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(54) Title: METHOD OF CONTROLLING PLANT PATHOGENIC FUNGI

(57) Abstract

Acidic osmotin-like proteins control fungal damage to plants. Genes encoding for these proteins may be cloned into vectors for transformation of plant-colonizing microorganisms or plants, thereby providing a method of inhibiting fungal growth on plants.

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METHOD OF CONTROLLING PLANT PATHOGENIC FUNGI

This application is a continuation-in-part of U.S.S.N. 07/953,495, filed September 28, 1992.

FIELD OF THE INVENTION

This invention relates to a method of controlling plant pathogenic fungi by a protein which may be applied directly to the plant or produced thereon by microorganisms or by genetically modifying the plant to produce the protein, and to genes, microorganisms, and plants useful in that method.

BACKGROUND OF THE INVENTION

The use of natural products, including proteins, is a known method of controlling plant pathogens. For example, chitinases are recognized to inhibit the growth of certain fungi. European Patent Application 0 392 225 (Ciba-Geigy AG) discloses pathogenesis-related (PR) proteins and transgenic plants having resistance to attack by fungi.

European Patent Application 0 460 753 (Mogen International, 12/91) discloses antifungal PR proteins isolated from plants pretreated with a pathogen. Leaf extracts from tobacco and tomato yielded proteins of about 24 kD and having a high degree of homology to the known stress-induced protein osmotin. EP 0 460 753 (incorporated herein by reference) defines osmotins or osmotin-like proteins as proteins with an amino-acid sequence homology of more than 70% compared to osmotin from tobacco, preferably more than 80%, and a basic isoelectric point, hereinafter referred to as "pI", synthesis of which is correlated with osmotic adaptation of plant cells to high NaCl containing media, and which have an antifungal effect on at least one fungus. Sources of such proteins were reported to be maize, millet, soybean, carrot, cotton, potato, alfalfa, bean, and *Thaumatococcus daniellii*, the source of the sweet protein, thaumatin. The antifungal osmotin-like proteins disclosed in EP 0 460 753 are naturally expressed as intracellular proteins accumulated in vacuoles. A manipulation of the gene for this wild-type protein resulting in a deletion of

approximately 20 C-terminal amino acids was required for extracellular expression, which was advantageous for fungal resistance.

Woloshuk et al. in a journal article ("Pathogen-Induced Proteins with Inhibitory Activity toward *Phytophthora infestans*," The Plant Cell, Vol. 3, pp 619-628, 1991) report that an acidic PR-5 protein from TMV-induced tobacco plants is not antifungal.

SUMMARY OF THE INVENTION

It has been found that proteins having some homology to osmotin, but having an acidic pI, also have antifungal properties. It is an object of the present invention to provide acidic osmotin-like proteins capable of reducing or eliminating the damage caused by plant fungal pathogens and genes useful in producing such proteins. It is a further object of the present invention to provide genetic constructs for and methods of inserting such genetic material into microorganisms and plant cells. It is another object of the present invention to provide transformed microorganisms and plants containing such genetic material.

Additionally, the plant may also be transformed to co-express other antifungal proteins or insecticidal proteins, for example, using *Bacillus thurengiensis* (*B.t.*) genes. Examples of plants transformed to express *B.t.* genes are disclosed in European Patent Publication No. 0 385 962, which corresponds to U.S. Serial Number 07/476,661, filed February 12, 1990 [Fischhoff et al.], which is incorporated herein by reference. An advantage of co-expressing other antifungal proteins lies in the use of more than one mode of action for controlling fungal damage which minimizes the possibility of the development of resistant fungal strains.

In accomplishing the foregoing, there is provided, in accordance with one aspect of the present invention, a method of controlling fungal damage to plants by providing an acidic osmotin-like protein to the plant locus.

In accordance with another aspect of the present invention, there is provided a recombinant, double-stranded DNA molecule comprising in operative sequence:

a) a promoter which functions in plant cells to cause the production of an RNA sequence; and

b) a structural coding sequence that encodes for production of an acidic osmotin-like protein;

c) a 3' non-translated region which functions in plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the RNA sequence,

said promoter being heterologous with respect to the structural coding sequence.

