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(21) International Application Number: PCT/US99/18067 (22) International Filing Date: 10 August 1999 (10.08.99) (30) Priority Data: 09/132,290 11 August 1998 (11.08.98) US (71) Applicant (for all designated States except US): THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 12th floor, 1111 Franklin Street, Oakland, CA 94607-5200 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): GILCHRIST, David, G. [US/US]; 3575 Digger Pines Ridge, Winters, CA 95694 (US). LINCOLN, James, E. [US/US]; 1860 Olvera Drive, Woodland, CA 95776 (US). RICHAEAL, Craig [US/US]; 1108 Caversham Way, Roseville, CA 95747 (US). (74) Agents: WEBER, Kenneth, A. et al.; Townsend and Townsend and Crew LLP, Two Embarcadero Center, 8th floor, San Francisco, CA 94111-3834 (US).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: INHIBITING APOPTOSIS IN PLANTS USING A CASPASE INHIBITOR (57) Abstract This invention relates to methods of inhibiting apoptosis in plants using a caspase inhibitor. This method can impart improved pathogen resistance to pathogens that exploit apoptosis as a part of the infection process.		

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INHIBITING APOPTOSIS IN PLANTS USING A CASPASE INHIBITOR

BACKGROUND OF THE INVENTION

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FIELD OF THE INVENTION

This invention relates to methods of inhibiting apoptosis in plants using a caspase inhibitor. This method can impart improve pathogen resistance to pathogens that exploit apoptosis as a part of the infection process.

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CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part and claims priority to U.S. Patent Application No. 09/132,290, filed August 11, 1998, the specification of which is incorporated herein by reference.

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STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

This invention was made with Government support under National Science Foundation Cooperative Agreement BIR-8920216. The Government has certain rights in this invention.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. (a) is a map of the pBI121 which is a shuttle vector. (b) is a map of pPRM.35K-ORF from which p35 was obtained. This plasmid was described by P.D. Friesen in *J. Virol.* 68:3467-3477 (1994).

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SUMMARY OF THE INVENTION

This invention provides for a novel method of inhibiting apoptosis in a plant said method comprising the administration of a caspase inhibitor to the plant in an

amount sufficient to inhibit apoptosis with the proviso that the caspase inhibitor is not Baculovirus p35 protease inhibitor. In one embodiment, the inhibitor is an amino blocked peptide with a reduced carboxy end. Examples of peptide inhibitors include Ac-DEVD-CHO, Ac-YVAD-CHO, Ac-DMQD-CHO and Ac-DQMD-CHO. Plants in which this
5 invention has application include members of the family of *graminaceae*, *solanaceae*, *rosaceae*, *compositae*, *leguminaceae*, *brassicaceae*, and *cucurbitaceae*.

The invention is applicable to fruit trees or vines. The inhibitors can be exogenously applied or supplied to a plant by genetic transformation. When exogenously applied the administration can be via foliar spraying.

10 The invention further provides for a composition suitable for horticultural application which comprises a caspase inhibitor and a surfactant where the inhibitor is present in an amount sufficient to inhibit apoptosis in a plant. The surfactant can be a detergent. The composition can be combined with other additives used in horticulture. The invention further provides for apparatus or systems including a spraying device and the
15 inhibitor formulated to be sprayed upon a plant. The inhibitors can be peptides as described above.

This invention further provides for a method of increasing non-viral plant disease resistance in a plant said method comprising the expressing of a p35 protease inhibitor of baculovirus in the plant in an amount sufficient to increase non-viral plant
20 disease resistance in the plant. Tobacco plants may be optionally excluded. The p35 or other peptides may be operably linked to an inducible plant promoter. The inhibitor gene is preferably expressed from a stably incorporated gene encoding p35 protease inhibitor wherein the gene has codons selected to be those preferably used by the host plant. The pathogens include both bacteria and fungi.

25 This invention further provides for a plant having increased plant disease resistance against non-viral pathogens wherein the increased plant disease resistance is due to the stable expression of a caspase protease inhibitor including the p35 protease inhibitor of baculovirus in the plant in an amount sufficient to increase non-viral plant disease resistance in the plant. Preferred plants are those described above.

30 Finally, this invention provides for a method for increasing the transformation rate of a plant cell with a heterologous gene by transfecting the cell with a gene encoding a caspase inhibitor such as p35 prior to transformation with the heterologous gene and transfecting the cell with the heterologous gene.

DETAILED DESCRIPTION

A. Introduction.

This invention provides for method of inhibiting apoptosis in plants. Inhibiting apoptosis has importance from a practical and from a research perspective. Thinning of fruit, weed control and disease resistance in plant host interactions are practical uses. Research uses involve the controlled studies of apoptosis to define its role in germination, growth, development, flowering, seed production and infection by pathogens. Among the discoveries herein are the fact that caspase inhibitors are useful for preventing apoptosis, especially apoptosis induced by pathogens. It has been surprisingly discovered that caspase inhibitors and p35 protease inhibitors from baculoviruses will delay or suppress or inhibit an apoptosis response in plants to a number of stimuli. The suppressive effect on cell death extends to a wide range of fungal and bacterial pathogens. This research has identified a major paradigm shift in terms of the critical importance of apoptosis (induction of programmed cell death) in the infection process by most, if not all, pathogens that have traditionally been classified as necrotrophs. Conversely, the research leading to this discovery indicates that any method that blocks the ability of potential pathogens to trigger the induction of apoptosis has potential for disease control. This discovery has particular relevance in circumstances where non-viral plant pathogens induce and exploit apoptosis in plants as a part of the infection process. When a plant is transformed with caspase inhibitors, the plant will not be as susceptible to pathogen induced apoptosis and a resistance phenotype is generated.

This discovery has general application across the plant kingdom. It has been demonstrated by recombinant genetics and by exogenous application of the inhibitors. The use of caspase inhibitors to generate disease resistance is particularly useful for disease in which the plant has no known resistance gene.

In particular the examples provide specific plant/bacterial combinations and plant/fungal combinations where the invention is applicable. It is a diverse grouping of plants and pathogens. Bacterial pathogens include *Psuedomonas* and *Xanthomonas*. Fungal pathogens include *Sclerotinia sclerotiorum*, *Alternaria species* such as stem canker disease of tomato, and *Fusarium sps*.

B. Definitions.

“Amino acid” refers to naturally occurring amino acids, amino acid analogs, and amino acid mimetics that function in a manner similar to the naturally occurring and analog amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ -carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to synthetic amino acids that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group (*e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium). Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Both naturally occurring and analog amino acids can be made synthetically. Amino acid mimetics refer to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that function in a manner similar to a naturally occurring amino acid.

Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission.

"Apoptosis" refers to programmed cell death. It is an essential process for normal development and homeostasis in multicellular organisms such as mammals, insects and plants. Apoptosis differs from general cell death as from a toxin or ischemic event in that the cell is shutting down according to a controlled pattern of events. Apoptosis is accompanied by chromosome condensation, by DNA degradation and by its ability to be inhibited by administration of p35.

"Apoptosis-inducing chemical" refers to a chemical that promotes cell programmed cell death. The chemical can be administered directly to the plant or indirectly by secretion of a pathogen.

“Caspase” refers to a family of cysteine proteases bearing an active site with a conserved amino acid sequence and which cleave specifically following aspartate

residues. These proteases were dubbed C(ysteine dependent) ASP(artate cleaving prote)ASE because of the shared structural and functional features. caspase-1 (formerly called ICE, interleukin 1 β -converting enzyme, see table 2) was the first member to be identified as a protease involved in mammalian apoptosis because of its homology with CED-3. The latter gene product was known for its requirement in the execution of apoptosis in the nematode *C. elegans*. To date 10 mammalian members of this family have been identified by structure and substrate specificity. The caspases cleave their substrates after an Aspartate in a recognition sequence of 4 amino acids with the conserved Aspartate at position S1 and variability in positions S4-S2 (XXXD). On basis of the recognition sequence the caspase family can be subdivided into three groups. The active site is conserved across the family and has a conserved QACXG (where X is R, Q or G) pentapeptide active site motif. For a general review see Cohen, G. M. 1997. Caspases: the executioners of apoptosis. *Biochem J.* 326:1-16

“Caspase inhibitors” refers to compounds or peptides which have an inhibitory effect on caspase protease activity. Inhibitor activity is based on concentration-dependent reduction in catalytic activity of a caspase due to substrate-site specific inhibition of the enzyme. For example, inhibition of substrate cleavage by caspase 3 is inhibitor concentration dependent with an IC₅₀ of 25 nM for the prototype tetrapeptide inhibitor AC-DEVD-CHO. The activity of this caspase can be completely blocked by 0.1 μ M AC-DEVD-CHO. Inhibition of caspase activity is dependent on the presence of the aldehyde residue on the alpha carbon at the carboxy terminal amino acid. Minimal inhibition is defined by IC₅₀ of 100 nM or less. Selectivity for caspase is meant that the inhibitor is at least twice as potent against a caspase as it is against calpain.

“Exogenous” refers to administration of an inhibitor where the inhibitor is not produced by the plants genes. Soil treatments or foliar sprays are exogenous applications or administrations.

“Heterologous gene” refers to a gene introduced into a host cell via recombinant technology where that gene is either not naturally present in the cell or represents an additional copy of an endogenous gene or is operably linked to a promoter that is not normally found in association with the gene in the host cell.

"Inducible plant promoter" refers to a promoter which directs expression of a gene where the level of expression is alterable by factors such as temperature, pH, transcription factors and chemicals.

5 "Insecticidal protein" refers to a diverse group of compounds that are lethal to insects when ingested. Examples include the crystal proteins or delta endotoxins from *Bacillus thuringiensis*.

