



(86) Date de dépôt PCT/PCT Filing Date: 1995/04/13
(87) Date publication PCT/PCT Publication Date: 1995/10/26
(45) Date de délivrance/Issue Date: 2012/06/05
(85) Entrée phase nationale/National Entry: 1996/10/09
(86) N° demande PCT/PCT Application No.: US 1995/004570
(87) N° publication PCT/PCT Publication No.: 1995/028478
(30) Priorité/Priority: 1994/04/13 (US08/227,360)

(51) Cl.Int./Int.Cl. *C12N 15/29* (2006.01),
A01H 5/00 (2006.01), *C07K 14/415* (2006.01),
C12N 15/82 (2006.01), *C12N 5/10* (2006.01),
C12Q 1/68 (2006.01)

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(54) Titre : GENE RPS2 ET UTILISATIONS DE CE DERNIER
(54) Title: RPS2 GENE AND USES THEREOF

(57) **Abrégé/Abstract:**

Disclosed is substantially pure DNA encoding an Arabidopsis thaliana Rps2 polypeptide; substantially pure Rps2 polypeptide; and methods of using such DNA to express the Rps2 polypeptide in plant cells and whole plants to provide, in transgenic plants, disease resistance to pathogens.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶: C12N 5/04, 15/29, 15/31, 15/82, C07K 14/415, A01H 4/00	A1	(11) International Publication Number: WO 95/28478 (43) International Publication Date: 26 October 1995 (26.10.95)
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RPS2 GENE AND USES THEREOFBackground of the Invention

The invention relates to recombinant plant nucleic acids and polypeptides and uses thereof to confer disease
5 resistance to pathogens in transgenic plants.

Plants employ a variety of defensive strategies to combat pathogens. One defense response, the so-called hypersensitive response (HR), involves rapid localized necrosis of infected tissue. In several host-pathogen
10 interactions, genetic analysis has revealed a gene-for-gene correspondence between a particular avirulence (avr) gene in an avirulent pathogen that elicits an HR in a host possessing a particular resistance gene.

15 Summary of the Invention

Various embodiments of this invention provide a substantially pure DNA encoding a polypeptide comprising a P-loop and an LRR domain, said polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant
20 pathogen.

Other embodiments of this invention provide substantially pure DNA having the sequence of SEQ ID NO:1 as shown in Fig. 2, or degenerate variants thereof, and encoding the amino acid sequence of SEQ ID NOs: 2-5 of open
25 reading frame "a" of Fig. 2.

Other embodiments of this invention provide substantially pure DNA having about 50% or greater sequence identity to the DNA sequence of SEQ ID NO:1 as shown in Fig. 2.

30 Other embodiments of this invention provide a cell transformed with DNA of this invention. The cell may be a plant cell and the plant cell may be resistant to disease caused by a plant pathogen carrying an avirulence gene generating a signal recognized by a polypeptide comprising a

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P-loop and an LRR domain. The plant pathogen may carry a gene comprising SEQ ID NO:105.

Various embodiments of this invention provide a transgenic plant cell transformed with the DNA of this invention integrated into the genome of said plant, wherein
5 said DNA is expressed in said transgenic plant cell, including plant cells generated from such transgenic plant cells. A plant cell of this invention may be a seed cell.

Various embodiments of this invention provide a method
10 of isolating a disease resistance gene or portion thereof from a plant, said method comprising: (a) amplifying by PCR said disease resistance gene or portion thereof using oligonucleotide primers wherein said primers (i) are each greater than 13 nucleotides in length; (ii) each have
15 regions of complementarity to opposite DNA strands in a region of the nucleotide sequence of SEQ ID NO:1 of Fig. 2; and (iii) optionally contain sequences capable of producing restriction enzyme cut sites in the amplified product; and
20 (b) isolating said disease resistance gene or portion thereof.

Various embodiments of this invention provide a method of detecting a resistance gene in a plant cell, said method comprising: contacting the DNA of this invention or a portion thereof greater than about 18 nucleic acids in
25 length with a preparation of genomic DNA from said plant cell under hybridization conditions providing detection of DNA sequences having about 50% or greater sequence identity to the DNA sequence of SEQ ID NO:1 as shown in Fig. 2.

Various embodiments of this invention provide a method
30 of producing a plant disease resistance polypeptide comprising: providing a cell transformed with DNA of this invention positioned for expression in said cell; culturing said transformed cell under conditions for expressing said

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DNA; and isolating a polypeptide comprising a P-loop and an LRR domain.

Various embodiments of this invention provide a method of providing, in a transgenic plant, resistance to a plant pathogen, said method comprising: growing said transgenic plant from the plant cell of this invention, wherein said DNA and said gene comprising SEQ ID NO: 105 are expressed in said transgenic plant.

Various embodiments of this invention provide a substantially pure polypeptide comprising a P-loop and an LRR domain, said polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen.

Various embodiments of this invention provide a vector comprising the DNA of this invention, said vector being capable of directing expression of the peptide encoded by said DNA in a vector-containing cell. The vector may comprise the aforementioned DNA and DNA of SEQ ID NO:105, operably linked to regulatory sequences wherein said regulatory sequences comprise a promoter.

Various embodiments of this invention provide substantially pure DNA having about 85% or greater sequence identity to a DNA sequence of Fig. 2.

Various embodiments of this invention provide a vector comprising the DNA of SEQ ID NO. 105 gene operably linked to plant regulatory sequences wherein said plant regulatory sequences comprise a promoter.

In general, the invention features substantially pure DNA (for example, genomic DNA, cDNA or synthetic DNA) encoding an Rps polypeptide as defined below. In related aspects, the invention also features a vector, a cell (e.g., a plant cell), and a transgenic plant or seed thereof which includes such a substantially pure DNA encoding an Rps polypeptide.

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In preferred embodiments, an *RPS* gene [SEQ ID NO:5] is the *RPS2* gene of a plant of the genus *Arabidopsis*. In various preferred embodiments, the cell is a transformed plant cell derived from a cell of a transgenic plant. In
5 related aspects, the invention features a transgenic plant containing a transgene which encodes an *Rps* polypeptide that is expressed in plant tissue susceptible to infection by pathogens expressing the *avrRpt2* avirulence gene [SEQ ID NO:105] or pathogens expressing an avirulence signal
10 similarly recognized by an *Rps* polypeptide.

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In a second aspect, the invention features a substantially pure DNA which includes a promoter capable of expressing the *RPS2* gene [SEQ. ID. NO:1] in plant tissue susceptible to infection by bacterial pathogens
5 expressing the *avrRpt2* avirulence gene [SEQ. ID NO:105].

In preferred embodiments, the promoter is the promoter native to an *RPS* gene. Additionally, transcriptional and translational regulatory regions are preferably native to an *RPS* gene.

10 The transgenic plants of the invention are preferably plants which are susceptible to infection by a pathogen expressing an avirulence gene, preferably the *avrRpt2* avirulence gene [SEQ ID. NO:105]. In preferred embodiments the transgenic plant is from the group of
15 plants consisting of but not limited to *Arabidopsis*, tomato, soybean, bean, maize, wheat and rice.

In another aspect, the invention features a method of providing resistance in a plant to a pathogen which involves: (a) producing a transgenic plant cell having a
20 transgene encoding an *Rps2* polypeptide wherein the transgene is integrated into the genome of the transgenic plant and is positioned for expression in the plant cell; and (b) growing a transgenic plant from the transgenic plant cell wherein the *RPS2* transgene is expressed in the
25 transgenic plant.

In another aspect, the invention features a method of detecting a resistance gene in a plant cell involving: (a) contacting the *RPS2* gene [SEQ ID NO:1] or a portion thereof greater than 18 nucleic acids in length with a
30 preparation of genomic DNA from said plant cell under hybridization conditions providing detection of DNA sequences having about 50% or greater sequence identity to the DNA sequence of Fig. 2 encoding the *Rps2* polypeptide [SEQ. ID NOS:2-5].

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In another aspect, the invention features a method of producing an Rps2 polypeptide which involves: (a) providing a cell transformed with DNA encoding an Rps2 polypeptide positioned for expression in the cell; (b) 5 culturing the transformed cell under conditions for expressing the DNA; and (c) isolating the Rps2 polypeptide.

In another aspect, the invention features substantially pure Rps2 polypeptide. Preferably, the 10 polypeptide includes a greater than 50 amino acid sequence substantially identical to a greater than 50 amino acid sequence shown in Fig. 2, open reading frame "a". Most preferably, the polypeptide is the *Arabidopsis thaliana* Rps2 polypeptide [SEQ. ID NOS:2-5].

15 In another aspect, the invention features a method of providing resistance in a transgenic plant to infection by pathogens which do not carry the *avrRpt2* avirulence gene wherein the method includes: (a) producing a transgenic plant cell having transgenes 20 encoding an Rps2 polypeptide as well as a transgene encoding the *avrRpt2* gene product [SEQ ID. NO:106] wherein the transgenes are integrated into the genome of the transgenic plant; are positioned for expression in the plant cell; and the *avrRpt2* transgene and, if 25 desired, the *RPS2* gene, are under the control of regulatory sequences suitable for controlled expression of the gene(s); and (b) growing a transgenic plant from the transgenic plant cell wherein the *RPS2* and *avrRpt2* transgenes are expressed in the transgenic plant.

30 In another aspect, the invention features a method of providing resistance in a transgenic plant to infection by pathogens in the absence of avirulence gene expression in the pathogen wherein the method involves: (a) producing a transgenic plant cell having integrated 35 in the genome a transgene containing the *RPS2* gene under

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the control of a promoter providing constitutive expression of the *RPS2* gene; and (b) growing a transgenic plant from the transgenic plant cell wherein the *RPS2* transgene is expressed constitutively in the transgenic
5 plant.

In another aspect, the invention features a method of providing controllable resistance in a transgenic plant to infection by pathogens in the absence of avirulence gene expression in the pathogen wherein the
10 method involves: (a) producing a transgenic plant cell having integrated in the genome a transgene containing the *RPS2* gene under the control of a promoter providing controllable expression of the *RPS2* gene; and (b) growing a transgenic plant from the transgenic plant cell wherein
15 the *RPS2* transgene is controllably expressed in the transgenic plant. In preferred embodiments, the *RPS2* gene is expressed using a tissue-specific or cell type-specific promoter, or by a promoter that is activated by the introduction of an external signal or agent, such as
20 a chemical signal or agent.

By "disease resistance gene" is meant a gene encoding a polypeptide capable of triggering the plant defense response in a plant cell or plant tissue. An *RPS* gene is a disease resistance gene having about 50% or
25 greater sequence identity to the *RPS2* sequence [SEQ ID. NO:1] of Fig. 2 or a portion thereof. The gene, *RPS2*, is a disease resistance gene encoding the *Rps2* disease resistance polypeptide [SEQ. ID NOS:2-5] from *Arabidopsis thaliana*.

30 By "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation).

By "substantially identical" is meant a polypeptide or nucleic acid exhibiting at least 50%,
35 preferably 85%, more preferably 90%, and most preferably

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95% homology to a reference amino acid or nucleic acid sequence. For polypeptides, the length of comparison sequences will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids. For nucleic acids, the length of comparison sequences will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides.

10 Sequence identity is typically measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Such software
15 matches similar sequences by assigning degrees of homology to various substitutions, deletions, substitutions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine alanine; valine, isoleucine,
20 leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

By a "substantially pure polypeptide" is meant an Rps2 polypeptide which has been separated from components
25 which naturally accompany it. Typically, the polypeptide is substantially pure when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more
30 preferably at least 90%, and most preferably at least 99%, by weight, Rps2 polypeptide. A substantially pure Rps2 polypeptide may be obtained, for example, by extraction from a natural source (e.g., a plant cell); by expression of a recombinant nucleic acid encoding an Rps2
35 polypeptide; or by chemically synthesizing the protein.

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Purity can be measured by any appropriate method, e.g., those described in column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

A protein is substantially free of naturally associated components when it is separated from those contaminants which accompany it in its natural state. Thus, a protein which is chemically synthesized or produced in a cellular system different from the cell from which it naturally originates will be substantially free from its naturally associated components. Accordingly, substantially pure polypeptides include those derived from eukaryotic organisms but synthesized in *E. coli* or other prokaryotes.

By "substantially pure DNA" is meant DNA that is free of the genes which, in the naturally-occurring genome of the organism from which the DNA of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

By "transformed cell" is meant a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding (as used herein) an Rps2 polypeptide.