In accordance with another aspect of the present invention, there is provided a method of producing genetically transformed plants which express an antifungal amount of an acidic osmotin-like protein, comprising the steps of:

a) inserting into the genome of a plant cell a recombinant, double-stranded DNA molecule comprising

(i) a promoter which functions in plant cells to cause the production of an RNA sequence;

(ii) a structural coding sequence that causes the production of an acidic osmotin-like protein;

(iii) a 3' non-translated region which functions in said plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the RNA sequence,

said promoter being heterologous with respect to the structural coding sequence;

b) obtaining transformed plant cells; and

c) regenerating from the transformed plant cells genetically transformed plants which express an fungal inhibitory amount of acidic osmotin-like protein.

There is also provided, in accordance with another aspect of the present invention, bacterial and transformed plant cells that contain DNA comprised of the above-mentioned elements (i), (ii), and (iii).

As used herein, the term "osmotin-like" is used relative only to the amino acid sequence, that is, a limitation only as to homology to osmotin, which is preferably above 60% similarity, more preferably above 70%

similarity. It is not a limitation as to the pI of any protein or any other characteristic of a protein except its homology to osmotin.

As used herein, the term "acidic" is used to indicate a protein having an isoelectric point of less than 7.

As used herein, the term "controlling fungal damage" is used to indicate causing a reduction in damage to a crop due to infection by a fungal pathogen.

As used herein, the term "structural coding sequence" means a DNA sequence which encodes for a polypeptide, which may be made by a cell following transcription of the DNA to mRNA, followed by translation to the desired polypeptide.

DETAILED DESCRIPTION OF THE INVENTION

The method of the present invention may be carried out in a variety of ways. The antifungal protein, prepared by various techniques, may be directly applied to plants in a mixture with carriers or other additives, including other antifungal agents. The protein may be expressed by bacterial or yeast cells which have been applied to the plant. Preferably plant cells are transformed by one or more means to contain the gene encoding an acidic osmotin-like protein which is expressed constitutively or in certain plant parts or upon exposure of the plant to the fungal infection.

One embodiment of the present invention comprises a protein isolated from the overripe fruits of a wild persimmon, *Diospyros texana*. This protein, designated P139, has been purified to homogeneity by ammonium sulfate precipitation, DEAE-Sepharose and C_{18} reverse phase column chromatography. The purified protein has a molecular weight of approximately 22.1 kD and an acidic pI, predicted to be approximately 4.1. It inhibited the growth of the agronomically important pathogen causing potato late blight (*Phytophthora infestans*) with an amount of less than 1 μg under the assay conditions. NH_2-terminal amino acid sequence analysis of the purified protein indicates that it has some degree of homology to osmotin, but unlike the osmotin-like proteins previously

reported, it is acidic and was isolated from a woody plant not under any known extraordinary stresses.

Other acidic osmotin-like proteins may be obtained by preparing a crude plant tissue extraction using an acidic grinding buffer; desalting the tissue extract and incubating at 4 °C; and centrifuging to remove insoluble material. An aliquot of the supernatant can be tested for the presence of antifungal activity, as described below, and, if found, the protein further purified by known methods for isolating a protein with an acidic pI. The proteinaceous nature of the antifungal activity can be tested by heating and/or protease treatment and similar testing. After sufficient purification of the proteinaceous antifungal activity some of its physical parameters may be determined, such as molecular weight, isoelectric point, hydrophobicity and the like, to aid in the choice of the next fractionation technique, in order to optimize purification. The choice of the optimal purification techniques, based on physical parameters, is well within the reach of a person skilled in the art of protein purification.

The plants that may be protected by the methods of the present invention will depend on the level of protection from the fungal pathogens for that plant. For example, many vegetables such as potatoes and tomatoes may be protected from *P. infestans* by the present methods. However, other *Phytophthora* species are pathogenic to many other plants, such as fruit trees or turf, and thus these plants may also be protected by the methods of the present invention.

As noted above, the antifungal proteins of the present invention may be used in combination with other antifungal proteins so as to provide a broad spectrum of activity, i.e., control additional pathogens, and/or provide multiple modes of action for the same fungal pathogen. Sources of such antifungal proteins might be plants, such as the proteins of the present invention, or may be microbial or other nonplant organisms.