10 "Non-viral plant disease resistance" refers to the ability of a plant to prevent or inhibit a fungal or bacterial (non-viral) pathogen from successfully infecting plant tissue and causing the symptoms of disease.

15 "Operably linked" refers to nucleotide sequences which are joined in such a manner that their individual function complements each other. Examples are promoters, transcription terminators, enhancers or activators and heterologous genes which when transcribed and if appropriate to translate will produce a functional product, *i.e.* a protein, ribozyme or anti-sense construct.

20 "p35 protease inhibitors" refer to a family of cysteine protease inhibitors derived from the genome of baculoviruses as well as artificially manipulated forms of the inhibitor such as biologically active, fusion proteins, truncated forms and forms with conserved substitutions of the wild-type amino acids.

25 "Plant" includes whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds and plant cells and progeny of same. The class of plants which can be used in the methods of the invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants.

30 "Plant pathogens" refer to fungi, bacteria and viruses that infect plants and have negative effects on their appearance, growth, production and nutritional quality

"Plant pathogens" refer to fungi, bacteria, virus and insects that infect plants and have negative effects on their growth and health.

"Promoter" refers to a region of DNA involved in binding the RNA polymerase to initiate transcription.

"Transformation rate" refers to the percent of cells that are successfully incorporate a heterologous gene into its genome and survive.

"Transfecting" refers to the process of introducing a heterologous gene into a cell.

10 C. Caspase Inhibitors

Caspase inhibitors are those compositions which have the ability to inhibit caspases, in particular caspase-3 which is also known as CPP32, apopain and Yama. Caspases are a family of related proteases. To date they have not been reported in plants which makes this discovery very unexpected.

15 In mammals, caspases are members of the interleukin -1 β converting enzyme family of cysteine proteases. The enzymes have been generally described in Nicholson *et al. Nature*, 1995, **376**:37; Femandes-Alnemri *et al. J. Biol. Chem.* 1994, **269**:30761; and, Tewari *et al., Cell* 1995, **81**:801. Substrate targets include poly(ADP-ribose) polymerase (Darmon *et al. Nature*, 1995, **377**:446), nuclear lamins (Lazebnik *et al. P.N.A.S. USA*, 1995, **92**:9042) and others.

20 Assays for studying caspases and the effect of inhibitors such as p35 inhibitors are described in the above-recited literature. Conveniently there also is a commercially available kit; BIOMOL QuantiZyme Assay System. This is a Caspase-3 Assay kit designed for Drug Discovery -AK-700 that is available from BIOMOL Research Laboratories, Inc., 5100 Campus Drive; Plymouth Meeting, PA 19462.

25 The BIOMOL caspase assay employs the colorimetric substrate DEVD-p-nitroanilide which upon cleavage has increased absorption at 405nm. Inhibitors of caspase are assayed by the ability to specifically block DEVD-p-nitroanilide cleavage. For example in a 100ul reaction containing 30 units of caspase-3 (1 unit is 1pmol of DEVD-p-nitroanilide cleaved per minute at 25°C), 0.2 mM DEVD-p-nitroanilide, the activity can be completely blocked by 0.1uM Ac-DEVD-CHO. Inhibition of caspase 3 is inhibitor concentration dependent with an IC₅₀ of 25 nM of the prototype tetrapeptide Ac-DEVD-CHO.

i. Peptide inhibitors.

Caspase inhibitors are well known. and are commercially available (Stratagene La Jolla, CA). In general they are peptides or peptide analogs that bind to the active site of the caspase enzyme. Among the commercially available tetrapeptides there is: Ac-YVAD-CHO (caspase 1), Ac-WEHD-CHO (caspase 1), Ac-YVKD-CHO (caspase 1 with low specificity), Ac-DEVD-CHO (caspase 3), Ac-DMQD-CHO (caspase 3), Ac-LEVD-CHO (caspase 4), Ac-VEID-CHO (caspase 6), Ac-IETD-CHO (caspase 8), Ac-LEHD-CHO (caspase 9) and Ac-ESMD-CHO. It is possible to synthesize longer peptides or peptides with non-natural linkages such as L peptides, and peptide mimetics with residues that are chemically similar to but are not naturally occurring amino acids such as BAF - boc-aspartyl(OMe)-fluoromethylketone - BAF is a modified amino acid produced by Enzyme Systems Products

Ac is a acetyl group that blocks the alpha amino group at the amino terminus. However, Ac can be replaced with other functionally equivalent groups such as t-butoxycarbonyl (tBOC). CHO is the aldehyde derivative of the alpha carboxyl group on the carboxy-terminal aspartate acid. CHO can be replaced with other functionally equivalent groups such as fluoromethylketone (FMK) or chloromethylketone (CMK) or dimethylbenzoyloxymethylketone or aldehyde dimethylacetal.

There are commercially available assays for determining the relative potency or selectivity of any novel caspase inhibitor. In general one would expect that a caspase-specific inhibitor to be at least twice as selective as an inhibitor of caspase as for any other cysteine protease such as calpain. Calpain inhibitor (leupeptin) was not effective in blocking apoptotic death or disease in bean or tobacco in experiments we have conducted. Most preferably, the inhibitor should be at least 10x more selective against caspase than other non-caspase enzymes. A conventional means to assay such selectivity by relative potency as measured by IC_{50} where IC is inhibitory concentration.

ii. p35 inhibitors.

There is a known natural source of caspase inhibitors. That source is the insect virus designated baculovirus. Two examples are the p35 inhibitors from *Bombyx mori* nuclear polyhedrosis virus (BmNPV) described in Kamita *et al.*, 1993, *J.Virology*, 67:455-463 and *Autographa californica* NPV (AcNPV) described in Bump *et al.* *Science*,

1995, 269:1885. The nucleic acid sequence encoding p35 of AcNPV is provided in Seq. ID No. 1. Seq. ID. No. 2 provides the amino acid sequence of p35.

In addition to the above functional means to define p35, these proteins can be structurally defined as having at least 70% homology to native forms of protein from wild-type baculovirus. Alternatively they can be defined as being able to bind under stringent conditions to a probe generated from the p35 from AcNPV by PCR using PCR primers:

Seq. ID No. 3: 5'GGCAATAAATTTTAACATTTATTTAATTGTG 3'

Seq. ID No. 4: 5'TGTGTAATTTTCCGGTAGAAATCGAC 3'

10

Sources of p35.

Protein 35 can be obtained from members of the baculovirus family. Members reported as having p35 include *Bombyx mori* nuclear polyhedrosis virus (BmNPV) described in Kamita *et al.*, *supra*, and *Autographa californica* NPV (AcNPV) described in Bump *et al. supra* and the sequence is available from GenBank Accession No. L22858 which provides the entire AcNPV genome and annotates p35.

The p35 nucleic acids of interest can be amplified from viral nucleic acid samples using amplification techniques. For instance, polymerase chain reaction (PCR) technology can be used amplify p35 sequences from any of the baculoviruses identified above.

Appropriate primers and probes for obtaining the p35 sequences from baculovirus are Seq. ID Nos. 1 and 2 listed above.

For a general overview of PCR see PCR Protocols: A Guide to Methods and Applications. (Innis, M, Gelfand, D., Sninsky, J. and White, T., eds.), *Academic Press*, San Diego (1990), incorporated herein by reference.

Polynucleotides encoding p35 may also be synthesized by well-known techniques as described in the technical literature. See, *e.g.*, Carruthers *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* 47:411-418 (1982), and Adams *et al.*, *J. Am. Chem. Soc.* 105:661 (1983). Double stranded DNA fragments may then be obtained either by synthesizing the complementary strand and annealing the strands together under appropriate conditions, or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

Making Conservative Modifications of caspase inhibitors.

The present invention provides a novel use for caspase inhibitors which includes synthetic peptides and naturally occurring forms, mimetics and analogs. It also includes recombinantly manipulated forms of p35.

Conservatively modified variations of the p35 gene refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given polypeptide. For instance, the codons CGU, CGC, CGA, CGG, AGA, and AGG all encode the amino acid arginine. Thus, at every position where an arginine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are termed silent variations.

Silent variations are particularly important when expressing a heterologous gene where that gene is from a species that does not share the same codon preference as the host cell. Thus it is anticipated that those of skill will want to and can with routine means optimize the p35 gene for best expression in the particular plant host being used to express the p35.

Every nucleic acid sequence herein which encodes a p35 polypeptide also describes every possible silent variation. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule by standard techniques.

Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence. Furthermore, one of skill will recognize that individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 5%, more typically less than 1%) in an encoded sequence are also included so long as the biological properties are essentially unaltered. For example, one of skill could study the two available wild-type proteins and readily ascertain a large number of amino acid changes that could be introduced into either form of p35.

Conservatively modified variations of peptides and p35 also include where the alterations result in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following six groups each contain amino acids that are conservative substitutions for one another:

- 1) Alanine (A), Serine (S), Threonine (T);
- 2) Aspartic acid (D), Glutamic acid (E);
- 3) Asparagine (N), Glutamine (Q);
- 4) Arginine (R), Lysine (K);
- 5 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
- 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

The nucleic acid sequences are preferably optimized for expression in a particular plant host cell used to produce the protein (e.g., a plant cell such as a tomato, or a cloning and expression system such as a yeast cell).

10 Similarly, conservative amino acid substitutions include those in which one or a few amino acids in an amino acid sequence are substituted with different amino acids with highly similar properties as described above in the listing of related amino acids.

