS
1051 AKLYNLETLS TLYFSRVEDA ELMLRKTPNL RKLICEVECL EYPPQYHVLN
1101 FPIRLEILKL YRSKFKTIPF CISAPNLKYL KLCGFSLDSQ YLSETADHLK
1151 HLEVILLYKV EFGDHRWKV SNGKFPQLKI LKLEYLSLVK WIVADDAFPN
1201 LEQLVLRGCQ DLMEIPSCFM DILSLKYGV EYCNESVKS ALNIQETQVE
1251 DYQNTNFKLY LIEFSLQKKA WKLNLDAED MHNAVKNILA EIR*

Figure 4

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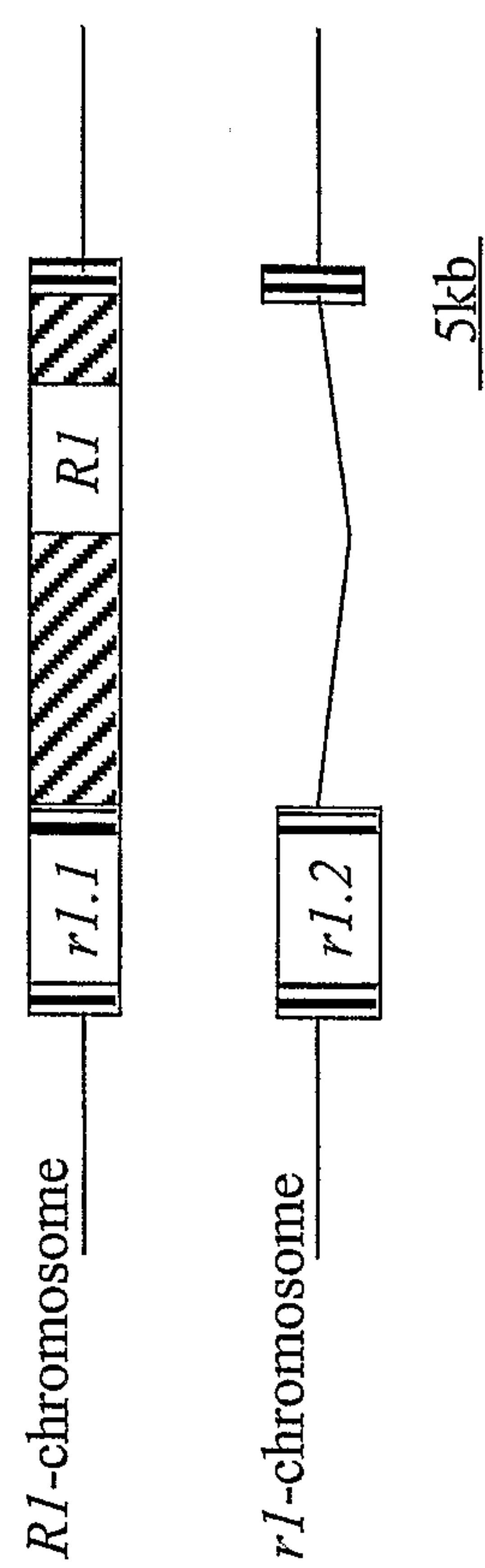


Figure 5