BIOEFFICACY ASSAYS

Phytophthora infestans

Assays for activity against *P. infestans* were conducted with P139. The growth medium for the *P. infestans* assay was made from 100 mL V8 vegetable juice, 2 g calcium carbonate, 15 g bacto agar, and 900

mL water. The calcium carbonate was added to the V8 juice; then the mixture was decanted and combined with the rest of the ingredients. The medium was then autoclaved for 30 minutes. All reagents used were of the highest grade commercially available.

Antifungal activity of the protein was determined using a hyphal extension-inhibition assay as described by Roberts and Selitrennikoff. Typically, fungal mycelium was harvested from actively growing fungus and placed in center of a sterile Petri dish containing nutrient agar. After incubation of the dish at 20 °C for 48-72 hr to allow for mycelial growth in a symmetrically circular shape, sterile paper discs (Difco concentration disc, 1/4) were positioned on the agar approximately 1.5 cm from the mycelium. 35 µL of a Tris buffer solution (25 mM, pH 8.0) containing <1 µg of the protein was applied to each disc. The plate was incubated at 20 °C overnight. The antifungal activity was determined based on the zone of hyphal extension inhibition exhibited in the vicinity of the discs. P139 protein demonstrated inhibition of *P. infestans*. This activity showed up as the formation of crescent shaped curve at the edge of the mycelial growth that was approaching the disc.

PROTEIN IDENTIFICATION

The active protein P139 from *Diospyros texana* was isolated, purified, partially sequenced, and identified as having homology to osmotin, but unlike osmotin it is acidic.

Protein Isolation

All purification procedures were carried out at 4 °C unless otherwise noted. A total of 250 g of overripe fruits from *Diospyros texana* were extracted with a Tekmar® Tissumizer® homogenizer (Cincinnati, OH) in 750 mL 50 mM sodium acetate buffer, pH 5.0, for five 1 min intervals. The extraction was centrifuged at 10,000 x g for 30 minutes to separate debris. The supernatant was then subjected to a 75% ammonium sulfate precipitation. The precipitate was collected from the suspension by centrifugation at 40,000 x g for 45 min, resuspended in 50 mM sodium acetate buffer, pH 5.0, and concentrated using Centriprep 10. Only soluble

materials that could not pass through the filter membrane of 10 kDa cutoff were selected for further purification. The sample then was loaded on a PD-10 column according to the manufacture's instructions for desalting and also to remove any remaining small molecular weight molecules. The high molecular weight fraction was dialyzed overnight against 25 mM Tris-HCl buffer, pH 8.0, and applied to a DEAE-sepharose column (2 x 10 cm) equilibrated with the same buffer. Active fractions were eluted with a gradient of NaCl (0 to 0.75 M) in the above buffer. The active fractions were pooled and further purified at room temperature by RP-HPLC using a Vydac C_{18} analytical column equilibrated with 0.1 % trifluoroacetic acid (TFA).

Amino Acid Sequences

Protein samples were hydrolyzed for 24 hrs in 6 N HCl at 110 °C *in vacuo* and analyzed on a Beckman 630 High Performance Amino Acid Analyzer. Automated Edman degradation was carried out on an Applied Biosystems model 470A Protein Sequenator [Hunkpillar, 1983]. The respective PTH-amino acid derivatives were identified by reversed phase analysis in an on-line fashion employing an Applied Biosystems model 120 PTH Analyzer. Purity of the purified protein was confirmed by SDS-PAGE with silver staining and by amino acid sequencing.

GENETIC IDENTIFICATION

A gene for an acidic osmotic-like protein, designated P139, was isolated from *Diospyros texana* and its sequence determined.

Cloning of the P139 Gene

The P139 gene was isolated from a genomic DNA library, which was made using DNA isolated from leaf tissue of *Diospyros texana*. The genomic library was constructed from genomic DNA partially digested with MboI ligated into the BamHI site of the lambda EMBL3 vector essentially described by Sambrook et al. The library was screened using degenerate oligonucleotides designed to hybridize to the P139 gene (based on N-terminal amino acid sequence). A hybridizing clone, designated lambda clone #20, was identified as possibly carrying the P139 gene. A

2.2 kb SmaI fragment was subcloned from lambda clone #20 into pUC119 [Vieira] resulting in pMON8982. This subclone contained the entire coding region of the P139 gene as determined by DNA sequencing. The P139 gene sequence was thus determined to be that shown in SEQ ID NO:1. The translated protein sequence is shown as SEQ ID NO:2.