One of skill will recognize many ways of generating alterations in a given
15 nucleic acid sequence of p35, which optionally provides alterations to the encoded protein. Such well-known methods include site-directed mutagenesis, PCR amplification using degenerate oligonucleotides, exposure of cells containing the nucleic acid to mutagenic agents or radiation, chemical synthesis of a desired oligonucleotide (e.g., in conjunction with ligation and/or cloning to generate large nucleic acids) and other well-
20 known techniques. See, Gilman & Smith, *Gene* 8:81 (1979); Roberts, *et al.*, *Nature* 328:731 (1987) and Sambrook, Innis, Ausubel, Berger, and Mullis (*supra*) and F.M. Ausubel *et al.*, eds., *Current Protocols*, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1998 Supplement) (Ausubel).

Most commonly, amino acid sequences are altered by altering the
25 corresponding nucleic acid sequence and expressing the polypeptide. However, polypeptide sequences are also optionally generated synthetically on commercially available peptide synthesizers to produce any desired polypeptide (see, Merrifield, *Science* 232:341-347 (1986), Barany & Merrifield, *THE PEPTIDES*, Gross & Meienhofer, eds., Academic Press, N.Y., pp. 1-284 (1979); and Stewart & Young, *SOLID*
30 *PHASE PEPTIDE SYNTHESIS*, 2d Ed., Pierce Chemical Co., Rockford, Ill., (1984).

Moreover, general knowledge regarding the nature of proteins and nucleic acids allows one of skill to select appropriate sequences with activity similar or equivalent to the nucleic acids, vectors and polypeptides disclosed herein. Exemplar conservative amino acid substitutions are described, *supra*.

Finally, most modifications to nucleic acids and polypeptides are evaluated by routine screening techniques in suitable assays for the desired characteristic. For instance, changes in the immunological character of a polypeptide can be detected by an appropriate immunological assay. Modifications of other properties such as nucleic acid hybridization to a target nucleic acid, redox or thermal stability of a protein, hydrophobicity, susceptibility to proteolysis, or the tendency to aggregate are all assayed according to standard techniques.

Variants of p35 can also be determined by their ability to bind to Seq. ID. No. 1 under stringent conditions. These variants are said to hybridize specifically to their targets, *i.e.* p35 encoding DNA. The phrases "hybridizing specifically to" or "specific hybridization", refers to the binding, duplexing, or hybridizing of a nucleic acid molecule preferentially to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (*e.g.*, total cellular) DNA or RNA.

The term "stringent conditions" refers to conditions under which a probe will hybridize preferentially to its target subsequence, and to a lesser extent to, or not at all to, other sequences. "Stringent hybridization" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments such as Southern and northern hybridizations are sequence dependent, and are different under different environmental parameters. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes* part I chapter 2 "overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York. Generally, highly stringent hybridization and wash conditions are selected to be about 5° C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the T_m for a particular probe.

An example of stringent hybridization conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or northern blot is 50% formalin with 1 mg of heparin at 42°C, with the hybridization being carried out overnight. An example of highly stringent wash conditions is .15M NaCl at 72°C for about 15 minutes. An example of stringent wash conditions is a .2x SSC wash at 65°C for 15 minutes (*see*, Sambrook, *supra* for a

description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, *e.g.*, more than 100 nucleotides, is 1x SSC at 45°C for 15 minutes. An example low stringency wash for a duplex of, *e.g.*, more than 100 nucleotides, is 4-6x
5 SSC at 40°C for 15 minutes. In general, a signal to noise ratio of 2x (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization. Nucleic acids which do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, *e.g.*, when a copy of a nucleic acid is created
10 using the maximum codon degeneracy permitted by the genetic code.

Where a variant is defined by homology to a DNA or amino acid sequence encoding p35. The following terms will assist in defining the limits of the homology defining p35.

The terms "identical" or percent "identity," in the context of two or more
15 nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection.

The phrase "substantially identical," in the context of two nucleic acids or
20 polypeptides, refers to two or more sequences or subsequences that have at least 60%, preferably 80%, most preferably 90-95% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection. Preferably, the substantial identity exists over a region of the sequences that is at least about 50 residues
25 in length, more preferably over a region of at least about 100 residues, and most preferably the sequences are substantially identical over at least about 150 residues. In a most preferred embodiment, the sequences are substantially identical over the entire length of the coding regions.

For sequence comparison, typically one sequence acts as a reference
30 sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence

identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* **2**:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* **48**:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* **85**:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection (see generally Ausubel *et al.*, *supra*).

One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity. It also plots a tree or dendrogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, *J. Mol. Evol.* **35**:351-360 (1987). The method used is similar to the method described by Higgins & Sharp, *CABIOS* **5**:151-153 (1989). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. For example, a reference sequence can be compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps.

Another example of algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul *et al.*, *J. Mol. Biol.* **215**:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence,

which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then
5 extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in
10 each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences)
15 uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* **89**:10915 (1989)).

In addition to calculating percent sequence identity, the BLAST algorithm also
20 performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* **90**:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a
25 reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

A further indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is
30 immunologically cross reactive with the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially

identical is that the two molecules hybridize to each other under stringent conditions, as described below.

CONSTRUCTION OF THE P35 EXPRESSION VECTOR FOR PLANTS.

5 i. Basic Methods For Constructing Recombinant Plasmids for Expressing Peptide Inhibitors of Caspase.

Recombinant expression techniques involve the construction of recombinant nucleic acids and the expression of genes in transfected cells. Molecular cloning techniques to achieve these ends are known in the art. A wide
10 variety of cloning and *in vitro* amplification methods suitable for the construction of recombinant nucleic acids are well-known to persons of skill. Examples of these techniques and instructions sufficient to direct persons of skill through many cloning exercises are found in Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology*, Volume 152, Academic Press,
15 Inc., San Diego, CA (Berger); and Current Protocols in Molecular Biology, F.M. Ausubel *et al.*, eds., *Current Protocols*, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1998 Supplement) (Ausubel).

Generally, the definitions of nomenclature and descriptions of
20 general laboratory procedures used in this application can be found in the above identified manuals.

All enzymes are used according to the manufacturer's instructions.

Nucleotide sizes are given in either kilobases (kb) or basepairs (bp). These are estimates derived from agarose gel electrophoresis or from published
25 DNA sequences.

Oligonucleotides that are not commercially available can be chemically synthesized according to the solid phase phosphoramidite triester method first described by Beaucage S.L. and Caruthers, *M.H. Tetrahedron Letts.* **22(20)**:1859-1862 (1981) using an automated synthesizer, as described in
30 Needham-VanDevanter, D.R., *et al.*, *Nucleic Acids Res.*, **12**:6159-6168 (1984). Purification of oligonucleotides is by either native acrylamide gel electrophoresis or by anion-exchange HPLC as described in Pearson, J.D. and Regnier, F.E., *J. Chrom.*, **255**:137-149 (1983).

The cloning of p35 requires the use of vectors able to replicate in
35 bacteria. Where *Agrobacterium* is the means of transformation, shuttle vectors are constructed. A plethora of kits are commercially available for the purification of plasmids from bacteria. For their proper use, follow the manufacturer's

instructions (see, for example, EasyPrepJ, FlexiPrepJ, both from Pharmacia Biotech; StrataCleanJ, from Stratagene; and, QIAexpress Expression System, Qiagen). The isolated and purified plasmids can then be further manipulated to produce other plasmids, used to transfect plant cells or incorporated into

5 *Agrobacterium tumefaciens* to infect plants. Cloning in *Streptomyces* or bacillus is possible.

In order to select the transformed bacteria, selectable markers must be incorporated into the cloning vectors. These markers permit the selection of bacterial colonies containing the vectors which one desires to replicate.

10 Examples of selectable markers include for *E. coli*: genes specifying resistance to antibiotics, *i.e.*, ampicillin, tetracycline, kanamycin, erythromycin, or genes conferring other types of selectable enzymatic activities such as beta-galactosidase, or the lactose operon.

The *in vitro* delivery of nucleic acids into bacterial hosts can be to

15 any cell grown in culture. Contact between the cells and the genetically engineered nucleic acid constructs, when carried out *in vitro*, takes place in a biologically compatible medium. The concentration of nucleic acid varies widely depending on the particular application, but is generally between about 1 mM and about 10 mM. Treatment of the cells with the nucleic acid is generally carried

20 out at physiological temperatures (about 37°C) for periods of time of from about 1 to 48 hours, preferably of from about 2 to 4 hours.

ii. Components of the plant expression cassette

In general, the expressed gene must be driven by a plant promoter

25 (CaMV 35S here) and followed by a transcription terminator (nopaline synthase terminator here). Control of expression is a preferred mode of practice. Control of expression is most easily achieved by selection of a promoter. The transcription terminator is not as critical and a variety of known elements may be used so long as they are recognized by the plant.

30 There is a wide variety of promoters available to express peptide and p35 protease inhibitors. Those of skill are familiar with the need to optimize each system in accordance with the plant being used to express p35. Expression of the inhibitors can be achieved in all tissues of a regenerated plant using "constitutive" promoters. Constitutive promoters are active under most

35 environmental conditions and states of development or cell differentiation. Examples of constitutive promoters include the cauliflower mosaic virus (CaMV) 35S transcription initiation region, the 1'- or 2'- promoter derived from T-DNA of

Agrobacterium tumefaciens, and other transcription initiation regions from various plant genes known to those of skill.

Alternatively, the plant promoter may direct expression of the inhibitor gene in a specific tissue or may be otherwise under more precise environmental or developmental control. Such promoters are referred to here as "inducible" promoters. Examples of environmental conditions that may effect transcription by inducible promoters include pathogen attack, anaerobic conditions, ethylene or the presence of light.

Examples of promoters under developmental control include promoters that initiate transcription only in certain tissues, such as leaves, roots, fruit, seeds, or flowers. The operation of a promoter may also vary depending on its location in the genome. Thus, an inducible promoter may become fully or partially constitutive in certain locations.