By "positioned for expression" is meant that the DNA molecule is positioned adjacent to a DNA sequence which directs transcription and translation of the sequence (i.e., facilitates the production of, e.g., an

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Rps2 polypeptide, a recombinant protein or a RNA molecule).

By "reporter gene" is meant a gene whose expression may be assayed; such genes include, without
5 limitation, β -glucuronidase (GUS), luciferase, chloramphenicol transacetylase (CAT), and β -galactosidase.

By "promoter" is meant minimal sequence sufficient to direct transcription. Also included in the invention
10 are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell-type specific, tissue-specific or inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the native gene.

15 By "operably linked" is meant that a gene and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequence(s).

20 By "plant cell" is meant any self-propagating cell bounded by a semi-permeable membrane and containing a plastid. Such a cell also requires a cell wall if further propagation is desired. Plant cell, as used herein includes, without limitation, algae, cyanobacteria, seeds
25 suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores.

By "transgene" is meant any piece of DNA which is inserted by artifice into a cell, and becomes part of the
30 genome of the organism which develops from that cell. Such a transgene may include a gene which is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism.

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By "transgenic" is meant any cell which includes a DNA sequence which is inserted by artifice into a cell and becomes part of the genome of the organism which develops from that cell. As used herein, the transgenic
5 organisms are generally transgenic plants and the DNA (transgene) is inserted by artifice into the nuclear or plastidic genome.

By "pathogen" is meant an organism whose infection into the cells of viable plant tissue elicits a disease
10 response in the plant tissue.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Detailed Description

15 The drawings will first be described.

Drawings

Figs. 1A - 1F are a schematic summary of the physical and RFLP analysis that led to the cloning of the RPS2 locus.

20 Fig. 1A is a diagram showing the alignment of the genetic and the RFLP maps of the relevant portion of *Arabidopsis thaliana* chromosome IV adapted from the map published by Lister and Dean (1993) Plant J. 4:745-750. The RFLP marker L11F11 represents the left arm of the
25 YUP11F11 YAC clone.

Fig. 1B is a diagram showing the alignment of relevant YACs around the RPS2 locus. YAC constructs designated YUP16G5, YUP18G9 and YUP11F11 were provided by J. Ecker, University of Pennsylvania. YAC constructs
30 designated EW3H7, EW11D4, EW11E4, and EW9C3 were provided by E. Ward, Ciba-Geigy, Inc.

Fig. 1C is a diagram showing the alignment of cosmid clones around the RPS2 locus. Cosmid clones with the designation H are derivatives of the EW3H7 YAC clone
35 whereas those with the designation E are derivatives of

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the EW11E4 YAC clone. Vertical arrows indicate the relative positions of RFLP markers between the ecotypes La-er and the *rps2-101N* plant. The RFLP markers were identified by screening a Southern blot containing more than 50 different restriction enzyme digests using either the entire part or pieces of the corresponding cosmid clones as probes. The cosmid clones described in Fig. 1C were provided by J. Giraudat, C.N.R.S., Gif-sur-Yvette, France.

10 Figs. 1D and 1E are maps of *EcoRI* restriction endonuclease sites in the cosmids E4-4 and E4-6, respectively. The recombination break points surrounding the *RPS2* locus are located within the 4.5 and 7.5 kb *EcoRI* restriction endonuclease fragments.

15 Fig. 1F is a diagram showing the approximate location of genes which encode the RNA transcripts which have been identified by polyA⁺ RNA blot analysis. The sizes of the transcripts are given in kilobase pairs below each transcript.

20 Fig. 2 is the complete nucleotide sequence of cDNA-4 comprising the *RPS2* [SEQ. ID. NO: 1] gene locus. The three reading frames are shown below the nucleotide sequence. The deduced amino acid sequence [SEQ. ID NOS:2-5] of reading frame "a" is provided and contains 909 amino acids. The methionine encoded by the ATG start codon is circled in open reading frame "a" of Fig. 2. The A of the ATG start codon is nucleotide 31 of Fig. 2.

Fig. 3 is the nucleotide sequence of the *avrRpt2* gene [SEQ. ID NO:105] and its deduced amino acid sequence [SEQ. ID NO:106]. A potential ribosome binding site is underlined. An inverted repeat is indicated by horizontal arrows at the 3' end of the open reading frame. The deduced amino acid sequence is provided below the nucleotide sequence of the open reading frame.

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Fig. 4 is a schematic summary of the complementation analysis that allowed functional confirmation that the DNA carried on p4104 and p4115 (encoding cDNA-4) confers RPS2 disease resistance activity to *Arabidopsis thaliana* plants previously lacking RPS2 disease resistance activity. Small vertical marks along the "genome" line represent restriction enzyme *EcoRI* recognition sites, and the numbers above this line represent the size, in kilobasepairs (kb), of the resulting DNA fragments (see also Fig. 1E). Opposite "cDNAs" are the approximate locations of the coding sequences for RNA transcripts (See also Fig. 1F); arrowheads indicate the direction of transcription for cDNAs 4, 5, and 6. For functional complementation experiments, *rps2-201C/rps2-201C* plants were genetically transformed with the *Arabidopsis thaliana* genomic DNA sequences indicated; these sequences were carried on the named plasmids (derivatives of the binary cosmid vector pSLJ4541) and delivered to the plant via *Agrobacterium*-mediated transformation methods. The disease resistance phenotype of the resulting transformants following inoculation with *P. syringae* expressing *avrRpt2* is given as "Sus." (susceptible, no resistance response) or "Res." (disease resistant).

25 The Genetic Basis for Resistance to Pathogens

An overview of the interaction between a plant host and a microbial pathogen is presented. The invasion of a plant by a potential pathogen can have a range of outcomes delineated by the following outcomes: either the pathogen successfully proliferates in the host, causing associated disease symptoms, or its growth is halted by the host defenses. In some plant-pathogen interactions, the visible hallmark of an active defense response is the so-called hypersensitive response or "HR". The HR

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involves rapid necrosis of cells near the site of the infection and may include the formation of a visible dry brown lesion. Pathogens which elicit an HR on a given host are said to be avirulent on that host, the host is
5 said to be resistant, and the plant-pathogen interaction is said to be incompatible. Strains which proliferate and cause disease on a particular host are said to be virulent; in this case the host is said to be susceptible, and the plant-pathogen interaction is said
10 to be compatible

"Classical" genetic analysis has been used successfully to help elucidate the genetic basis of plant-pathogen recognition for those cases in which a series of strains (races) of a particular fungal or
15 bacterial pathogen are either virulent or avirulent on a series of cultivars (or different wild accessions) of a particular host species. In many such cases, genetic analysis of both the host and the pathogen revealed that many avirulent fungal and bacterial strains differ from
20 virulent ones by the possession of one or more avirulence (avr) genes that have corresponding "resistance" genes in the host. This avirulence gene-resistance gene correspondence is termed the "gene-for-gene" model (Crute, et al., (1985) pp 197-309 in: *Mechanisms of*
25 *Resistance to Plant Disease*. R.S.S. Fraser, ed.; Ellingboe, (1981) *Annu. Rev. Phytopathol.* 19:125-143; Flor, (1971) *Annu. Rev. Phytopathol.* 9:275-296; Keen and Staskawicz, (1988) supra; and Keen et al. in: *Application of Biotechnology to Plant Pathogen Control*. I. Chet, ed.,
30 John Wiley & Sons, 1993, pp. 65-88). According to a simple formulation of this model, plant resistance genes encode specific receptors for molecular signals generated by avr genes. Signal transduction pathway(s) then carry the signal to a set of target genes that initiate the HR
35 and other host defenses (Gabriel and Rolfe, (1990) *Annu.*

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Rev. Phytopathol. 28:365-391). Despite this simple predictive model, the molecular basis of the avr-resistance gene interaction is still unknown.

One basic prediction of the gene-for-gene hypothesis has been convincingly confirmed at the molecular level by the cloning of a variety of bacterial avr genes (Innes, et al., (1993) J. Bacteriol. 175:4859-4869; Dong, et al., (1991) Plant Cell 3:61-72; Whelan et al., (1991) Plant Cell 3:49-59; Staskawicz et al., (1987) J. Bacteriol. 169:5789-5794; Gabriel et al., (1986) P.N.A.S., USA 83:6415-6419; Keen and Staskawicz, (1988) Annu. Rev. Microbiol. 42:421-440; Kobayashi et al., (1990) Mol. Plant-Microbe Interact. 3:94-102 and (1990) Mol. Plant-Microbe Interact. 3:103-111). Many of these cloned avirulence genes have been shown to correspond to individual resistance genes in the cognate host plants and have been shown to confer an avirulent phenotype when transferred to an otherwise virulent strain. The avrRpt2 locus was isolated from *Pseudomonas syringae* pv. *tomato* and sequenced by Innes et al. (Innes, R. et al. (1993) J. Bacteriol. 175:4859-4869). Fig. 3 is the nucleotide sequence [SEQ. ID NO:105] and deduced amino acid sequence [SEQ. ID NO:6] of the avrRpt2 gene.

Examples of known signals to which plants respond when infected by pathogens include harpins from *Erwinia* (Wei et al. (1992) Science 257:85-88) and *Pseudomonas* (He et al. (1993) Cell 73:1255-1266); avr4 (Joosten et al. (1994) Nature 367:384-386) and avr9 peptides (van den Ackerveken et al (1992) Plant J. 2:359-366) from *Cladosporium*; PopA1 from *Pseudomonas* (Arlat et al. (1994) EMBO J. 13:543-553); avrD-generated lipopolysaccharide (Midland et al. (1993) J. Org. Chem. 58:2940-2945); and NIP1 from *Rhynchosporium* (Hahn et al. (1993) Mol. Plant-Microbe Interact. 6:745-754).

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Compared to avr genes, considerably less is known about plant resistance genes that correspond to specific avr-generated signals. The plant resistance gene, *RPS2* (rps for resistance to *Pseudomonas syringae*), the first
5 gene of a new, previously unidentified class of plant disease resistance genes corresponds to a specific avr gene (*avrRpt2*). Some of the work leading up to the cloning of *RPS2* is described in Yu, et al., (1993), *Molecular Plant-Microbe Interactions* 6:434-443 and in
10 Kunkel, et al., (1993) *Plant Cell* 5:865-875.

An apparently unrelated avirulence gene which corresponds specifically to plant disease resistance gene, *Pto*, has been isolated from tomato (*Lycopersicon esculentum*) (Martin et al., (1993) *Science* 262:1432-1436).
15 Tomato plants expressing the *Pto* gene are resistant to infection by strains of *Pseudomonas syringae* pv. *tomato* that express the *avrPto* avirulence gene. The amino acid sequence inferred from the *Pto* gene DNA sequence displays strong similarity to serine-threonine protein kinases,
20 implicating *Pto* in signal transduction. No similarity to the tomato *Pto* locus or any known protein kinases was observed for *RPS2*, suggesting that *RPS2* is representative of a new class of plant disease resistance genes.

The isolation of a race-specific resistance gene
25 from *Zea mays* (corn) known as *Hm1* has been reported (Johal and Briggs (1992) *Science* 258:985-987). *Hm1* confers resistance against specific races of the fungal pathogen *Cochliobolus carbonum* by controlling degradation of a fungal toxin, a strategy that is mechanistically
30 distinct from the avirulence-gene specific resistance of the *RPS2-avrRpt2* resistance mechanism.

The cloned *RPS2* gene of the invention can be used to facilitate the construction of plants that are resistant to specific pathogens and to overcome the
35 inability to transfer disease resistance genes between

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species using classical breeding techniques (Keen et al., (1993), supra). There now follows a description of the cloning and characterization of an *Arabidopsis thaliana* RPS2 genetic locus, the RPS2 genomic DNA, and the RPS2
5 cDNA. The *avrRpt2* gene and the RPS2 gene, as well as mutants *rps2-101C*, *rps2-102C*, and *rps2-201C* (also designated *rps2-201*), are described in Dong, et al., (1991) Plant Cell 3:61-72; Yu, et al., (1993) supra; Kunkel et al., (1993) supra; Whalen et al., (1991),
10 supra; and Innes et al., (1993), supra). A mutant designated *rps2-101N* has also been isolated. The identification and cloning of the RPS2 gene is described below.