The P139 gene has a ORF (open reading frame) of 690 bp encoding a full length peptide of 230 amino acids. The ORF contains a 5' DNA sequence encoding a 23 amino acid amino-terminal signal sequence. This signal sequence is removed during protein processing and is not present in the mature native protein. An initiation codon has been engineered in front of the first codon of the DNA sequence for the mature protein for heterologous expression. The resulting DNA sequence encoding the mature protein is given in SEQ ID NO:3.

GENETIC TRANSFORMATION

An acidic osmotin-like protein gene can be isolated from plants such as *Diospyros texana*. This gene may then be used to transform bacterial cells or plant cells to enable the production of an acidic osmotin-like protein and carry out methods of this invention. Examples of how this may be done with the gene for P139 are given below.

Mutagenesis of the P139 Gene

In order to incorporate the P139 gene (SEQ ID NO:1) into vectors appropriate for expression of an acidic osmotin-like protein in heterologous bacterial or plant hosts, it was necessary to introduce appropriate restriction sites near the ends of the gene. The goals of this mutagenesis were to create cassettes that included the protein coding sequence with minimal noncoding flanking sequences and to incorporate useful restriction sites to mobilize these cassettes. Cassettes were designed that would allow mobilization of the intact coding sequence including the signal peptide or just the mature coding sequence. To incorporate these cassettes into appropriate bacterial or plant expression vectors, a BglII restriction site was engineered at the N-terminus of the intact protein sequence. A HindIII site and a BglII site were engineered just after the

termination codon of the intact coding sequence. In a separate mutagenesis, a NcoI restriction site was engineered at the N-terminus of the mature protein sequence, and a HindIII site and a BglII site were engineered just after the termination codon of the intact coding sequence.

Three mutagenesis primers were designed to create these cassettes, as shown below. Mutagenesis with primer GBP139-19 (SEQ ID NO:5) added one amino acid at the N-terminus of the mature protein. This was necessary to allow incorporation of the NcoI restriction site and to give the mature gene an initiation codon. The NcoI-HindIII fragment was needed only for transformation of *E. coli*, discussed below.

GBP139-18 (SEQ ID NO:4): GTTGATTGAGATCTAAAATGAGG
(BglII site underlined)

GBP139-19 (SEQ ID NO:5): CCTTCCGCCATGGCCACCTTCG
(NcoI site underlined)

GBP139-20 (SEQ ID NO:6):
CCCGTGAAGCTTATGAAGATCTTCTCCATCG
(HindIII and BglII sites underlined)

Expression of P139 protein in *E. coli*

A NcoI-HindIII fragment containing only the mature protein coding sequence (SEQ ID NO:3) was inserted into pKK233-2 (Pharmacia, Piscataway, NJ), a vector designed for protein expression in *E. coli*. This cloning placed the P139 gene under the control of the *E. coli* tac promoter. Expression in *E. coli* led to the production of P139 protein as determined by western blot analysis.

Expression of P139 protein in Yeast

A BglII-BglII fragment containing the full length protein coding sequence (SEQ ID NO:1) was inserted into pYES2 (Invitrogen, San Diego, CA), a vector designed for protein expression in yeast. This cloning placed the P139 gene under control of the yeast gal10 promoter. Expression in yeast led to the production of P139 protein as determined by western blot analysis. However, the P139 protein was not efficiently excreted by the

yeast cells; a majority of the P139 protein was found to be retained in the yeast cells.

Expression of P139 in Baculovirus

A BglII-BglII fragment containing the full length protein coding sequence (SEQ ID NO:1) was inserted into pVL1392, a vector for transfection of baculovirus with a heterologous gene [Luckow]. This cloning placed the P139 gene under the control of the Baculovirus polyhedrin promoter. The P139 gene was detected in the transfected virus using dot blot analysis. P139 protein was expressed by the baculovirus as detected by western blot analysis.