Specific types of plant promoters of use in this invention include the dexamethasone inducible promoter described in McNellis, *et al.*, Glucocorticoid-inducible expression of a bacterial avirulence gene in transgenic *Arabidopsis* induces hypersensitive cell death, *Plant Journal*, 1998 APR, **14(2)**:247-257; and a Tet inducible promoter described in GATZ C., *et al.* Regulation of a Modified CAMV 35S Promoter by the TN10-Encoded TET Repressor in Transgenic Tobacco, *Mol. & Gen. Genetics*, 1991 June, **227(2)**:229-237.

Other promoters of use in this invention would include the tissue or light regulated promoter described in JofreGarfias, AE; *et al.*, *Agrobacterium*-mediated transformation of *Amaranthus hypochondriacus*: light- and tissue-specific expression of a pea chlorophyll a/b-binding protein promoter, *Plant Cell Reports*, 1997 Oct, **16(12)**:847-852; Liu, ZR *et al.* Identification of an *Arabidopsis thaliana* Ribulose-1,5-bisphosphate Carboxy-lase Oxygenase Activase (RCA) Minimal Promoter Regulated by Light and the Circadian Clock, *Plant Physiol.*, 1996 Sept, **112(1)**:43-51; and, Kaiser T., *et al.*, Promoter Elements of the Mustard CHS1 Gene are Sufficient for Light Regulation in Transgenic Plants, *Plant Mol. Biol.*, 1995 May, **28(2)**:219-229.

Because apoptosis is a beneficial process, it is preferred that the p35 be induced under controlled conditions. In general it is preferred that the p35 be either placed under an inducible promoter or in an organ specific promoter such as those identified above.

iii. Shuttle Vectors

Agrobacterium-mediated transformation is a convenient means to transfect p35 genes into a plant. To transform plants using *Agrobacterium* shuttle vectors are constructed. Shuttle vectors must be able to replicate in each of the
5 hosts and have a selectable marker. There are commercially available vectors for this process and the example section describes such a vector. In brief, the shuttle vectors has one origin of replication for *E.coli* and one for *Agrobacterium*. They may have selectable markers to differentiate between the bacteria. In the example, the selectable marker is kamomycin for both *E.coli* and *Agrobacterium*.

TRANSFECTING PLANT CELLS WITH CASPASE INHIBITORS

The DNA constructs of the invention are introduced into plant cells, either in culture or in the organs of a plant by a variety of conventional techniques. For example, the DNA construct can be introduced directly into the
15 genomic DNA of the plant cell using techniques such as electroporation and microinjection of plant cell protoplasts, or the DNA constructs can be introduced directly to plant cells using ballistic methods, such as DNA particle bombardment. Alternatively, the DNA constructs are combined with suitable T-DNA flanking regions and introduced into a conventional *Agrobacterium*
20 *tumefaciens* host vector. The virulence functions of the *A. tumefaciens* host directs the insertion of the construct and adjacent marker into the plant cell DNA when the cell is infected by the bacteria.

Microinjection techniques are known in the art and well described in the scientific and patent literature. The introduction of DNA constructs using
25 polyethylene glycol precipitation is described in Paszkowski, *et al.*, *EMBO J.* **3**:2717 (1984). Electroporation techniques are described in Fromm, *et al.*, *Proc. Natl. Acad. Sci. USA*, **82**:5824 (1985). Ballistic transformation techniques are described in Klein, *et al.*, *Nature*, **327**:70-73 (1987).

Alternatively and preferred, the DNA constructs may be combined
30 with suitable T-DNA flanking regions and introduced into a conventional *Agrobacterium tumefaciens* host vector. The virulence functions of the *A. tumefaciens* host will direct the insertion of the inhibitor genes such as the p35 construct and adjacent marker gene(s) (if present) into the plant cell DNA when the cell is infected by the bacteria. For a review of gene transfer methods for
35 plant and cell cultures see, Fisk *et al.* (1993) *Scientia Horticulturae* **55**:5-36 (1993) and Potrykus (1990) CIBA Found. Symp. **154**:198.

Agrobacterium tumefaciens-mediated transformation techniques are the most commonly used techniques for transferring genes into plants. These techniques are well described in the scientific literature. See, for example Horsch *et al.* (1984) *Science*, **233**:496-498, Fraley *et al.* (1983) *Proc. Natl. Acad. Sci. USA*, **80**:4803, and Hooykaas (1989) *Plant Mol. Biol.*, **13**:327-336, Bechtold *et al.* (1993). *Comptes Rendus De L Academie Des Sciences Serie Iii-Sciences De La Vie-Life Sciences*, **316**:1194-1199, Valvekens *et al.* (1988) *Proc. Natl. Acad. Sci. USA*, **85**:5536-5540.

All species which are natural plant hosts for *Agrobacterium* are transformable in vitro. Most dicotyledonous species can be transformed by *Agrobacterium*. Monocotyledonous plants, and in particular, cereals, have not previously been regarded as natural hosts to *Agrobacterium*. There is, however, growing evidence that monocots can be transformed by *Agrobacterium*. Using recently developed approaches cereal species such as rye (de la Pena *et al.* (1987) *Nature*, **325**:274-276), corn (Rhodes *et al.* (1988) *Science*, **240**:204-207), and rice (Shimamoto *et al.*, (1989) *Nature*, **338**:274-276) may now be transformed.

Transformation of a number of woody plants using *Agrobacterium* and other methods has also been described. (Shuerman *et al.* (1993) *Scientia Horticulturae*, **55**:101-124). For instance, regeneration and transformation of apples is described in James *et al.* (1989) *Plant Cell Rep.*, **7**:658-661.

After transformation, transformed plant cells or plants comprising the introduced DNA must be identified. A selectable and/or scorable marker gene is typically used. Such genes are well known. Transformed plant cells can be selected by growing the cells on growth medium containing the appropriate antibiotic. In some instances, the presence of opines can also be used if the plants are transformed with *Agrobacterium*.

After selecting the transformed cells, one can confirm expression of the introduced caspase-inhibitor gene(s). Simple detection of mRNA encoded by the inserted DNA can be achieved by well known methods in the art, such as Northern blot hybridization. The inserted sequence can be identified using the polymerase chain reaction (PCR) and Southern blot hybridization, as well (see, e.g., Sambrook, *et al.*, *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 1989.).

REGENERATION OF TRANSGENIC PLANTS EXPRESSING INHIBITOR PEPTIDES AND P35.

Transformed plant cells, which are derived by any of the above transformation techniques can be cultured to regenerate a whole plant which possesses the transformed genotype and thus the desired phenotype. Such

regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker which has been introduced together with the desired nucleotide sequences. Plant regeneration from cultured protoplasts is described in Evans, *et al.*, Protoplasts Isolation and Culture, *Handbook of Plant Cell Culture*, pp. 124-176, Macmillian Publishing Company, New York, (1983); and in Binding, Regeneration of Plants, *Plant Protoplasts*, pp. 21-73, CRC Press, Boca Raton, (1985). Regeneration can also be obtained from plant callus, explants, somatic embryos (Dandekar, *et al.*, *J. Tissue Cult. Meth.*, **12**:145 (1989); McGranahan, *et al.*, *Plant Cell Rep.*, **8**:512 (1990)), organs, or parts thereof. Such regeneration techniques are described generally in Klee, *et al.*, *Ann. Rev. of Plant Phys.*, **38**:467-486 (1987).

PLANTS THAT CAN BE RENDERED DISEASE RESISTANCE.

Nucleic acids expressing caspase inhibitors can be used to confer plant disease resistance on essentially any plant. Thus, the invention has use over a broad range of plants, including species from the genera *Asparagus*, *Avena*, *Brassica*, *Citrus*, *Citrullus*, *Capsicum*, *Cucumis*, *Cucurbita*, *Daucus*, *Fragaria*, *Glycine*, *Hordeum*, *Lactuca*, *Lycopersicon*, *Malus*, *Manihot*, *Nicotiana*, *Oryza*, *Persea*, *Pisum*, *Pyrus*, *Prunus*, *Raphanus*, *Secale*, *Solanum*, *Sorghum*, *Triticum*, *Vitis*, *Vigna*, and *Zea*.

One of skill will recognize that after the expression cassette is stably incorporated in transgenic plants and confirmed to be operable, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

D. EXOGENOUS ADMINISTRATION OF CASPASE INHIBITORS.

Protein agents for agricultural use are formulated like chemical agents. They are usually applied through existing sprayer or spreader technology. The horticultural or agricultural compositions of the invention will include conventional agriculturally accepted agents and additives. Such agents include but are not limited to surface-active agents; such as octylphenol ethoxylate, stabilizers; such as propionic acid, filling agents; such as de-fatted soyflour, flowability or anti-caking agents; such as synthetic precipitated silica, dispersants; such as sodium salt of condensed naphthalene sulfonic acid and the like. The quantity of surface-active agents can vary over a wide range. For convenience such agents may comprise from about 0.1% to about 50% by

weight of the composition, more preferably about 0.5% to about 40% and most preferably 1% to about 20%.

5 The compositions of the present invention may be further formulated, and one skilled in the art is aware of many methods for producing formulated bioagricultural products. These methods are described in the technical and patent literature and include methods for forming granules, wettable powders, water-based and oil-based flowables, concentrates and the like. The following examples are provided for illustration and are not meant to limit the invention in any way.

10 Wettable powder formulation: 63% active insecticidal ingredients, 10% flow agent (precipitated silica), 4% surfactant (naphthalene sulfonate), 13% dispersant (alkylaryl polyoxy acetate), 7% carrier (attapulgitic clay), and 3% sequestrant (EDTA).

Granule: 63% active insecticidal ingredients, 20% flow agent (precipitated silica), 3% surfactant (linear alkyl benzene sulfonate), 5% dispersant (blend of anionic surfactant and sodium lignosulfonate), and 9% carrier (kaolin clay).