15 RPS2 Overcomes Sensitivity to Pathogens Carrying the *avrRpt2* Gene.

To demonstrate the genetic relationship between an avirulence gene in the pathogen and a resistance gene in the host, it was necessary first to isolate an avirulence gene. By screening *Pseudomonas* strains that are known
20 pathogens of crop plants related to *Arabidopsis*, highly virulent strains, *P. syringae* pv. *maculicola* (*Psm*) ES4326, *P. syringae* pv. *tomato* (*Pst*) DC3000, and an avirulent strain, *Pst* MM1065 were identified and analyzed as to their respective abilities to grow in wild type
25 *Arabidopsis thaliana* plants (Dong et al., (1991) Plant Cell, 3:61-72; Whalen et al., (1991) Plant Cell 3:49-59; MM1065 is designated JL1065 in Whalen et al.). *Psm* ES4326 or *Pst* DC3000 can multiply 10^4 fold in *Arabidopsis thaliana* leaves and cause water-soaked lesions that
30 appear over the course of two days. *Pst* MM1065 multiplies a maximum of 10 fold in *Arabidopsis thaliana* leaves and causes the appearance of a mildly chlorotic dry lesion after 48 hours. Thus, disease resistance is

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associated with severely inhibited growth of the pathogen.

An avirulence gene (*avr*) of the *Pst* MM1065 strain was cloned using standard techniques as described in Dong et al. (1991), Plant Cell 3:61-72; Whalen et al., (1991) supra; and Innes et al., (1993), supra. The isolated avirulence gene from this strain was designated *avrRpt2*. Normally, the virulent strain *Psm* ES4326 or *Pst* DC3000 causes the appearance of disease symptoms after 48 hours as described above. In contrast, *Psm* ES4326/*avrRpt2* or *Pst* DC3000/*avrRpt2* elicits the appearance of a visible necrotic hypersensitivity response (HR) within 16 hours and multiplies 50 fold less than *Psm* ES4326 or *Pst* DC3000 in wild type *Arabidopsis thaliana* leaves (Dong et al., (1991), supra; and Whalen et al., (1991), supra). Thus, disease resistance in a wild type *Arabidopsis* plant requires, in part, an avirulence gene in the pathogen or a signal generated by the avirulence gene.

The isolation of four *Arabidopsis thaliana* disease resistance mutants has been described using the cloned *avrRpt2* gene to search for the host gene required for disease resistance to pathogens carrying the *avrRpt2* gene (Yu et al., (1993), supra; Kunkel et al., (1993), supra). The four *Arabidopsis thaliana* mutants failed to develop an HR when infiltrated with *Psm* ES4326/*avrRpt2* or *Pst* DC3000/*avrRpt2* as expected for plants having lost their disease resistance capacity. In the case of one of these mutants, approximately 3000 five to six week old *M₂* ecotype Columbia (Col-0 plants) plants generated by ethyl methanesulfonic acid (EMS) mutagenesis were hand-inoculated with *Psm* ES4326/*avrRpt2* and a single mutant, *rps2-101C*, was identified (resistance to *Pseudomonas syringae*) (Yu et al., (1993), supra).

The second mutant was isolated using a procedure that specifically enriches for mutants unable to mount an

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HR (Yu et al., (1993), supra). When 10-day old *Arabidopsis thaliana* seedlings growing on petri plates are infiltrated with *Pseudomonas syringae* pv. *phaseolicola* (Psp) NPS3121 versus Psp NPS3121/avrRpt2, about 90% of the plants infiltrated with Psp NPS3121 survive, whereas about 90%-95% of the plants infiltrated with Psp NPS3121/avrRpt2 die. Apparently, vacuum infiltration of an entire small *Arabidopsis thaliana* seedling with Psp NPS3121/avrRpt2 elicits a systemic HR which usually kills the seedling. In contrast, seedlings infiltrated with Psp NPS3121 survive because Psp NPS3121 is a weak pathogen on *Arabidopsis thaliana*. The second disease resistance mutant was isolated by infiltrating 4000 EMS-mutagenized Columbia M₂ seedlings with Psp NPS3121/avrRpt2. Two hundred survivors were obtained. These were transplanted to soil and re-screened by hand inoculation when the plants reached maturity. Of these 200 survivors, one plant failed to give an HR when hand-infiltrated with Psm ES4326/avrRpt2. This mutant was designated rps2-102C (Yu et al., (1993), supra).

A third mutant, rps2-201C, was isolated in a screen of approximately 7500 M₂ plants derived from seed of *Arabidopsis thaliana* ecotype Col-0 that had been mutagenized with diepoxybutane (Kunkel et al., (1993), supra). Plants were inoculated by dipping entire leaf rosettes into a solution containing Pst DC3000/avrRpt2 bacteria and the surfactant Silwet L-77 (Whalen et al., (1991), supra), incubating plants in a controlled environment growth chamber for three to four days, and then visually observing disease symptom development. This screen revealed four mutant lines (carrying the rps2-201C, rps2-202C, rps2-203C, and rps2-204C alleles), and plants homozygous for rps2-201C were a primary subject for further study (Kunkel et al., (1993), supra and the instant application).

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Isolation of the fourth *rps2* mutant, *rps2-101N*, has not yet been published. This fourth isolate is either a mutant or a susceptible *Arabidopsis* ecotype. Seeds of the *Arabidopsis* Nossen ecotype were gamma-irradiated and then sown densely in flats and allowed to germinate and grow through a nylon mesh. When the plants were five to six weeks old, the flats were inverted, the plants were partially submerged in a tray containing a culture of *Psm* ES4326/*avrRpt2*, and the plants were vacuum infiltrated in a vacuum desiccator. Plants inoculated this way develop an HR within 24 hours. Using this procedure, approximately 40,000 plants were screened and one susceptible plant was identified. Subsequent RFLP analysis of this plant suggested that it may not be a Nossen mutant but rather a different *Arabidopsis* ecotype that is susceptible to *Psm* ES4326/*avrRpt2*. This plant is referred to as *rps2-101N*. The isolated mutants *rps2-101C*, *rps2-102C*, *rps2-201C*, and *rps2-101N* are referred to collectively as the "*rps2* mutants".

20 The *rps2* Mutants Fail to Specifically Respond to the Cloned Avirulence Gene, *avrRpt2*.

The *RPS2* gene product is specifically required for resistance to pathogens carrying the avirulence gene, *avrRpt2*. A mutation in *Rps2* polypeptide that eliminates or reduces its function would be observable as the absence of a hypersensitive response upon infiltration of the pathogen. The *rps2* mutants displayed disease symptoms or a null response when infiltrated with *Psm* ES4326/*avrRpt2*, *Pst* DC3000/*avrRpt2* or *Psp* NPS3121/*avrRpt2*, respectively. Specifically, no HR response was elicited, indicating that the plants were susceptible and had lost resistance to the pathogen despite the presence of the *avrRpt2* gene in the pathogen.

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Pathogen growth in *rps2* mutant plant leaves was similar in the presence and absence of the *avrRpt2* gene. *Psm* ES4326 and *Psm* ES4326/*avrRpt2* growth in *rps2* mutants was compared and found to multiply equally well in the
5 *rps2* mutants, at the same rate that *Psm* ES4326 multiplied in wild-type *Arabidopsis* leaves. Similar results were observed for *Pst* DC3000 and *Pst* DC3000/*avrRpt2* growth in *rps2* mutants.

The *rps2* mutants displayed a HR when infiltrated
10 with *Pseudomonas* pathogens carrying other *avr* genes, *Psm* ES4326/*avrB*, *Pst* DC3000/*avrB*, *Psm* ES4326/*avrRpm1*, *Pst* DC3000/*avrRpm1*. The ability to mount an HR to an *avr* gene other than *avrRpt2* indicates that the *rps2* mutants isolated by selection with *avrRpt2* are specific to
15 *avrRpt2*.

Mapping and Cloning of the RPS2 Gene.

Genetic analysis of *rps2* mutants *rps2-101C*, *rps2-102C*, *rps-201C* and *rps-101N* showed that they all corresponded to genes that segregated as expected for a
20 single Mendelian locus and that all four were most likely allelic. The four *rps2* mutants were mapped to the bottom of chromosome IV using standard RFLP mapping procedures including polymerase chain reaction (PCR)-based markers (Yu et al., (1993), supra; Kunkel et al., (1993), supra).

25 Segregation analysis showed that *rps2-101C* and *rps2-102C* are tightly linked to the PCR marker, PG11, while the RFLP marker M600 was used to define the chromosome location of the *rps2-201C* mutation (Fig. 1A) (Yu et al., (1993), supra; Kunkel et
30 al., (1993), supra). *RPS2* has subsequently been mapped to the centromeric side of PG11.

Heterozygous *RPS2/rps2* plants display a defense response that is intermediate between those displayed by the wild-type and homozygous *rps2/rps2* mutant plants (Yu,

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et al., (1993), supra; and Kunkel et al., (1993), supra). The heterozygous plants mounted an HR in response to *Psm* ES4326/*avrRpt2* or *Pst* DC3000/*avrRpt2* infiltration; however, the HR appeared later than in wild type plants and required a higher minimum inoculum (Yu, et al., (1993), supra; and Kunkel et al., (1993), supra).

High Resolution Mapping of the RPS2 Gene and RPS2 cDNA Isolation.

To carry out map-based cloning of the *RPS2* gene, *rps2-101N/rps2-101N* was crossed with Landsberg erecta *RPS2/RPS2*. Plants of the F_1 generation were allowed to self pollinate (to "self") and 165 F_2 plants were selfed to generate F_3 families. Standard RFLP mapping procedures showed that *rps2-101N* maps close to and on the centromeric side of the RFLP marker, PG11. To obtain a more detailed map position, *rps2-101N/rps2-101N* was crossed with a doubly marked Landsberg erecta strain containing the recessive mutations, *cer2* and *ap2*. The genetic distance between *cer2* and *ap2* is approximately 15 cM, and the *rps2* locus is located within this interval. F_2 plants that displayed either a *CER2 ap2* or a *cer2 AP2* genotype were collected, selfed, and scored for *RPS2* by inoculating at least 20 F_3 plants for each F_2 with *Psm* ES4326/*avrRpt2*. DNA was also prepared from a pool of approximately 20 F_3 plants for each F_2 line. The *CER2 ap2* and *cer2 AP2* recombinants were used to carry out a chromosome walk that is illustrated in Figure 1.

As shown in Figure 1, *RPS2* was mapped to a 28-35 kb region spanned by cosmid clones E4-4 and E4-6. This region contains at least six genes that produce detectable transcripts. There were no significant differences in the sizes of the transcripts or their level of expression in the *rps2* mutants as determined by RNA blot analysis. cDNA clones of each of these

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transcripts were isolated and five of these were sequenced. As is described below, one of these transcripts, cDNA-4, was shown to correspond to the *RPS2* locus. From this study, three independent cDNA clones
5 (cDNA-4-4, cDNA-4-5, and cDNA-4-11) were obtained corresponding to *RPS2* from Columbia ecotype wild type plants. The apparent sizes of *RPS2* transcripts were 3.8 and 3.1 kb as determined by RNA blot analysis.

A fourth independent cDNA-4 clone (cDNA-4-2453)
10 was obtained using map-based isolation of *RPS2* in a separate study. Yeast artificial chromosome (YAC) clones were identified that carry contiguous, overlapping inserts of *Arabidopsis thaliana* ecotype Col-0 genomic DNA from the M600 region spanning approximately 900 kb in the
15 *RPS2* region. *Arabidopsis* YAC libraries were obtained from J. Ecker and E. Ward, supra and from E. Grill (Grill and Somerville (1991) Mol. Gen. Genet. 226:484-490). Cosmids designated "H" and "E" were derived from the YAC inserts and were used in the isolation of *RPS2* (Fig. 1).

20 The genetic and physical location of *RPS2* was more precisely defined using physically mapped RFLP, RAPD (random amplified polymorphic DNA) and CAPS (cleaved amplified polymorphic sequence) markers. Segregating populations from crosses between plants of genotype
25 *RPS2/RPS2* (No-0 wild type) and *rps2-201/rps2-201* (Col-0 background) were used for genetic mapping. The *RPS2* locus was mapped using markers 17B7LE, PG11, M600 and other markers. For high-resolution genetic mapping, a set of tightly linked RFLP markers was generated using
30 insert end fragments from YAC and cosmid clones (Fig. 1) (Kunkel et al. (1993), supra; Konieczny and Ausubel (1993) Plant J. 4:403-410; and Chang et al. (1988) PNAS USA 85:6856-6860). Cosmid clones E4-4 and E4-6 were then used to identify expressed transcripts (designated cDNA-

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4, -5, -6, -7, -8 of Fig 1F) from this region, including the cDNA-4-2453 clone.

RPS2 DNA Sequence Analysis.