15 The inhibitors can be sprayed in an aqueous solution with agriculturally accepted oils, emulsifiers or surfactants to enhance spreadability. Surfactants include detergents such as Triton-X 100 and non-detergent based silane surfactants.

It is expected that the amount and rate of inhibitor will need to be determined on a crop by crop basis. Potency of the inhibitor will also play a role in the determination of application rates. In general, applications rates should produce an application of 1-1000 ug/ml per square meter of leaf surface.

20 To facilitate the application to large area of plant growth, the inhibitors will be prepared in large scale and sold as a liquid in volumes of a pint or greater, eg. a quart, gallon or 5 gallon containers. Dry solids would be in amounts of a pound or greater.

25 The compositions of the present invention containing the active ingredients and any further formulations may be used in a method of controlling bacterial, fungi and other plant pathogens on a plant where control is desired comprising applying an effective amount of the composition to the plants.

30 This invention further anticipates the use of the inhibitors in a spray apparatus where a nozzle emits the inhibitor at a uniform volume under pressure. Such spray apparatus are well known in the industry and are commercially available. The combination of inhibitor and sprayer as a system is considered an invention.

E. BIOCHEMICAL METHODS OF ASSAYING FOR INHIBITION OF APOPTOSIS AND PLANT RESISTANCE BY CASPASE INHIBITORS

General methods

5 Apoptosis in plants follows an analogous pathway as in animal cells. Apoptosis can be induced in plants using conventional means such as exposure to cyanide, mannose (1%) or if the plant is susceptible using the various toxins including Fumonisin B₁ and AAL-Toxin TA from *Alternaria sps.* and as described in the examples below.

10 Two molecular analytic tools are available and established to determine when apoptosis is occurring in plants as distinguished from cell death due to lethal toxins (such as toxic levels of Fe₂SO₄) or hypoxic conditions. The first is demonstration of an orderly fragmentation of the cellular DNA. These are called DNA ladders and are readily analyzed using a variety of electrophoretic
15 techniques. The specific gel or running conditions are not critical. Suitable electrophoretic conditions are provided by Wang *et al.* 1996, Apoptosis: A Functional Paradigm for Programmed Plant Cell Death Induced by a Host-selective Phytotoxin and Invoked During Development, *Cell*, 8:375-391.

Alternatively, fragmentation of DNA during apoptosis can be
20 detected *in situ* by reagents that react with exposed 3'hydroxyl groups on the nucleosomal units. The assay procedure involves end labeling the DNA fragments by terminal deoxynucleotidyl transferase (TdT) with UTP conjugated to a detectable marker. The method is termed (TUNEL) and more details of this method can be found in Wang *et al.* (*supra*). Finally one can visually identify
25 apoptotic bodies containing fragmented DNA which is one of the hallmarks of apoptosis.

Finally, one can determine if apoptosis is a controlling event in the pathogenicity process by administering an effective amount of a caspase inhibitor in the presence of a pathogen. If the inhibitor imparts or increases
30 disease resistance, one can conclude that the pathogen/host interaction depends upon apoptosis to be successful.

Assaying for plant disease resistance in plants using morphological and other visual indicators.

35 Once it is established that apoptosis inhibition is due to administration of a caspase inhibitor, those of skill will recognize that it is convenient to assay for disease resistance by traditional methods. These methods involve visual observation of the effects of a pathogen on a host plant.

Observable symptoms include wilt, rot, stunted growth, color changes and mycelial growth. The symptoms are scored for their degree of intensity in sufficient repeated trials until statistically meaningful information is generated. The generic experimental parameters for reproducible study of infection and resistance in plants is well known.

Methods for assaying tetrapeptide caspase inhibitors' effect on necrotrophic bacterial diseases of plants

A leaf of the susceptible plant of interest is syringe or vacuum infiltrated with a concentration of virulent bacteria sufficient to cause disease and tissue death. A portion of the same leaf or another leaf on the same plant of similar position and age is infiltrated with bacteria and the tetrapeptide inhibitor. Usually a 0.5 mM concentration of inhibitor is sufficient to inhibit disease but may vary according to pathogen and host plant. Disease is permitted to develop under conditions suitable for the plant/pathogen combination. Symptoms are viewed over the course of the experiment to assess the level of symptom (death) inhibition by the caspase inhibitor compared to the control. The affect of the caspase inhibitor on bacterial growth can be assayed by taking samples of the tissue and performing growth curves of the pathogen as described in the literature.

For p35 transformed plants, one simply exposes the plant to the pathogen and compare the disease resistance with appropriate controls such as non-transformed plants.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. Those of skill will readily recognize a variety of noncritical parameters which could be changed or modified to yield essentially similar results.

Example 1. Exogenous administration of caspase inhibitor peptides and the Method for screening efficacy of peptide inhibitors of apoptosis

Host plant leaves are infiltrated with bacteria at a suitable concentration (10^4 - 10^8 colony forming units per ml) to cause disease. Leaves of the same age and position on the same or a different plant are infiltrated with bacteria of the same concentration with the desired peptide inhibitor added. All solutions are infiltrated directly into the plant leaf lamina with a syringe. Disease symptoms in the form of dead areas of cells are quantified by time lapse color image capture and lesion area measurements determined by digital image analysis. Samples of the control tissue and inhibitor treated tissues are analyzed on a time course basis for bacterial growth rate.

Pathogen growth is measured by grinding leaf disc samples in a suitable buffer followed by serial dilutions and plating of samples onto agar plates to determine the relative number of colonies produced. Colony counts are then related back to the original undiluted samples. Growth values are reported as means of several samples with appropriate statistical measures of mean separation. Differential effects of the inhibitors thus are measured both in terms of the amount of apoptotic cell death induced (symptoms) and by the effect of altering cell death induction on the amount of pathogen growth in the tissue.

Following the above identified procedure, the following bacterial pathogens have been tested and the caspase inhibitors blocked the disease induced by these pathogens.

- 1) *Pseudomonas syringae* pv. *phaseolicola* in bean leaves
This is a compatible interaction normally resulting in disease and cell death.
Ac-DEVD-CHO blocks cell death
- 2) *Pseudomonas syringae* pv. *tabaci* in tobacco leaves
This is a compatible interaction normally resulting in disease and cell death.
Ac-DEVD-CHO blocks cell death
Ac-DMQD-CHO blocks cell death
Ac-YVAD-CHO blocks cell death but less well than others
Ac-LRR-CHO (a calpain protease inhibitor) does not block cell death and is used as a control on specificity of the tetrapeptide caspase inhibitors.
- 3) *Pseudomonas syringae* pv. *angulata* in tobacco leaves
This is a compatible interaction normally resulting in disease and cell death.
Ac-DEVD-CHO blocks cell death
- 4) *Pseudomonas syringae* pv. *phaseolicola* in tobacco leaves
This is an incompatible interaction normally resulting in no disease but does involve cell death (non-host hypersensitive response).

Ac-DEVD-CHO blocks cell death
 Ac-DMQD-CHO blocks cell death
 Ac-YVAD-CHO blocks cell death but less well than others

- 5 5) *Pseudomonas syringae* pv. *tomato* (avrPto) in tomato (Pto/Pto) leaves
 This is an incompatible interaction normally resulting in no disease but does involve cell death (host specific "gene for gene" hypersensitive response)
 Ac-DEVD-CHO blocks cell death
 Ac-DQMD-CHO blocks cell death
- 10 6) *Pseudomonas syringae* pv. *syringae* in tomato leaves.
 This is a compatible interaction normally resulting in disease and cell death.
 Ac-DEVD-CHO blocks cell death
- 15 7) *Pseudomonas syringae* pv. *tomato* in tomato leaves.
 This is a compatible interaction normally resulting in disease and cell death.
 Ac-DEVD-CHO blocks cell death
 Ac-DMQD-CHO blocks cell death
 Ac-YVAD-CHO blocks cell death but less well than others
- 20 8) *Xanthomonas campestris* pv. *tomato* in tomato leaves.
 This is a compatible interaction normally resulting in disease and cell death.
 Ac-DEVD-CHO blocks cell death.

25 Hence, these caspase specific inhibitors not only block the diseases associated with each these pathogens but also confirm that the induction of apoptosis is a critical and dependent requirement for disease. This is a basis for the novel discovery that each of these pathogens expresses virulence by inducing apoptosis or programmed cell death and that the blocking of cell death can effectively block disease.

30

EXAMPLE 2. Construction of a p35 plant expression vector for tomatoes.

The p35 ORF was obtained from plasmid pPRM-35K-ORF from Paul Friesen (*J. Virol.*, **68**:3467-3477 (1994)). The plasmid pPRM-35K-ORF was digested with XbaI and SacI to release the p35 ORF. This fragment was

35 ligated to XbaI and SacI digested pBI121 (available from Clontech located in Palo Alto, California) so as to replace the GUS gene with the p35 gene. This construct was transformed into *E. coli* and selected on kanamycin. The plasmid was then purified and used to transform *Agrobacterium tumefaciens* to kanamycin resistance. The resulting *Agrobacterium* strain was used to transform

40 tomato cotyledons as described below. The plasmids are described in Figure 1(a) and (b), respectively.

EXAMPLE 3. Transformation of a tomato plant with p35.

The transformation of tomatoes is in accordance with protocols described in McCormick *et al.* 1986, *Plant Cell Rpts*, 5:81:84 and Fillate *et al.* 1987, *Biotechnology*, July 5, 1987. In brief, tomato seeds are surfaced sterilized with bleach and a wetting agent. The seeds are rinsed in distilled water and allowed to germinate on a basic media including Murshige and Skoog salts, sucrose Nitsh's vitamins, inositol and agar at pH 5.8. The seeds are kept moist and dark for 48 hours and germinated in the light at 25C.

Cotyledon explants are cut from the seedlings in placed upside down on a co-cultivation plate which has tobacco callus tissue.

Agrobacterium in a fresh medium free of antibiotic at concentration of about 5×10^8 cells per ml is poured over the explants. The excess bacteria is decanted and the explants are removed and dried and replated. After about 48hrs. at 25C°, the explants are replated on basic media with Zeatin riboside and allowed to regenerate into shoots. The shoots are cut and placed into rooting media. Rooting is done in the presence of the selection and control antibiotics. The plants are then hardened and tested for genotype and phenotype.

EXAMPLE 4. Genotype testing.

To determine if the plants were expressing p35, DNA was extracted using the CTAB method of DNA isolation described by Ausubel *et al.* (1995). Short protocols in molecular biology: a compendium of methods from Current protocols in molecular biology / editorial board, Frederick M. Ausubel *et al.*, 3rd ed. [New York]: Wiley, c1995. PCR analysis using primers specific to p35 revealed which segregants were positive for p35 insert.

For Northern analysis, plant RNA was extracted using Trizol (GibcoBRL) and transferred onto a Nytran Plus membrane (Scheicher & Schuell). The dot blot was hybridized with a ^{32}P -labeled, full-length p35 probe. With rare exceptions, plants having the p35 inhibitor gene demonstrated higher levels of disease resistance than non-p35 transformed plants (see Table 1).

EXAMPLE 5. Assaying for increased plant disease resistance in the p35 transformed tomato.

Tomato (*Lycopersicon esculentum*) plants bearing p35 were used for the bioassay were derived from a self-pollinated primary transformant (T1) shown to express p35 transcript. The p35 gene was expressed in plants normally

susceptible (asc/asc) to Alternaria stem canker (*Alternaria alternata* f. sp. *lycopercisi*) and Anthracnose (*Colletotrichum coccodes*).

Plants were grown under greenhouse conditions at 22-25°C and a 16/8 hr. photoperiod until the plants were treated at the 5-6 leaf stage with *A. alternata* f. sp. *lycopercisi*. Replications of assays of p35 activity were accomplished on cuttings derived from the mother plants. All fruit harvested for assays were from greenhouse-grown plants.

Cultures of *A. alternata* f. sp. *lycopercisi* were grown on potato dextrose agar under fluorescent bulbs on a 12 hr. light/ 12 hr. dark cycle. Eight to ten day old cultures were immersed in water with 0.01% Tween 20 and scraped with a glass rod to dislodge conidia. Conidia were filtered through a double layer of cheesecloth and adjusted to a concentration of 1×10^5 conidia/ml. Conidia concentration was ascertained with a hemacytometer.

15 i. LEAF ASSAYS.

All plants of the segregating population were treated by brushing the spore suspension down the entire length of one side of the stem with a camel hair brush. The lowest, oldest leaf of every plant was also brushed on the adaxial surface with the spore suspension. Several control plants were treated in the same fashion. Control plants included wild-type, non-transgenic susceptible controls (asc/asc), wild-type, non-transgenic resistant controls (Asc/Asc) and GUS-transformed susceptible controls (asc/asc).

All treated plants were placed in a moist chamber (98-100% RH) at 24°C on a 16/8 hr. photoperiod for 2 days. Plants were removed from the moist chamber and observed for 21 days. Plants were treated in the above manner to ensure that each plant received approximately the same high concentration of inoculum and diminish the possibility of escapes. Any plant recessive at the Asc locus would always die when treated in such a way. Plants were scored for resistance phenotype before knowledge of the genotype of each plant was ascertained.

Spore concentrations of 1×10^4 , 1×10^5 and 1×10^6 spores/ml were used to understand the dosage dependent contribution of p35 to resistance. This assay was performed by spraying the entire upper surface of the plants with spore suspension until run-off. Inoculated plants were treated as were the brush-inoculated plants. Spray treatments are likely more typical of a situation occurring in the field.

Segregation analysis revealed a role of p35 in conditioning plant response to the necrotrophic pathogen *Alternaria alternata* f. sp. *lycopercisi* (AAL). Resistance to AAL co-segregates with the presence of the transgene in

T2 progeny derived from a self of the p35-transformed parent. In plants containing p35 mRNA, a reduction in the rate of symptom development and rate at which the pathogen spreads is evident (Table 1). All susceptible controls (asc/asc) and GUS-transformed susceptible controls died under experimental conditions applied to all individuals. Northern analysis revealed the number of segregants with p35 transcript equals 53 and those without p35 transcript number 11. This ratio suggests that one p35 locus is controlling the interaction between plant and pathogen. The ratio of strongly insensitive to moderately sensitive to susceptible plants scored at 21 days post-inoculation also suggests that one locus is involved (16 strongly insensitive: 32 moderately sensitive: 16 susceptible). The pathogen likely overwhelmed the protective influence of p35 in some hemizygotes which would account for the discrepancy between the number of p35 minus plants (11) and the number of plants dying at 21 days (16).

ii. FRUIT ASSAY

The interaction of p35 and *A. alternata* f. *sp. lycopersici* was further defined by inoculating green fruit. Green fruit was wounded with a hypodermic needle in several locations on the blossom end. Five ml of spore suspension derived as above was placed at the wound site. Spore concentrations of 1×10^5 and 5×10^5 were used. Treated fruit were placed in a clear plastic box on a wire mesh that held the fruit above a layer of water in the bottom of the box. The plastic box was covered and placed in a growth chamber at 24°C and 16/8 light/dark cycle for the duration of the experiment. Lesions on fruit developed as circular depressions, the diameters of which were easily measured with a ruler. Mean lesion diameter of lesion on p35-expressing fruit was compared to mean lesion diameter on susceptible control fruit. As with the studies in the tomato leaf sections, the fruits expressing p35 were more resistant to the fungi with p35 expressing fruit showing a 2 to 3 times smaller lesion size than control fruit.

iii. ASSAYS WITH *COLLETOTRICHUM COCCODES*

Assays with *C. coccodes* were performed on ripe fruit. Fungal cultures were maintained on 30% V8 agar. Eight to ten day old cultures were immersed in water and scraped to dislodge conidia. Conidia suspensions were filtered through 2 layers of cheesecloth and adjusted to 1×10^5 spores/ml with a hemacytometer. Ripe fruit was surface sterilized with 70 % ethanol and rinsed in sterile water. Wounds in the epidermis of the fruit made with a needle were inoculated with 5 µl of spore suspension. Inoculated fruit were placed in clear plastic boxes on wire mesh above a layer of warm water. The plastic box was covered and placed in a growth chamber at 24°C and 16/8 light/dark cycle for the

duration of the experiment. Lesions on fruit developed as circular depressions, the diameters of which were easily measured with a ruler. Mean lesion diameter of lesion on p35-expressing fruit was compared to mean lesion diameter on susceptible control fruit. The p35 expressing fruit showed lesions that were one-

5 half the size of control fruit.

The leaf assay for *Alternaria* toxin was as follows: Young fully expanded leaves of greenhouse grown tomato plants were cut from the plant and incubated for 48 to 72 hours with the cut end immersed in 250 nanomolar TA toxin. Sensitivity to the toxin was visually scored for interveinal regions of death. Comparisons to untransformed tomato showed p35 transformed leaves

10 were much slower to show death.