DNA sequence analysis of cDNA-4 from wild-type Col-0 plants and from mutants *rps2-101C*, *rps2-102C*, *rps2-201C* and *rps2-101N* showed that cDNA-4 corresponds to RPS2. DNA sequence analysis of *rps2-101C*, *rps2-102C* and *rps2-201C* revealed changes from the wild-type sequence as shown in Table 1. The numbering system in Table 1 starts at the ATG start codon encoding the first methionine where A is nucleotide 1. DNA sequence analysis of cDNA-4 corresponding to mutant *rps2-102C* showed that it differed from the wild type sequence at amino acid residue 476. Moreover, DNA sequence analysis of the cDNA corresponding to cDNA-4 from *rps2-101N* showed that it contained a 10 bp insertion at amino acid residue 581, a site within the leucine-rich repeat region which causes a shift in the RPS2 reading frame. Mutant *rps2-101C* contains a mutation that leads to the formation of a chain termination codon. The DNA sequence of mutant allele *rps2-201C* revealed a mutation altering a single amino acid within a segment of the LRR region that also has similarity to the helix-loop-helix motif, further supporting the designation of this locus as the RPS2 gene. The DNA and amino acid sequences are shown in Figure 2 [SEQ. ID NO:1 and SEQ ID NOS:2-5, respectively].

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Table 1.

Mutant	Wild type	position of	Change mutation
<i>rps2-101C</i>	703 TGA 705	704	TAA Stop Codon
5 <i>rps2-101N</i>	1741 GTG 1743	1741	GTGGAGTTGTATG Insertion
<i>rps2-102C</i> 476	1426 AGA 1428 arg	1427	AAA Amino acid lys
10 <i>rps2-201C</i>	2002 ACC 2004 thr	2002	CCC Amino acid pro

DNA sequence analysis of cDNA-4 corresponding to *RPS2* from wild-type Col-0 plants revealed an open reading frame (between two stop codons) spanning 2,751 bp. There are 2,727 bp between the first methionine codon of this reading frame and the 3'-stop codon, which corresponds to a deduced 909 amino acid polypeptide (See open reading frame "a" of Fig. 2). The amino acid sequence has a relative molecular weight of 104,460 and a pI of 6.51.

RPS2 belongs to a new class of disease resistance genes; the structure of the *Rps2* polypeptide does not resemble the protein structure of the product of the only previously cloned and publicized avirulence gene-specific plant disease resistance gene, *Pto*, which has a putative protein kinase domain. From the above analysis of the deduced amino acid sequence, *RPS2* contains several distinct protein domains conserved in other proteins from both eukaryotes and prokaryotes. These domains include but are not limited to Leucine Rich Repeats (LRR) (Kobe and Deisenhofer, (1994) *Nature* 366:751-756); P-loop (Saraste et al. (1990) *Trends in Biological Sciences* TIBS 15:430-434; Helix-Loop-Helix (Murre et al. (1989) *Cell* 56:777-783; and Leucine Zipper (Rodrigues and Park (1993) *Mol. Cell Biol.* 13:6711-6722). The amino acid sequence

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of Rps2 contains a LRR motif (LRR motif from amino acid residue 505 to amino acid residue 867), which is present in many known proteins and which is thought to be involved in protein-protein interactions and may thus allow interaction with other proteins that are involved in plant disease resistance. The N-terminal portion of the Rps2 polypeptide LRR is, for example, related to the LRR of yeast (*Saccharomyces cerevisiae*) adenylate cyclase, CYR1. A region predicted to be a transmembrane spanning domain (Klein et al. (1985) Biochim., Biophys. Acta 815:468-476) is located from amino acid residue 350 to amino acid residue 365, N-terminal to the LRR. An ATP/GTP binding site motif (P-loop) is predicted to be located between amino acid residue 177 and amino acid residue 194, inclusive.

From the above analysis of the deduced amino acid sequence, the Rps2 polypeptide may have a membrane-receptor structure which consists of an N-terminal extracellular region and a C-terminal cytoplasmic region. Alternatively, the topology of the Rps2 may be the opposite: an N-terminal cytoplasmic region and a C-terminal extracellular region. LRR motifs are extracellular in many cases and the Rps2 LRR contains five potential N-glycosylation sites.

25 Identification of RPS2 by Functional Complementation.

Complementation of *rps2-201* homozygotes with genomic DNA corresponding to *Arabidopsis thaliana* functionally confirmed that the genomic region encoding cDNA-4 carries RPS2 activity. Cosmids were constructed that contained overlapping contiguous sequences of wild type *Arabidopsis thaliana* DNA from the RPS2 region contained in YACs EW11D4, EW9C3, and YUP11F1 of Fig. 1 and Fig. 4. The cosmid vectors were constructed from pSLJ4541 (obtained from J. Jones, Sainsbury Institute,

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Norwich, England) which contains sequences that allow the inserted sequence to be integrated into the plant genome via *Agrobacterium*-mediated transformation (designated "binary cosmid"). "H" and "E" cosmids (Fig. 1) were used to identify clones carrying DNA from the *Arabidopsis thaliana* genomic RPS2 region.

More than forty binary cosmids containing inserted RPS2 region DNA were used to transform *rps2-201* homozygous mutants utilizing *Agrobacterium*-mediated transformation (Chang et al. ((1990) p. 28, Abstracts of the Fourth International Conference on *Arabidopsis* Research, Vienna, Austria). Transformants which remained susceptible (determined by methods including the observed absence of an HR following infection to *P. syringae* pv. *phaseolicola* strain 3121 carrying *avrRpt2* and Psp 3121 without *avrRpt2*) indicated that the inserted DNA did not contain functional RPS2. These cosmids conferred the "Sus." or susceptible phenotype indicated in Fig. 4. Transformants which had acquired *avrRpt2*-specific disease resistance (determined by methods including the display of a strong hypersensitive response (HR) when inoculated with Psp 3121 with *avrRpt2*, but not following inoculation with Psp 3121 without *avrRpt2*) suggested that the inserted DNA contained a functional RPS2 gene capable of conferring the "Res." or resistant phenotype indicated in Fig. 4. Transformants obtained using the pD4 binary cosmid displayed a strong resistance phenotype as described above. The presence of the insert DNA in the transformants was confirmed by classical genetic analysis (the tight genetic linkage of the disease resistance phenotype and the kanamycin resistance phenotype conferred by the cotransformed selectable marker) and Southern analysis. These results indicated that RPS2 is encoded by a segment of the 18 kb *Arabidopsis thaliana* genomic region carried on cosmid pD4 (Fig. 4).

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To further localize the *RPS2* locus and confirm its ability to confer a resistance phenotype on the *rps2-201* homozygous mutants, a set of six binary cosmids containing partially overlapping genomic DNA inserts were tested. The overlapping inserts pD2, pD4, pD14, pD15, pD27, and pD47 were chosen based on the location of the transcription corresponding to the five cDNA clones in the *RPS2* region (Fig. 4). These transformation experiments utilized a vacuum infiltration procedure (Bechtold et al. (1993) C.R. Acad. Sci. Paris 316:1194-1199) for *Agrobacterium*-mediated transformation. *Agrobacterium*-mediated transformations with cosmids pD2, pD14, pD15, pD39, and pD46 were performed using a root transformation/regeneration protocol (Valveekens et al. (1988), PNAS 85:5536-5540). The results of pathogen inoculation experiments assaying for *RPS2* activity in these transformants is indicated in Fig. 4.

Additional transformation experiments utilized binary cosmids carrying the complete coding region and more than 1 kb of upstream genomic sequence for only cDNA-4 or cDNA-6. Using the vacuum infiltration transformation method, three independent transformants were obtained that carried the wild-type cDNA-6 genomic region in a *rps2-201c* homozygous background (pAD431 of Fig. 4). None of these plants displayed *avrRpt2*-dependent disease resistance. Homozygous *rps2-201c* mutants were transformed with wild-type genomic cDNA-4 (p4104 and p4115, each carrying Col-0 genomic sequences corresponding to all of the cDNA-4 open reading frame, plus approximately 1.7 kb of 5' upstream sequence and approximately 0.3 kb of 3' sequence downstream of the stop codon). These p4104 and p4115 transformants displayed a disease resistance phenotype similar to the wild-type *RPS2* homozygotes from which the *rps2* were derived. Additional mutants (*rps2-101N* and *rps2-101C*

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homozygotes) also displayed *avrRpt2*-dependent resistance when transformed with the cDNA-4 genomic region.

RPS2 Sequences Allow Detection of Other Resistance Genes.

DNA blot analysis of *Arabidopsis thaliana* genomic
5 DNA using RPS2 cDNA as the probe showed that *Arabidopsis* contains several DNA sequences that hybridize to RPS2 or a portion thereof, suggesting that there are several related genes in the *Arabidopsis* genome.

From the aforementioned description and the
10 nucleic acid sequence [SEQ. ID. NO:1] shown in Fig. 2, it is possible to isolate other plant disease resistance genes having about 50% or greater sequence identity to the RPS2 gene. Detection and isolation can be carried out with an oligonucleotide probe containing the RPS2
15 gene or a portion thereof greater than about 18 nucleic acids in length. Probes to sequences encoding specific structural features of the Rps2 polypeptide [SEQ. ID NOS:2-5] are preferred as they provide a means of isolating disease resistance genes having similar
20 structural domains. Hybridization can be done using standard techniques such as are described in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, (1989).

For example, high stringency conditions for
25 detecting the RPS2 gene include hybridization at about 42°C, and about 50% formamide; a first wash at about 65°C, about 2X SSC, and 1% SDS; followed by a second wash at about 65°C and about 0.1% SSC. Lower stringency conditions for detecting RPS genes having about 50%
30 sequence identity to the RPS2 gene are detected by, for example, hybridization at about 42°C in the absence of formamide; a first wash at about 42°C, about 6X SSC, and about 1% SDS; and a second wash at about 50°C, about 6X SSC, and about 1% SDS. An approximately 350 nucleotide

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DNA probe encoding the middle portion of the LRR region of Rps2 was used as a probe in the above example. Under lower stringency conditions, a minimum of 5 DNA bands were detected in BamHI digested *Arabidopsis thaliana* genomic DNA as sequences having sufficient sequence identity to hybridize to DNA encoding the middle portion of the LRR motif of Rps2. Similar results were obtained using a probe containing a 300 nucleotide portion of the RPS2 gene encoding the extreme N-terminus of Rps2 outside of the LRR motif.

Isolation of other disease resistance genes is performed by PCR amplification techniques well known to those skilled in the art of molecular biology using oligonucleotide primers designed to amplify only sequences flanked by the oligonucleotides in genes having sequence identity to RPS2. The primers are optionally designed to allow cloning of the amplified product into a suitable vector.

RPS2 Expression in Transgenic Plant Cells and Plants

The expression of the RPS2 gene in plants susceptible to pathogens carrying avrRpt2 is achieved by introducing into a plant a DNA sequence containing the RPS2 gene for expression of the Rps2 polypeptide. A number of vectors suitable for stable transfection of plant cells or for the establishment of transgenic plants are available to the public; such vectors are described in, e.g., Pouwels et al., *Cloning Vectors: A Laboratory Manual*, 1985, Supp. 1987); Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academic Press, 1989; and Gelvin et al., *Plant Molecular Biology Manual*, Kluwer Academic Publishers, 1990. Typically, plant expression vectors include (1) one or more cloned plant genes under the transcriptional control of 5' and 3' regulatory sequences and (2) a dominant selectable

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marker. Such plant expression vectors may also contain, if desired, a promoter regulatory region (e.g., a regulatory region controlling inducible or constitutive, environmentally- or developmentally-regulated, or cell-
5 or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

An example of a useful plant promoter which could
10 be used to express a plant resistance gene according to the invention is a caulimovirus promoter, e.g., the cauliflower mosaic virus (CaMV) 35S promoter. These promoters confer high levels of expression in most plant tissues, and the activity of these promoters is not
15 dependent on virally encoded proteins. CaMV is a source for both the 35S and 19S promoters. In most tissues of transgenic plants, the CaMV 35S promoter is a strong promoter (see, e.g., Odel et al., Nature 313:810, (1985)). The CaMV promoter is also highly active in
20 monocots (see, e.g., Dekeyser et al., Plant Cell 2:591, (1990); Terada and Shimamoto, Mol. Gen. Genet. 220:389, (1990)).

Other useful plant promoters include, without limitation, the nopaline synthase promoter (An et al.,
25 Plant Physiol. 88:547, (1988)) and the octopine synthase promoter (Fromm et al., Plant Cell 1:977, (1989)).