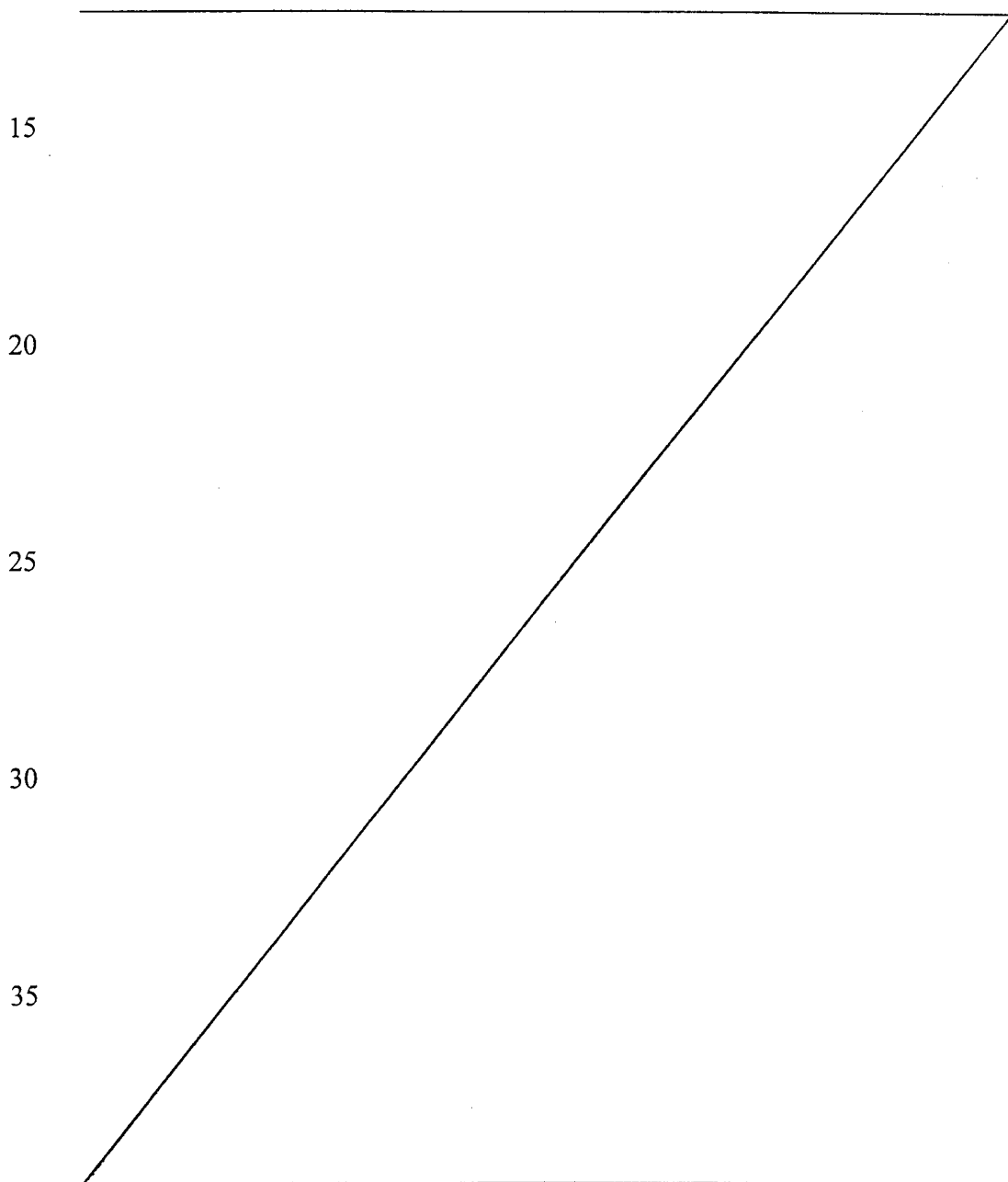


TABLE 1

AAL Disease		AAL Disease	
Genotype	p35 mRNA*	Genotype	p35 mRNA*
Severity Index**		Severity Index**	
114-2.1	y	114-2.35	y
114-2.2	y	114-2.36	y
114-2.3	y	114-2.37	y
114-2.4	n	114-2.38	y
114-2.5	y	114-2.39	y
114-2.6	y	114-2.40	y
114-2.7	y	114-2.41	n
114-2.8	y	114-2.42	y
114-2.9	y	114-2.43	y
114-2.10	y	114-2.44	y
114-2.11	n	114-2.45	y
114-2.12	y	114-2.46	y
114-2.13	y	114-2.47	y
114-2.14	y	114-2.48	n
114-2.15	y	114-2.49	y
114-2.16	y	114-2.50	y
114-2.17	y	114-2.51	y
114-2.18	y	114-2.52	y
114-2.19	y	114-2.53	y
114-2.20	y	114-2.54	n
114-2.21	n	114-2.55	y
114-2.22	n	114-2.56	y
114-2.23	y	114-2.57	y
114-2.24	y	114-2.58	y
114-2.25	n	114-2.59	y
114-2.26	n	114-2.60	n
114-2.27	y	114-2.61	y
114-2.28	y	114-2.62	y
114-2.29	n	114-2.63	y
114-2.30	y	114-2.64	y
114-2.31	y		
114-2.32	y	asc/asc	n
114-2.33	y	35S::GUS	n
114-2.34	y	ASC/ASC	n

Table 1. Affect of caspase inhibitor p35 on *Alternaria alternata* f.sp. *lycopersici* infection and spread in a population of transgenic tomato plants segregating at the p35 locus.

* Determined by Northern blot with full-length p35 insert as probe.

** Disease severity assessed by comparing treated plants with susceptible and resistant control plants 21 days post-inoculation: 0 = no disease symptoms; 1 = petiole lesions evident, < half stem with lesions; 2 = petiole lesions evident, all of stem with lesions; 3 = petiole and stem lesions expanding; 4 = petiole and stem lesions expanding, toxin symptoms on leaves; 5 = plant dead.

Sequence ID No. 1.

P35 sequence=

```

5  ATGTGTGTA  ATTTTCCGG  TAGAAATCGA  CGTGTCCCAG  ACGATTATTC
   GAGATTGTCA  GGTGGACAAA  CAAACCAGAG  AGTTGGTGTA  CATTAACAAG
   ATTATGAACA  CGCAATTGAC  AAAACCCGTT  CTCATGATGT  TTAACATTTC
   GGGTCCTATA  CGAAGCGTTA  CGCGCAAGAA  CAACAATTTG  CGCGACAGAA
   TAAAATCAAA  AGTCGATGAA  CAATTTGATC  AACTAGAACG  CGATTACAGC
   GATCAAATGG  ATGGATTCCA  CGATAGCATC  AAGTATTTTA  AAGATGAACA
10  CTATTCGGTA  AGTTGCCAAA  ATGGCAGCGT  GTTGAAAAGC  AAGTTTGCTA
   AAATTTTAAA  GAGTCATGAT  TATACCGATA  AAAAGTCTAT  TGAAGCTTAC
   GAGAAATACT  GTTTGCCCAA  ATTGGTCGAC  GAACGCAACG  ACTACTACGT
   GGCGGTATGC  GTGTTGAAGC  CGGGATTTGA  GAACGGCAGC  AACCAAGTGC
   TATCTTTCGA  GTACAACCCG  ATTGGTAACA  AAGTTATTGT  GCCGTTTGCT
   CACGAAATTA  ACGACACGGG  ACTTTACGAG  TACGACGTCG  TAGCTTACGT
15  GGACAGTGTG  CAGTTTGATG  GCGAACAATT  TGAAGAGTTT  GTGCAGAGTT
   TAATATTGCC  GTCGTCGTTT  AAAAATTCGG  AAAAGGTTTT  ATATTACAAC
   GAAGCGTCGA  AAAACAAAAG  CATGATCTAC  AAGGCTTTAG  AGTTTACTAC
   AGAATCGAGC  TGGGGCAAAT  CCGAAAAGTA  TAATTGGAAA  ATTTTTTGTA
   ACGGTTTTAT  TTATGATAAA  AAATCAAAAAG  TGTGTATGT  TAAATTGCAC
20  AATGTAACTA  GTGCACTCAA  CAAAAATGTA  ATATTAAACA  CAATTAAATA
   A

```

Seq. ID. No. 2

P35 translation=

```

25  MCVIFPVEIDVSQTIIRDCQVDKQTRELVIYINKIMNTQLTKPVL
   MMFNISGPIRSVTRKNNLRDRIKSKVDEQFDQLERDYSQMDGFHDSIKYFKDEHYSVS
   CQNGSVLKSKFAKILKSHDYTDKKSIEAYEKYCLPKLVDERNDYYVAVCVLKPGFENG
   NQVLSFEYNPIGNKVIVPFAHEINDTGLYEYDVVAYVDSVQFDGEQFEFVQSLILPSSFK
30  NSEKVLYYNEASKNKSIMYKALEFTTESSWGKSEKYNWKIFCNGFIYDKKSKVLYVKLH
   NVTALNKNVILNTIK

```

PCR Primers for p35 =

Seq. ID No. 3: 5'GGCAATAAAATTTTAACATTTATTTAATTGTG 3'

Seq. ID No. 4: 5'TGTGTAATTTTCCGGTAGAAATCGAC 3'

35

CLAIMS

What is claimed is:

- 5 1. A method of inhibiting apoptosis in a plant said method comprising the administration of a caspase inhibitor to the plant in an amount sufficient to inhibit apoptosis with the proviso that the caspase inhibitor is not Baculovirus p35 protease inhibitor.
2. A method of claim 1 wherein the inhibitor is an amino blocked peptide with a reduced carboxy end.
- 10 3. A method of claim 2 wherein the inhibitor is selected from the group consisting of Ac-DEVD-CHO, Ac-YVAD-CHO, Ac-DMQD-CHO and Ac-DQMD-CHO.
4. A method of claim 1 wherein the plant is selected from the group consisting of graminaceae, solanaceae, rosaceae, compositeae, leguminaceae, brassicaceae, and cucurbitaceae.
- 15 5. A method of claim 1 wherein the plants are fruit trees or vines.
6. A method of claim 1 where the administration is by transformation of the plant with a gene that expresses the inhibitor.
7. A method of claim 1 where the administration is exogenous.
8. A method of claim 7 where the exogenous administration is via foliar
20 spraying.
9. A composition suitable for horticultural application which comprises a caspase inhibitor and a surfactant where the inhibitor is present in an amount sufficient to inhibit apoptosis in a plant.
10. A composition of claim 9 wherein the surfactant is a detergent.
- 25 11. A composition of claim 9 wherein the inhibitor is an amino blocked peptide with a reduced carboxy end.
12. A composition of claim 9 contained within a sprayer.
13. A composition of claim 9 wherein the inhibitor is selected from the group consisting of Ac-DEVD-CHO, Ac-YVAD-CHO, Ac-DMQD-CHO and Ac-DQMD-CHO.

14. A method of increasing non-viral plant disease resistance in a plant said method comprising the expressing of a p35 protease inhibitor of baculovirus in the plant in an amount sufficient to increase non-viral plant disease resistance in the plant.

5 15. A method of claim 14 where the p35 is operably linked to an inducible plant promoter.

16. A method of claim 14 wherein the p35 protease inhibitor is expressed from a stably incorporated gene encoding p35 protease inhibitor wherein the gene has codons selected to be those preferably used by the host plant.

10 17. A method of inhibiting apoptosis in a plant or plant cell comprising transforming the cell with a p35 protease inhibitor of baculovirus and exposing the plant or plant cell to apoptosis-inducing chemicals.

18. A method of claim 17 wherein the apoptosis-inducing chemicals are from plant pathogens.

19. A method of claim 18 wherein the pathogens are fungal.

15 20. A plant having increased plant disease resistance against non-viral pathogens wherein the increased plant disease resistance is due to the stable expression of a p35 protease inhibitor of baculovirus in the plant in an amount sufficient to increase non-viral plant disease resistance in the plant.