For certain applications, it may be desirable to produce the RPS2 gene product or the avrRpt2 gene product in an appropriate tissue, at an appropriate level, or at
30 an appropriate developmental time. Thus, there are a variety of gene promoters, each with its own distinct characteristics embodied in its regulatory sequences, shown to be regulated in response to the environment, hormones, and/or developmental cues. These include gene
35 promoters that are responsible for (1) heat-regulated

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gene expression (see, e.g., Callis et al., Plant Physiol. 88: 965, (1988)), (2) light-regulated gene expression (e.g., the pea *rbcS*-3A described by Kuhlemeier et al., Plant Cell 1: 471, (1989); the maize *rbcS* promoter described by Schaffner and Sheen, Plant Cell 3: 997, (1991); or the chlorophyll a/b-binding protein gene found in pea described by Simpson et al., EMBO J. 4: 2723, (1985)), (3) hormone-regulated gene expression (e.g., the abscisic acid responsive sequences from the *Em* gene of wheat described Marcotte et al., Plant Cell 1:969, (1989)), (4) wound-induced gene expression (e.g., of *wun1* described by Siebertz et al., Plant Cell 1: 961, (1989)), or (5) organ-specific gene expression (e.g., of the tuber-specific storage protein gene described by Roshal et al., EMBO J. 6:1155, (1987); the 23-kDa zein gene from maize described by Schernthaner et al., EMBO J. 7: 1249, (1988); or the French bean β -phaseolin gene described by Bustos et al., Plant Cell 1:839, (1989)).

Plant expression vectors may also optionally include RNA processing signals, e.g, introns, which have been shown to be important for efficient RNA synthesis and accumulation (Callis et al., Genes and Dev. 1: 1183, (1987)). The location of the RNA splice sequences can influence the level of transgene expression in plants. In view of this fact, an intron may be positioned upstream or downstream of an *Rps2* polypeptide-encoding sequence in the transgene to modulate levels of gene expression.

In addition to the aforementioned 5' regulatory control sequences, the expression vectors may also include regulatory control regions which are generally present in the 3' regions of plant genes (Thornburg et al., Proc. Natl Acad. Sci USA 84: 744, (1987); An et al., Plant Cell 1: 115, (1989)). For example, the 3' terminator region may be included in the expression

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vector to increase stability of the mRNA. One such terminator region may be derived from the PI-II terminator region of potato. In addition, other commonly used terminators are derived from the octopine or
5 nopaline synthase signals.

The plant expression vector also typically contains a dominant selectable marker gene used to identify the cells that have become transformed. Useful selectable marker genes for plant systems include genes
10 encoding antibiotic resistance genes, for example, those encoding resistance to hygromycin, kanamycin, bleomycin, G418, streptomycin or spectinomycin. Genes required for photosynthesis may also be used as selectable markers in photosynthetic-deficient strains. Finally, genes
15 encoding herbicide resistance may be used as selectable markers; useful herbicide resistance genes include the *bar* gene encoding the enzyme phosphinothricin acetyltransferase, which confers resistance to the broad spectrum herbicide Basta® (Hoechst AG, Frankfurt,
20 Germany).

Efficient use of selectable markers is facilitated by a determination of the susceptibility of a plant cell to a particular selectable agent and a determination of the concentration of this agent which effectively kills
25 most, if not all, of the transformed cells. Some useful concentrations of antibiotics for tobacco transformation include, e.g., 75-100 µg/ml (kanamycin), 20-50 µg/ml (hygromycin), or 5-10 µg/ml (bleomycin). A useful strategy for selection of transformants for herbicide
30 resistance is described, e.g., in Vasil I.K., *Cell Culture and Somatic Cell Genetics of Plants*, Vol I, II, III Laboratory Procedures and Their Applications Academic Press, New York, 1984.

It should be readily apparent to one skilled in
35 the field of plant molecular biology that the level of

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gene expression is dependent not only on the combination of promoters, RNA processing signals and terminator elements, but also on how these elements are used to increase the levels of gene expression.

5 Plant Transformation

Upon construction of the plant expression vector, several standard methods are known for introduction of the recombinant genetic material into the host plant for the generation of a transgenic plant. These methods include (1) *Agrobacterium*-mediated transformation (*A. tumefaciens* or *A. rhizogenes*) (see, e.g., Lichtenstein and Fuller In: *Genetic Engineering*, vol 6, PWJ Rigby, ed, London, Academic Press, 1987; and Lichtenstein, C.P., and Draper, J., In: *DNA Cloning*, Vol II, D.M. Glover, ed, Oxford, IRI Press, 1985), (2) the particle delivery system (see, e.g., Gordon-Kamm et al., *Plant Cell* 2:603, (1990); or BioRad Technical Bulletin 1687, *supra*), (3) microinjection protocols (see, e.g., Green et al., *Plant Tissue and Cell Culture*, Academic Press, New York, 1987), (4) polyethylene glycol (PEG) procedures (see, e.g., Draper et al., *Plant Cell Physiol* 23:451, (1982); or e.g., Zhang and Wu, *Theor. Appl. Genet.* 76:835, (1988)), (5) liposome-mediated DNA uptake (see, e.g., Freeman et al., *Plant Cell Physiol* 25: 1353, (1984)), (6) electroporation protocols (see, e.g., Gelvin et al *supra*; Dekeyser et al. *supra*; or Fromm et al *Nature* 319: 791, (1986)), and (7) the vortexing method (see, e.g., Kindle, K., *Proc. Natl. Acad. Sci., USA* 87:1228, (1990)).

The following is an example outlining an *Agrobacterium*-mediated plant transformation. The general process for manipulating genes to be transferred into the genome of plant cells is carried out in two phases. First, all the cloning and DNA modification steps are done in *E. coli*, and the plasmid containing the gene

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construct of interest is transferred by conjugation into *Agrobacterium*. Second, the resulting *Agrobacterium* strain is used to transform plant cells. Thus, for the generalized plant expression vector, the plasmid contains
5 an origin of replication that allows it to replicate in *Agrobacterium* and a high copy number origin of replication functional in *E. coli*. This permits facile production and testing of transgenes in *E. coli* prior to transfer to *Agrobacterium* for subsequent introduction
10 into plants. Resistance genes can be carried on the vector, one for selection in bacteria, e.g., streptomycin, and the other that will express in plants, e.g., a gene encoding for kanamycin resistance or an herbicide resistance gene. Also present are restriction
15 endonuclease sites for the addition of one or more transgenes operably linked to appropriate regulatory sequences and directional T-DNA border sequences which, when recognized by the transfer functions of *Agrobacterium*, delimit the region that will be
20 transferred to the plant.

In another example, plant cells may be transformed by shooting into the cell tungsten microprojectiles on which cloned DNA is precipitated. In the Biolistic Apparatus (Bio-Rad, Hercules, CA) used for the shooting,
25 a gunpowder charge (22 caliber Power Piston Tool Charge) or an air-driven blast drives a plastic macroprojectile through a gun barrel. An aliquot of a suspension of tungsten particles on which DNA has been precipitated is placed on the front of the plastic macroprojectile. The
30 latter is fired at an acrylic stopping plate that has a hole through it that is too small for the macroprojectile to go through. As a result, the plastic macroprojectile smashes against the stopping plate and the tungsten microprojectiles continue toward their target through the
35 hole in the plate. For the instant invention the target

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can be any plant cell, tissue, seed, or embryo. The DNA introduced into the cell on the microprojectiles becomes integrated into either the nucleus or the chloroplast.

Transfer and expression of transgenes in plant
5 cells is now routine practice to those skilled in the art. It has become a major tool to carry out gene expression studies and to attempt to obtain improved plant varieties of agricultural or commercial interest.

Transgenic Plant Regeneration

10 Plant cells transformed with a plant expression vector can be regenerated, e.g., from single cells, callus tissue or leaf discs according to standard plant tissue culture techniques. It is well known in the art that various cells, tissues and organs from almost any
15 plant can be successfully cultured to regenerate an entire plant; such techniques are described, e.g., in Vasil supra; Green et al., supra; Weissbach and Weissbach, supra; and Gelvin et al., supra.

In one possible example, a vector carrying a
20 selectable marker gene (e.g., kanamycin resistance), a cloned RPS2 gene under the control of its own promoter and terminator or, if desired, under the control of exogenous regulatory sequences such as the 35S CaMV promoter and the nopaline synthase terminator is
25 transformed into *Agrobacterium*. Transformation of leaf tissue with vector-containing *Agrobacterium* is carried out as described by Horsch et al. (Science 227: 1229, (1985)). Putative transformants are selected after a few weeks (e.g., 3 to 5 weeks) on plant tissue culture media
30 containing kanamycin (e.g. 100 µg/ml). Kanamycin-resistant shoots are then placed on plant tissue culture media without hormones for root initiation. Kanamycin-resistant plants are then selected for greenhouse growth. If desired, seeds from self-fertilized transgenic plants

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can then be sowed in a soil-less media and grown in a greenhouse. Kanamycin-resistant progeny are selected by sowing surfaced sterilized seeds on hormone-free kanamycin-containing media. Analysis for the integration
5 of the transgene is accomplished by standard techniques (see, e.g., Ausubel et al. supra; Gelvin et al. supra).

Transgenic plants expressing the selectable marker are then screened for transmission of the transgene DNA by standard immunoblot and DNA and RNA detection
10 techniques. Each positive transgenic plant and its transgenic progeny are unique in comparison to other transgenic plants established with the same transgene. Integration of the transgene DNA into the plant genomic DNA is in most cases random and the site of integration
15 can profoundly effect the levels, and the tissue and developmental patterns of transgene expression. Consequently, a number of transgenic lines are usually screened for each transgene to identify and select plants with the most appropriate expression profiles.

20 Transgenic lines are evaluated for levels of transgene expression. Expression at the RNA level is determined initially to identify and quantitate expression-positive plants. Standard techniques for RNA analysis are employed and include PCR amplification
25 assays using oligonucleotide primers designed to amplify only transgene RNA templates and solution hybridization assays using transgene-specific probes (see, e.g., Ausubel et al., supra). The RNA-positive plants are then analyzed for protein expression by Western immunoblot
30 analysis using Rps2 polypeptide-specific antibodies (see, e.g., Ausubel et al., supra). In addition, *in situ* hybridization and immunocytochemistry according to standard protocols can be done using transgene-specific nucleotide probes and antibodies, respectively, to
35 localize sites of expression within transgenic tissue.

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Once the Rps2 polypeptide has been expressed in any cell or in a transgenic plant (e.g., as described above), it can be isolated using any standard technique, e.g., affinity chromatography. In one example, an anti-
5 Rps2 antibody (e.g., produced as described in Ausubel et al., supra, or by any standard technique) may be attached to a column and used to isolate the polypeptide. Lysis and fractionation of Rps2-producing cells prior to affinity chromatography may be performed by standard
10 methods (see, e.g., Ausubel et al., supra). Once isolated, the recombinant polypeptide can, if desired, be further purified, e.g., by high performance liquid chromatography (see, e.g., Fisher, *Laboratory Techniques In Biochemistry And Molecular Biology*, Work and Burdon,
15 eds., Elsevier, 1980).

These general techniques of polypeptide expression and purification can also be used to produce and isolate useful Rps2 fragments or analogs.

Use

20 Introduction of RPS2 into a transformed plant cell provides for resistance to bacterial pathogens carrying the avrRpt2 avirulence gene. For example, transgenic plants of the instant invention expressing RPS2 might be used to alter, simply and inexpensively, the disease
25 resistance of plants normally susceptible to plant pathogens carrying the avirulence gene, avrRpt2.

The invention also provides for broad-spectrum pathogen resistance by mimicking the natural mechanism of host resistance. First, the RPS2 transgene is expressed
30 in plant cells at a sufficiently high level to initiate the plant defense response constitutively in the absence of signals from the pathogen. The level of expression associated with plant defense response initiation is determined by measuring the levels of defense response

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gene expression as described in Dong et al., supra.
Second, the *RPS2* transgene is expressed by a controllable promoter such as a tissue-specific promoter, cell-type specific promoter or by a promoter that is induced by an
5 external signal or agent thus limiting the temporal and tissue expression of a defense response. Finally, the *RPS2* gene product is co-expressed with the *avrRpt2* gene product. The *RPS2* gene is expressed by its natural promoter, by a constitutively expressed promoter such as
10 the CaMV 35S promoter, by a tissue-specific or cell-type specific promoter, or by a promoter that is activated by an external signal or agent. Co-expression of *RPS2* and *avrRpt2* will mimic the production of gene products associated with the initiation of the plant defense
15 response and provide resistance to pathogens in the absence of specific resistance gene-avirulence gene corresponding pairs in the host plant and pathogen.

The invention also provides for expression in plant cells of a nucleic acid having the sequence [SEQ.
20 ID. NO:1] of Fig. 2 or the expression of a degenerate variant thereof encoding the amino acid sequence [SEQ. ID NOS:2-5] of open reading frame "a" of Fig. 2.

The invention further provides for the isolation of nucleic acid sequences having about 50% or greater
25 sequence identity to *RPS2* by using the *RPS2* sequence [SEQ. ID. NO:1] of Fig. 2 or a portion thereof greater than about 18 nucleic acids in length as a probe. Appropriate reduced hybridization stringency conditions are utilized to isolate DNA sequences having about 50% or
30 greater sequence identity to the *RPS2* sequence [SEQ. ID. NO: 1] of Fig. 2.

The invention will provide disease resistance to plants, especially crop plants, most especially important crop plants such as tomato, pepper, maize, wheat, rice
35 and legumes such as soybean and bean, or any plant which

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is susceptible to pathogens carrying an avirulence gene, e.g., the *avrRpt2* avirulence gene. Such pathogens include, but are not limited to, *Pseudomonas syringae* strains.

5 The invention also includes any biologically active fragment or analog of an Rps2 polypeptide. By "biologically active" is meant possessing any in vivo activity which is characteristic of the Rps2 polypeptide [SEQ. ID NOS:2-5] shown in Fig. 2. A useful Rps2
10 fragment or Rps2 analog is one which exhibits a biological activity in any biological assay for disease resistance gene product activity, for example, those assays described by Dong et al. (1991), supra; Yu et al. (1993) supra; and Kunkel et al. (1993) supra; and Whalen
15 et al. (1991). In particular, a biologically active Rps2 polypeptide fragment or analog is capable of providing substantial resistance to plant pathogens carrying the *avrRpt2* avirulence gene. By substantial resistance is meant at least partial reduction in susceptibility to
20 plant pathogens carrying the *avrRpt2* gene.

Preferred analogs include Rps2 polypeptides (or biologically active fragments thereof) whose sequences differ from the wild-type sequence only by conservative amino acid substitutions, for example, substitution of
25 one amino acid for another with similar characteristics (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-conservative amino acid substitutions, deletions, or insertions which do not abolish the polypeptide's biological activity.

30 Analogous can differ from naturally occurring Rps2 polypeptide in amino acid sequence or can be modified in ways that do not involve sequence, or both. Analogous of the invention will generally exhibit at least 70%, preferably 80%, more preferably 90%, and most preferably
35 95% or even 99%, homology with a segment of 20 amino acid

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residues, preferably 40 amino acid residues, or more preferably the entire sequence of a naturally occurring Rps2 polypeptide sequence [SEQ. ID NOS:2-5].

Alterations in primary sequence include genetic
5 variants, both natural and induced. Also included are analogs that include residues other than naturally occurring L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g., β or γ amino acids. Also included in the invention are Rps2
10 polypeptides modified by *in vivo* chemical derivatization of polypeptides, including acetylation, methylation, phosphorylation, carboxylation, or glycosylation.

In addition to substantially full-length polypeptides, the invention also includes biologically
15 active fragments of the polypeptides. As used herein, the term "fragment", as applied to a polypeptide, will ordinarily be at least 20 residues, more typically at least 40 residues, and preferably at least 60 residues in length. Fragments of Rps2 polypeptide can be generated
20 by methods known to those skilled in the art. The ability of a candidate fragment to exhibit a biological activity of Rps2 can be assessed by those methods described herein. Also included in the invention are Rps2 polypeptides containing residues that are not
25 required for biological activity of the peptide, e.g., those added by alternative mRNA splicing or alternative protein processing events.

Other embodiments are within the following claims.

DEMANDES OU BREVETS VOLUMINEUX

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CECI EST LE TOME __1__ DE __2__

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JUMBO APPLICATIONS / PATENTS

**THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE
THAN ONE VOLUME.**

THIS IS VOLUME __1__ OF __2__

NOTE: For additional volumes please contact the Canadian Patent Office.

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CLAIMS

1. A substantially pure DNA encoding a plant polypeptide comprising a P-loop and a leucine rich repeat (LRR) domain, said polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen.
2. The DNA of claim 1, wherein said DNA comprises the DNA of SEQ. ID. NO:1 of Fig. 2.
3. The DNA of claim 1, wherein said DNA is genomic DNA.
4. The DNA of claim 1, wherein said DNA is cDNA.
5. The DNA of claim 1, wherein said DNA is from a plant of the genus *Arabidopsis*.
6. Substantially pure DNA having the sequence of SEQ ID NO:1 as shown in Fig. 2, or degenerate variants thereof, and encoding the amino acid sequence of SEQ ID NOs: 2-5 of open reading frame "a" of Fig. 2, wherein said DNA encodes a polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen.
7. Substantially pure DNA having at least 50% or greater sequence identity to the DNA sequence of SEQ ID NO:1 as shown in Fig. 2, wherein said DNA encodes a polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen.

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8. The DNA of any one of claims 1 to 7, wherein said DNA is operably linked to expression regulatory sequences and wherein said regulatory sequences comprise a promoter.

9. The DNA of claim 8, wherein said promoter is a constitutive promoter.

10. The DNA of claim 8, wherein said promoter is inducible by one or more external agents.

11. The DNA of claim 8, wherein said promoter is cell-type specific.

12. A cell transformed with the DNA of any one of claims 1 to 11.

13. The cell of claim 12, said cell being a plant cell.

14. The plant cell of claim 13, said plant cell being resistant to disease caused by a plant pathogen carrying an avirulence gene generating a signal recognized by a polypeptide comprising a P-loop and a leucine rich repeat (LRR) domain.

15. The plant cell of claim 14, said plant pathogen carrying a gene comprising SEQ ID NO: 105.

16. The plant cell of claim 13, wherein said plant cell further includes a gene comprising SEQ ID NO: 105 operably linked to regulatory sequences; and wherein said regulatory sequences comprise a promoter.

17. The plant cell of claim 16, wherein said promoter is a constitutive promoter.

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18. The plant cell of claim 16, wherein said promoter is inducible by one or more external agents.

19. The plant cell of claim 16, wherein said promoter is cell-type specific.

20. The plant cell of any one of claims 13 to 19, said plant cell being from the group of plants comprising *Arabidopsis*, tomato, soybean, bean, maize, wheat, and rice.

21. The plant cell of any one of claims 13 to 19, said plant pathogen being *Pseudomonas syringae*.

22. A transgenic plant cell transformed with the DNA of any one of claims 1 to 11, integrated into the genome of said plant, wherein said DNA is expressed in said transgenic plant cell.

23. A transgenic plant cell generated from the plant cell of any one of claims 15 to 20, wherein said DNA and said gene comprising SEQ ID NO: 105 are expressed in said transgenic plant cell.

24. The transgenic plant cell of any one of claims 13 to 23, which is a seed cell.

25. A method of providing resistance to a plant pathogen in a plant, said method comprising: providing a plant cell transformed with the DNA of any one of claims 1 to 11 integrated into the genome of said plant cell and positioned for expression in said plant cell; and growing a

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transgenic plant from said plant cell wherein said DNA is expressed in said transgenic plant.

26. A method of detecting a resistance gene in a plant cell, said method comprising: contacting the DNA of any one of claims 1 to 7 or the complement thereof, or a portion thereof greater than about 18 nucleic acids in length with a preparation of genomic DNA from said plant cell under hybridization conditions providing detection of DNA sequences having at least 50% or greater sequence identity to the DNA sequence of SEQ ID NO:1 as shown in Fig. 2.

27. A method of producing a plant disease resistance polypeptide comprising: providing a cell transformed with the DNA of any one of claims 1 to 11, positioned for expression in said cell; culturing said transformed cell under conditions for expressing said DNA; and isolating a plant disease resistance polypeptide comprising a P-loop and a leucine rich repeat (LRR) domain encoded by said DNA.

28. A method of providing, in a transgenic plant, resistance to a plant pathogen, said method comprising: growing said transgenic plant from the plant cell of any one of claims 15 to 20, wherein said DNA and said gene comprising SEQ ID NO: 105 are expressed in said transgenic plant.

29. A method of isolating a disease resistance gene or portion thereof from a plant, said method comprising:

(a) amplifying by PCR said disease resistance gene or portion thereof using oligonucleotide primers wherein said primers

(i) are each greater than 13 nucleotides in length;

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(ii) each have regions of complementarity to opposite DNA strands in a region of the nucleotide sequence of SEQ ID NO:1 of Fig. 2; and

(iii) optionally contain sequences capable of producing restriction enzyme cut sites in the amplified product; and

(b) isolating said disease resistance gene or portion thereof.

30. A substantially pure polypeptide comprising a P-loop and a leucine rich repeat (LRR) domain, said polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen.

31. The polypeptide of claim 30, comprising an amino acid sequence having at least 50% sequence identity to an amino acid sequence of SEQ ID NOs: 2-5 as shown in Fig. 2.

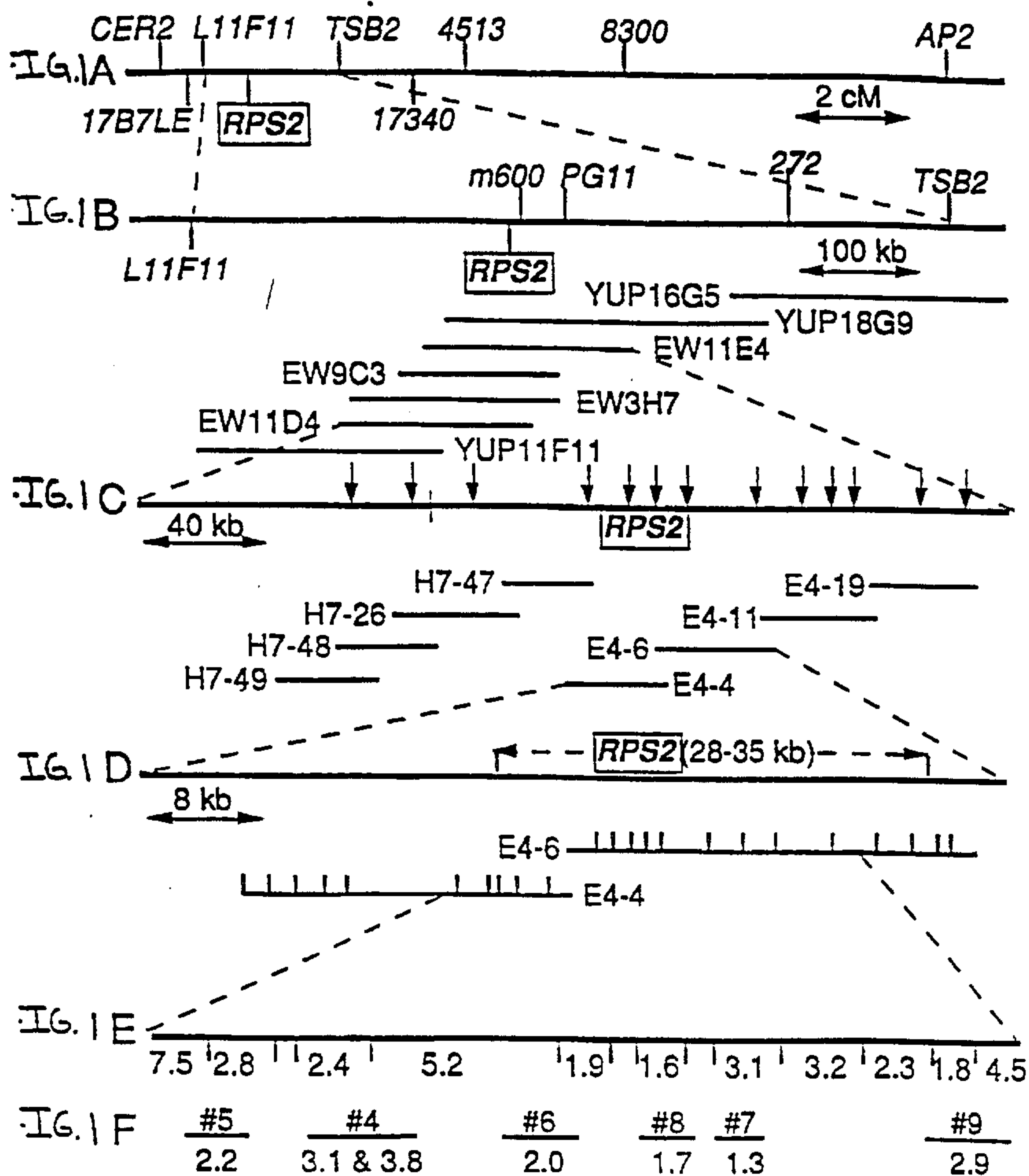
32. A vector comprising the DNA of any one of claims 1 to 11, said vector being capable of directing expression of the peptide encoded by said DNA in a vector-containing cell.