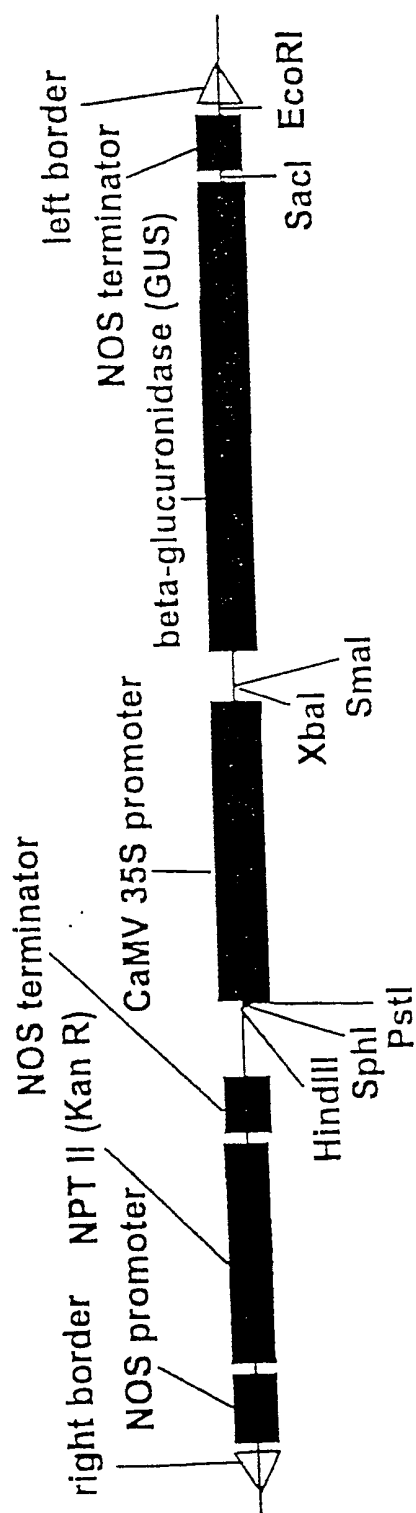
20 21. A plant of claim 20 wherein the plant is selected from the group consisting of graminaceae, solanaceae, rosaceae, compositeae, leguminaceae, brassicaceae, and cucurbitaceae.

22. A method for increasing the transformation rate of a plant cell with a heterologous gene by transfecting the cell with a gene encoding p35 prior to transformation with the heterologous gene and transfecting the cell with the heterologous gene.

25 23. A method of claim 22 wherein the plant is selected from the group consisting of graminaceae, solanaceae, rosaceae, compositeae, leguminaceae, brassicaceae, and cucurbitaceae.

24. A method of claim 22 wherein the gene encoding the p35 protease inhibitor has codons selected to be those preferably used by the host plant.

30



pBI121

Fig. 1(a)

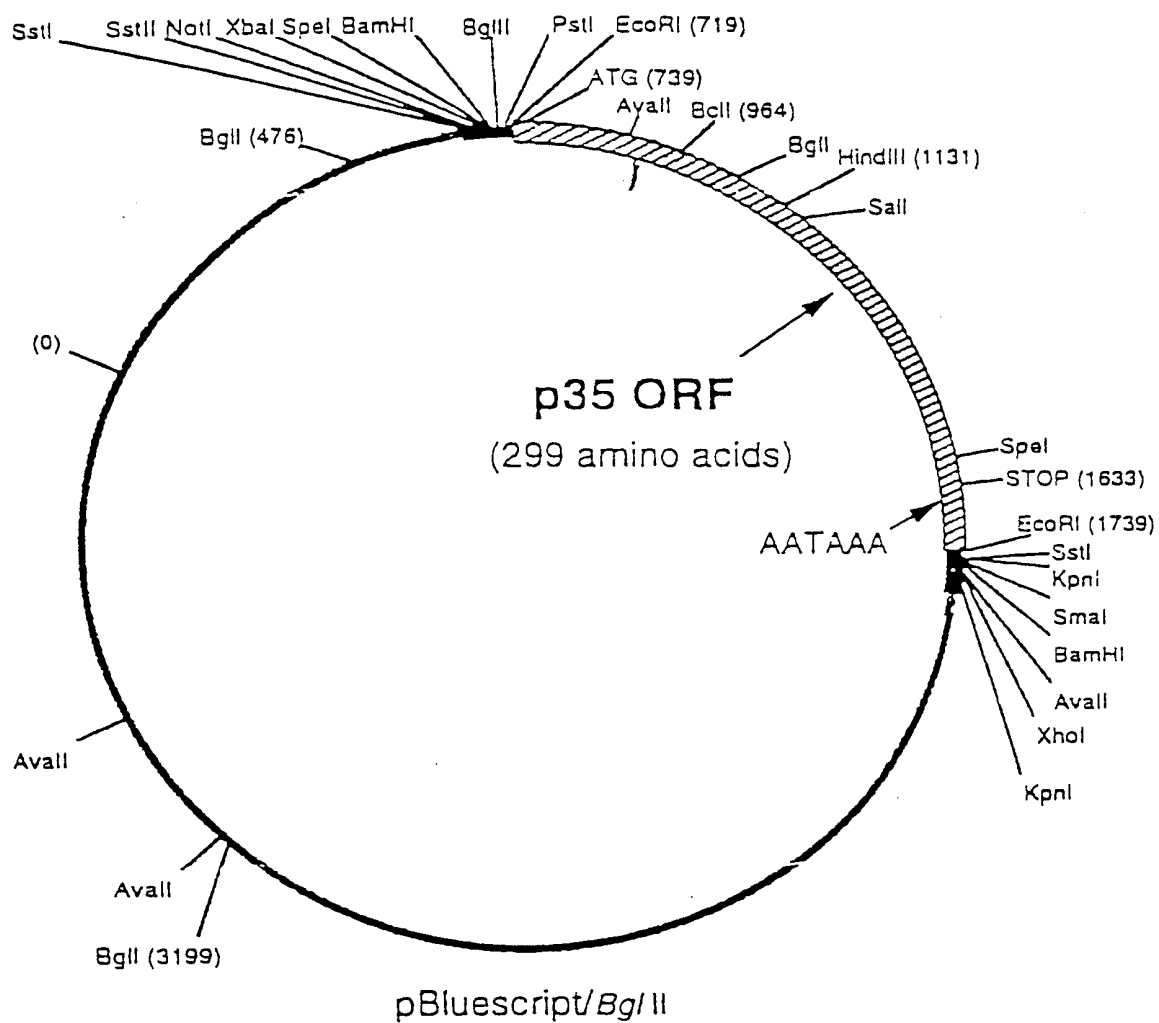
Plasmid pPRM⁻ 35K-ORF

Fig. 1(b)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/18067

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 5/04, 11/00; C07K 2/00; A01H 3/04, 5/00, 5/08

US CL : 435/410; 530/300; 800/295, 298, 306, 307, 316, 317, 320, 323

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/410; 530/300; 800/295, 298, 306, 307, 316, 317, 320, 323

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, Agricola, Biosis, Caplus

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HEATH, M. C. Apoptosis, Programmed Cell Death And The Hypersensitive Response. Eur. J. Plant Pathol. March 1998. Vol. 104. No. 2. pages 117-124, see entire document.	1-5
Y	D'SILVA et al. Activation Of Cysteine Proteases In Cowpea Plants During The Hypersensitive Response- A Form Of Programmed Cell Death. 1998. Exp. Cell Res. Vol. 245. pages 389-399, especially pages 392-394.	1-5, 7-13
Y	US 5,798,442 A (GALLANT et al) 25 August 1998, col. 26, line 47-col. 27, line 14, col. 28, line 54-col. 30, line 29, col. 32, line 16-col. 33, line 9.	1-5, 7-13

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

25 OCTOBER 1999

Date of mailing of the international search report

04 NOV 1999

Name and mailing address of the ISA/US
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/18067

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US 5,834,228 A (BECKER et al) 10 November 1998. col. 6, lines 47-61, col. 17, lines 8-64.	1-5, 7-13
Y,P	POZO et al. Caspases And Programmed Cell Death In The Hypersensitive Response Of Plants To Pathogens. Curr. Biol. 28 September 1998. Vol. 8. No. 20. pages 1129-1132, see entire document.	1-5, 7-13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/18067

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-5, 7-13

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/18067

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-5 and 7-13, drawn to a first method, comprising inhibiting apoptosis, comprising exogenous administration of a caspase inhibitor, not including Baculovirus p35 protease inhibitor, and a first product, a composition for horticultural application comprising a caspase inhibitor and surfactant.

Group II, claim(s) 6, drawn to a second method, comprising inhibiting apoptosis by plant transformation with a gene that expresses a caspase inhibitor, not including Baculovirus p35 protease inhibitor.

Group III, claim(s) 14-21, drawn to a third method, comprising increasing non-viral plant disease resistance, comprising expression of the Baculovirus p35 protease inhibitor; and a second product, comprising plants produced by the third method.

Group IV, claims 22-24, drawn to a fourth method, comprising increasing transformation rate of a plant cell with a heterologous gene, comprising first transfecting the cell with a p35 encoding gene, and then transfecting the cell again with the heterologous gene.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The composition and method of inhibiting apoptosis by exogenous application of a caspase inhibitor and surfactant of Group I is not shared by method of Group II, which requires genetic transformation; the method of disease resistance of Group III, which requires the Baculovirus p35 gene; nor the method of Group IV, which comprises two genetic transformation events. The transformation method of Group II is not shared with the exogenous application of Group I, the disease resistance method of Group III, nor the method of Group IV. The Baculovirus p35 gene of Group III is not shared with the methods and products of Groups I and II. The two transformation events and heterologous gene of Group IV are not shared with any of the other groups.