33. A vector comprising the DNA of any one of claims 1 to 7, and the DNA of SEQ ID NO:105, operably linked to regulatory sequences wherein said regulatory sequences comprise a promoter.

34. Substantially pure DNA having at least 85% or greater sequence identity to a DNA sequence of Fig. 2, wherein said DNA encodes a polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen.

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AAGTAAAGAAAGAGCGAGAAATCATCGAAATGGATTTCATCTCATCTCTTATCGTTGGC
1 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 60
TTCATTTTCTTTCTCGCTCTTTAGTAGCTTTACCTAAAGTAGAGTAGAGAATAGCAACCG

a K * K K E R E I I E (M) D F I S S L I V C -
b S K R K S E K S S K W I S S H L L S L A -
c V K E R A R N H R N G F H L I S Y R W L -

TGTGCTCAGGTGTTGTGTCGAATCTATGAATATGGCGGAGAGAAGAGGACATAAGACTGAT
61 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 120
ACACGAGTCCACAACACACTTAGATACTTATACCGCCTCTCTTCTCTCTGTATTCTGACTA

a C A Q V L C E S M N H A E R R G H K T D -
b V L R C C V N L * I W R R E E D I R L I -
c C S G V V * I Y E Y G G E K R T * D * S -

CTTAGACAAGCCATCACTGATCTTGAACAGCCATCGGTGACTTGAAGCCATACGTGAT
121 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 180
GAATCTGTTCCGTAGTGACTAGAACTTTGTCGGTAOCCACTGAACCTTCCGGTATGCCACTA

a L R Q A I T D L E T A I G D L R A I R D -
b L D K P S L I L K Q P S V T * R P Y V M -
c * T S H H * S * N S H R * L E G H T * * -

GACCTGACTTTACGGATCCAACAAGACGGTCTAGAGGGACGAAGCTGCTCAAATCGTGGC
181 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 240
CTGGACTGAAATGCCTAGGTTGTTCTGCCAGATCTCCCTGCTTCCGACGAGTTTAGCACCG

a D L T L R I Q Q D G L E G R S C S N R A -
b T * L Y G S N K T V * R D E A A Q I V P -
c P D F T D P T R R S R G T K L L K S C Q -

AGAGACTGGCTTAGTGCGGTGCAAGTAACGGAGACTAAAACAGCCCTACTTTTAGTGAGG
241 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 300
TCTCTCACCGAATCACGCCACGTTTATTGCTCTGATTTTGTCCGGAGGAAAATCACTCC

a R E W L S A V Q V T E T K T A L L L V R -
b E S G L V R C K * R R L K Q P Y F * * G -
c R V A * C G A S N G D * N S P T F S E V -

TTTAGGCGTCCGCAACAGAGGACGCGAATGAGGAGATACCTCAGTTGTTTCCGTTGT
301 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 360
AAATCCCGCAGCCCTTGTCTCCTGCGCTTACTCCTCCTCTATGGAGTCAACAAAGCCAAAC

a F R R R E Q R T R M R R R Y L S C F G C -
b L G V G N R G R E * G G D T S V V S V V -
c * A S G T E D A N E E E I P Q L F R L C -

GCCGACTACAAACTGTGCAAGAAGGTTTCTGCCATATTGAAGAGCATTCGTGAGCTGAGL

FIGURE 2

[illegible]

FIG. 2 CONTINUED

841 GAGAAACTGGAGTTCCTCGACCTGACAGGGAAAACAAATGCAAGGTGATGTTCCAGACA 900
CTCTTTTGACCTCAAGGAGCTGGACTGTCCCTTTTGTTTACGTTCCACTACAAGTGCTGT
a E K T G V P R P D R E N K C K V M F T T -
b R K L E F L D L T G K T N A R * C S R H -
c E N W S S S T * Q G K Q M Q G D V H D T -
901 CCGTCTATACCATTTATGCAACAATATGGGTGCGGAATACAAGTTGAGACTGGAGTTTCTC 960
GCCAGATATCGTAATACGTTGTTATACCCACCGCTTATGTTCAACTCTCACCTCAAAGAC
a R S I A L C N N M G A E Y K L R V E F L -
b G L * H Y A T I W V R N T S * E W S F W -
c V Y S I M Q Q Y G C G I Q V E S G V S G -
961 GAGAAGAAACACGCGTGGGAGCTGTTCTGTAGTAAGGTATGGAGAAAAGATCTTTTAGAG 1020
CTCTTCTTTGTGCGCACCCCTCGACAAGACATCATTCCATACCTCTTTTCTAGAAAATCTC
a E K K H A W E L F C S K V W R K D L L E -
b R R N T R G S C S V V R Y G E K I F * C -
c E E T R V G A V L * * G M E K R S F R V -
1021 TCATCATCAATTCCCGGCTCCCGGAGATTATAGTGAGTAAATGTGGAGGATTGCCACTA 1080
AGTAGTAGTTAAGCGGCGGAGCGCCTCTAATATCACTCATTACACCTCCTAACGGTGAT
a S S S I R R L A E I I V S K C G G L P L -
b H H Q F A G S R R L * * V N V E D C H * -
c I I N S P A R G D Y S E * M W R I A T S -
1081 CCGTTGATCACTTTAGGAGGAGCCATCGCTCATAGAGAGACAGAAGAAGAGTGGATCCAT 1140
CGCAACTAGTGAAATCCTCCTCGGTACCGAGTATCTCTCTGTCTTCTTCTCACCTAGGTA
a A L I T L G G A M A H R E T E E E W I H -
b R * S L * E E P W L I E R Q K K S G S M -
c V D H F R R S H G S * R D R R R V D P C -
1141 GCTAGTGAAGTTCTGACTAGATTTCCAGCAGAGATGAAGGGTATGAAGTATGTATTTGCC 1200
CGATCACTTCAAGACTGATCTAAAGGTCTGTCTCTACTTCCCATACTTGATACATAAAGCG
a A S E V L T R F P A E M K G M N Y V F A -
b L V K F * L D F Q Q R * R V * T M Y L P -
c * * S S D * I S S R D E G Y E L C I C P -
1201 CTTTGTAAATTCAGCTACGACAACCTCGAGAGTGATCTGCTTCCGTCTTGTCTTGTAC 1260
GAAACTTTAAGTGGATGCTGTTGGAGCTCTCACTAGACGAAGCCAGAACAAAGACATG
a L L K F S Y D N L E S D L L R S C F L Y -
b F * N S A T T T S R V I C F G L V S C T -
c F E I Q L R Q P R E * S A S V L F L V L -
1261 TCCGCTTTATTCCCAGAAGAACATTCTATAGAGATCCAGCAGCTTGTGTGACTACTGGGTC 1320
ACGCGAAATAAGGGTCTTCTTGTAAAGATATCTCTAGCTCGTCAACAACTCATGACCCAG
a C A L F P E E H S I E I E Q L V E Y W V -

FIG. 2 CONTINUED

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b A L Y S Q K N I L * R S S S L L S T G S -
c R F I P R R T F Y R D R A A C * V L G R -

GGCGAAGGGTTTCTCACCAGCTCCCATGGCGTTAACACCATTTACAAGGGATATTTTCTC
1321 -----+-----+-----+-----+-----+ 1380
CCGCTTCCCAAGAGTGCTCGAGGGTACCGCAATTGTGGTAAATGTTCCCTATAAAAGAG

a G E G F L T S S H G V N T I Y K G Y F L -
b A K G F S P A P M A L T P F T R D I F S -
c R R V S H Q L P W R * H H L Q G I F S H -

ATTGGGGATCTGAAAGCGGCATGTTTGTGGAAACCGGAGATGAGAAAACACAGGTGAAG
1381 -----+-----+-----+-----+-----+ 1440
TAACCCCTAGACTTTCCCGGTACAAACAACCTTTGGCCTCTACTCTTTTGTGTCCACTTC

a I G D L X A A C L L E T G D E K T Q V K -
b L G I * K R H V C W K P E M R K H R * R -
c W G S E S G M F V G N R R * E N T G E D -

ATGCATAATGTGGTCAGAAGCTTTGCATTGTGGATGGCATCTGAACAGGGGACTTATAAG
1441 -----+-----+-----+-----+-----+ 1500
TACGTATTACAGCCTCTCTCCGTAACACCTACCGTAGACTTGTCCCTGAATATTC

a M H N V V R S F A L W M A S E Q G T Y K -
b C I M W S E A L H C G W H L N R G L I R -
c A * C G Q K L C I V D G I * T G D L * G -

GAGCTGATCCTAGTTGAGCCTAGCATGGGACATACTGAAGCTCCTAAAGCAGAAAACCTGG
1501 -----+-----+-----+-----+-----+ 1560
CTCGACTAGCATCAACTCGGATCGTACCCTGTATGACTTCGAGGATTTCGTCTTTTGACC

a E L I L V E P S M G H T E A P K A E N W -
b S * S * L S L A W D I L K L L K Q K T C -
c A D P S * A * H G T Y * S S * S R K L A -

CGACAAGCGTTGGTGATCTCATTTAGATAACAGAATCCAGACCTTGCCTGAAAAACTC
1561 -----+-----+-----+-----+-----+ 1620
GCTGTTCCGAACCACTAGAGTAACAATCTATTGTCTTAGGTCTGGAACGGACTTTTGTAG

a R Q A L V I S L L D N R I Q T L P E K L -
b D K R W * S H C * I T E S R P C L K N S -
c T S V G D L I V R * Q N P D L A * K T H -

ATATCCCGAAACTGACAACACTGATGCTCCAACAGAACAGCTCTTTGAAGAAGATTCCA
1621 -----+-----+-----+-----+-----+ 1680
TATACGGGCTTTGACTGTTCTGACTACGAGGTTGTCTTGTCCGAGAAACTTCTTCTAAGGT

a I C P K L T T L M L Q Q N S S L K K I P -
b Y A R N * Q H * C S N R T A L * R R F Q -
c H P E T D N T D A P T E Q L F E E D S N -

ACAGGGTTTTTCATGCATATGCCTGTTCTCAGAGTCTTGGACTTGTCTGTTTCAAGTATC
1681 -----+-----+-----+-----+-----+ 1740
TGTCCCAAAAAGTACGTATACGGACAAGAGTCTCAGAACCTGAACAGCAAGTGTTCATAG

a T G F F M H M P V L R V L D L S F T S I -
b Q G F S C I C L F S E S W T C R S Q V S -
c R V F H A Y A C S Q S L G L V V H K Y H -

ACTGAGATTCCGTTGTCTATCAAGTATTTGGTGGAGTTGTATCATCTGCTATGTCAGGA
1741 -----+-----+-----+-----+-----+ 1800
TCACTCTAAGGCAACAGATAGTTTATAAACACCTCAACATAGTAGACAGATACAGTCCT

FIG. 2 CONTINUED

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FIG. 2 CONTINUED

2221 -----+-----+-----+-----+-----+-----+ 2280
CTCATGGACCAGTGTGGGCGTCTAAAACCTTTTACTAACCAGGCTCAGATCTCCAAGAC
a E Y L V T P A D F E N D W L P S L E V L -
b S T W S H P Q I L K M I G F R V * R P * -
c V P G H T R R F * K * L A S E S R G S D -
ACGTTACACAGCCTTCACAACCTTAACCAGAGTGTGGGGAAATTCTGTAAGCCAAGATTGT
2281 -----+-----+-----+-----+-----+ 2340
TGCAATGTGTGGGAAGTGTGAATTGGTCTCACACCCCTTTAAGACATTCGGTTCTAACA
a T L H S L H N L T R V W G N S V S Q D C -
b R Y T A F T T * P E C G E I L * A K I V -
c V T Q P S Q L N Q S V G K F C K P R L S -
CTGCGGAATATCCGTTGCATAAACATTTACACTGCAACAAGCTGAAGAATGTCTCATGG
2341 -----+-----+-----+-----+-----+ 2400
GACGCCTTATAAGCAACGTATTTGTAAAGTGTGACGTTGTTGACTTCTTACAGAGTACC
a L R N I R C I N I S H C N K L K N V S W -
b C G I S V A * T F H T A T S * R M S H G -
c A E Y P L H K H F T L Q Q A E E C L M G -
GTTCAGAACTCCCAAAGCTAGAGGTGATTGAAGTGTTCGACTGCAGAGAGATAGAGGAA
2401 -----+-----+-----+-----+-----+ 2460
CAAGTCTTTGAGGGTTTCGATCTCCACTAAGTGAACAAGCTGACCTCTCTCTATCTCCTT
a V Q K L P K L E V I E L F D C R E I E E -
b F R N S Q S * R * L N C S T A E R * R N -
c S E T P K A R G D * T V R L Q R D R G I -
TTGATAAGCGAACACGAGAGTCCATCCGTGGAAGATCCAACATTGTTCCCAAGCCTGAAG
2461 -----+-----+-----+-----+-----+ 2520
AACTATTGCTTGTGCTCTCAGGTAGGCAGCTTCTAGGTTGTAACAAGGGTTCCGACTTC
a L I S E H E S P S V E D P T L F P S L K -
b * * A N T R V H P S K I Q H C S Q A * R -
c D K R T R E S I R R R S N I V P K P E D -
ACCTTGAGAACTAGCGATCTGCCAGAACTAAACAGCATCCTCCCATCTCGATTTCATTC
2521 -----+-----+-----+-----+-----+ 2580
TGGAAGTCTTGATCCCTAGACGGTCTTGATTGTGCTAGGAGGCTAGAGCTAAAAGTAAG
a T L R T R D L P E L N S I L P S R F S F -
b P * E L G I C Q N * T A S S H L D F H S -
c L E N * G S A R T K Q H P P I S I F I P -
CAAAAAGTTGAAACATTAGTCATCACAATTTGCCCCAGAGTTAAGAACTGCCGTTTCAG
2581 -----+-----+-----+-----+-----+ 2640
GTTTTTCAACTTTGTAATCAGTAGTGTTTAACGGGCTCTCAATTCTTTGACGCCAAAGTC
a Q K V E T L V I T N C P R V K K L P F Q -
b K K L K H * S S Q I A P E L R N C R F R -
c K S * N I S H H K L P Q S * E T A V S G -
GAGAGGAGGACCCAGATGAACTTGCCAACAGTTTATTGTGAGGAGAAATGGTGGAAGCA
2641 -----+-----+-----+-----+-----+ 2700
CTCTCCTCCTGGGTCTACTTGAACGGTGTCAATAACACTCCTCTTTACCACCTTTTCGT
a E R R T Q M N L P T V Y C E E K W W K A -
b R G G P R * T C Q Q F I V R R N G C K H -
c E E D P D E L A N S L L * C E M V E S T -

FIG. 2 CONTINUED

2701 CTGCAAAAGATCAACCAAAAGAGAGCTTTTATTATTACCGCGCTTTTCTCCAAATTCA
-----+----- 2760
GACCTTTTCTAGTTGCTTTCTCTCCAAACATTAATGCGCGCAAGAGCTTTAACT
a L E K D Q P N E E L C Y L P R F V P N * -
b W K K I N Q T K S F V I Y R A L P Q I D -
c G X K S T K R R A L L F T A L C S K L I -
2761 TATAAGAGCTAAGAGCACTCTGTACAAATATGTCCATTCAATAGTAGCAGCAAGCCAGGA
-----+----- 2820
ATATTCTCGATTCTCTGTAGAGCATGTTTATACAGGTAAATATTCAATGCTCTCTCGCTCTCT
a Y K S * E H S V Q I C P F I S S R K X P G -
b I R A K S T L Y K Y V H S * V A G S Q E -
c * E L R A L C T N M S I H X * Q E A R K -
2821 AAGTTTCTCCAGTGAAGTCACTCACTTTCCACATAGCCACAAAGCTAGAGATTATGTAAT
-----+----- 2880
TCCAACAGGCTCACTTCACTAGTTGAAGGTGTATCGGTGTTTGAATCTCTAATACATTA
a R L F Q * E K Q L S T * P Q M * R L C M -
b G C S S E V I N P P H S H K T R D Y V I -
c V V P V K S S T F H I A T K L E I M * S -
2881 CATAAAAAGCAAGCTATCCGCGA (SEQ ID NO:1)
-----+----- 2903
GTATTTTCTGTTTGAATAGCGCT
a K K N Q T I R - (SEQ ID NO: 2-5)
b I K T K L S A - (SEQ ID NO: 6-59)
c * K P N Y P R - (SEQ ID NO: 60-104)

Enzymes that do cut:

NONE

Enzymes that do not cut:

XpnI

...TCATCTCTGGCTCAGTGGGAGTAGTCCATTTCAGAGCAACCTAGCCCCGCGTC -86
GGCATCATGGAGCTATTTGGAATTTTCGCAGGGTTATCCATTGCTAGTGGGAACCCATT -26
CATTGTTTGGAAACCACCAACGGACGACTTAACAAGCTCCCCGAGGTCCATGATGAAAATT 15
MetLysIle
GCTCCAGTTGCCATAAATCAGCCCCGCTCAGCAGGGAGGTCCCGTCACACGGCCGACCC 95
AlaProValAlaIleAsnHisSerProLeuSerArgGluValProSerHisAlaAlaPro
ACTCAGCCAAAGCAAACCAACCTTCAATCTCAAGCTGGCGATTAGATGCAAGAAAAAGT 155
ThrGlnAlaLysGlnThrAsnLeuGlnSerGluAlaGlyAspLeuAspAlaArgLysSer
AGCGCTTCAAGCCCCGAAACCCGCGATTACTCGCTACTAAGACAGTACTCCGGAGACAC 215
SerAlaSerSerProGluThrArgAlaLeuLeuAlaThrLysThrValLeuGlyArgHis
AAGATAGAGGTTCCGGCCTTTGGAGGGTGGTTCAAAAAGAAATCATCTAAGCAGGAGACG 275
LysIleGluValProAlaPheGlyGlyTrpPheLysLysLysSerSerLysHisGluThr
GGCGGTTCAAGTGCCAAACGCAGATAGTTCGAGCGTGGCTTCCGATTCCACCGAAAAACCT 335
GlyGlySerSerAlaAsnAlaAspSerSerSerValAlaSerAspSerThrGluLysPro
TTGTTCCGTCTCAGGCACGTTCTTACGTATCCCAAGGTAATGACCGAATGGGATGTTGG 395
LeuPheArgLeuThrHisValProTyrValSerGlnGlyAsnGluArgMetGlyCysTrp
TATGCTGCGCAAGAATGGTTGGCCATTCTGTCGAAGCTGGCCCTGGCCTAGGGCTGCCG 455
TyrAlaCysAlaArgMetValGlyHisSerValGluAlaGlyProArgLeuGlyLeuPro
GAGCTCTATGAGGGAAGGGAGCGCCAGCTGGGCTACAAGATTTTTCAGATGTAGAAAGG 515
GluLeuTyrGluGlyArgGluAlaProAlaGlyLeuGlnAspPheSerAspValGluArg
TTTATTACAATGAAGGATTAAGTCCGGTAGACCTTCCAGACAATGAGAGATTACACAC 575
PheIleHisAsnGluGlyLeuThrArgValAspLeuProAspAsnGluArgPheThrHis

Fig. 3

GAAGAGTTGGTGCACATGTTGTATAAGCACGGGCGGATTATATTTGGGTGGAAAACCTCG 615
GluGluLeuGlyAlaLeuLeuTyrLysHisGlyProIleIlePheGlyTyrLysThrPro

AATGACAGCTGGCACATGTCGGTCTCTACTGGTGTGGATAAAGAGACCTGGTCCATTACT 695
AsnAspSerTyrHisMetSerValLeuThrGlyValAspLysGluThrSerSerIleThr

TTTCACGATCCCCGACAGGGGGCGACCTAGCAATGCCGCTCGATTACTTTAATCAGCGA 755
PheHisAspProArgGlnGlyProAspLeuAlaMetProLeuAspTyrPheAsnGlnArg

TTCGATGGCAGGTTCCACACGCAATGCTCTACCGCTAAGTACGAGGTATCTTCACGTG 815
LeuAlaTyrGlnValProHisAlaMetLeuTyrArgEnd (SEQ ID NO: 106)

CGGGCATCATGACAAGCCCATGATGCCGCCAGCAGCTACCTCAATGCCGCTCGCTTTTT 875
← →

GGTCCCTATTGTCGTATCCGGAAGATGAGGTCAAAGAATCTCCCAAGAGCTTTCTTCT 935

CGACTCCTCAGCTTCCCGATCGATCAGGTGCTTGGCAGAGCGCGCTTGTCCATGAGCAT 995

CTGCCACAGCTGCTGGTGGATGGTGTCTCAGCTAAAGGATTTTGACGACAACCATGCG 1055

CAACTGCCCGTTCCGATACGCTCGATCCTGAAGCCCCCGTGTCCATGGCAGCCCCAAGAA 1115

AAAGACATAGTTGCCCCCTGTGAGCTTGTAGCTGTGCTGGCGGCGAGCTGGTCCCGAT 1175

AAACACCTGCAGTCCGATCTCTCTGAAAGCATCAATCGCTTCTGCGCTTCTTGG 1235

CGAGTCACTGCCCCACCAACCTCAGGACCCGACGCCAAGCTTGAGGAGTCTCCCGCAA 1295

CGTGGCCACGATTCCTGATACTCGCAGAAGAGGATCACCTTGTCTGTGAC 1346
(SEQ ID NO: 105)

Fig. 3 (continued)

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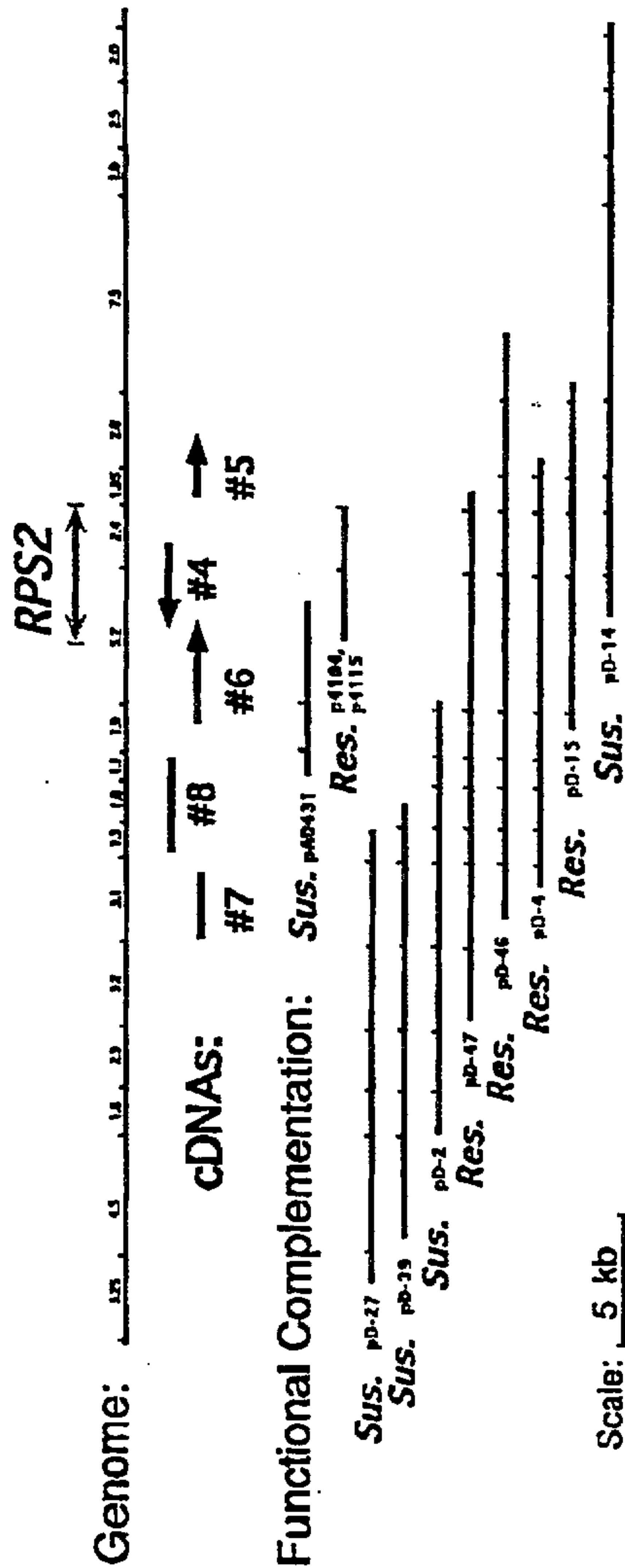


Figure 4.