Expression of an Acidic Osmotin-like Protein in Plant Colonizing Bacteria

To prevent fungal attack and damage to plants, it may be desirable to transform plant colonizing bacteria to express an acidic osmotin-like protein, and then apply these bacteria to the plant. When the fungus attacks the plant, it encounters a sufficient dose of the acidic osmotin-like protein produced by the plant colonizers which prevents the disease from damaging the plant. Plant colonizers can be either those that inhabit the plant surface, such as *Pseudomonas* or *Agrobacterium* species, or endophytes that inhabit the plant vasculature such as *Clavibacter* species. For surface colonizers, the acidic osmotin-like protein gene may be inserted into a broad host range vector capable of replicating in these Gram-negative hosts. Examples of these such vectors are pKT231 of the IncQ incompatibility group [Bagdasarian et al., 1981] or pVK100 of the IncP group [Knauf, 1982]. For endophytes the acidic osmotin-like protein gene can be inserted into the chromosome by homologous recombination or by incorporation of the gene onto an appropriate transposon capable of chromosomal insertion in these endophytic bacteria.

Plant Gene Construction

The expression of a plant gene which exists in double-stranded DNA form involves transcription of messenger RNA (mRNA) from one

strand of the DNA by RNA polymerase enzyme, and the subsequent processing of the mRNA primary transcript inside the nucleus. This processing involves a 3' non-translated region which adds polyadenylate nucleotides to the 3' end of the RNA. Transcription of DNA into mRNA is regulated by a region of DNA usually referred to as the "promoter." The promoter region contains a sequence of bases that signals RNA polymerase to associate with the DNA and to initiate the transcription of mRNA using one of the DNA strands as a template to make a corresponding strand of RNA.

A number of promoters which are active in plant cells have been described in the literature. These include the nopaline synthase (NOS) and octopine synthase (OCS) promoters (which are carried on tumor-inducing plasmids of *Agrobacterium tumefaciens*), the cauliflower mosaic virus (CaMV) 19S and 35S promoters, the Figwort Mosaic Virus (FMV) 35S promoter, and the light-inducible promoter from the small subunit of ribulose 1,5-bis-phosphate carboxylase (ssRUBISCO, a very abundant plant polypeptide). All of these promoters have been used to create various types of DNA constructs which have been expressed in plants (see e.g., PCT publication WO 84/02913).

Alternatively, the promoters utilized in the double-stranded DNA molecules may be selected to confer specific expression of the acidic osmotin-like protein gene protein in response to fungal infection. The infection of plants by fungal pathogens triggers the induction of a wide array of proteins, termed defense-related or pathogenesis-related (PR) proteins [Bowles; Bol et al.; Linthorst]. Such defense-related or PR genes may encode enzymes (such as phenylalanine ammonia lyase, chalcone synthase, 4-coumarate coA ligase, coumaric acid 4-hydroxylase) of phenylpropanoid metabolism, proteins that modify plant cell wall (such as hydroxyproline-rich glycoproteins, glycine-rich proteins, peroxidases), enzymes (such as chitinases and glucanases) that degrade the fungal cell wall, thaumatin-like proteins, or proteins of as yet unknown function. The defense-related or PR genes have been isolated and characterized from a number of plant species. The promoters of these genes may be used to attain expression of acidic osmotin-like protein gene protein in

transgenic potato plants when challenged with *P. infestans*. Such promoters may derive from defense-related or PR genes isolated from potato itself [Fritzemeier et al.; Cuypers et al.; Logemann et al.; Matton and Brisson; Taylor et al.; Matton et al.; Schroder et al.].

Alternatively, the promoters utilized in the double-stranded DNA molecules may be selected to confer specific expression in tissues where the protein is most effective, such as the flowering parts of the plant.

The particular promoter selected should be capable of causing sufficient expression of the enzyme coding sequence to result in the production of an effective amount of acidic osmotin-like protein. A preferred promoter is a constitutive promoter such as FMV35S.

The promoters used in the DNA constructs (i.e. chimeric plant genes) of the present invention may be modified, if desired, to affect their control characteristics. For example, the CaMV35S promoter may be ligated to the portion of the ssRUBISCO gene that represses the expression of ssRUBISCO in the absence of light, to create a promoter which is active in leaves but not in roots. The resulting chimeric promoter may be used as described herein. For purposes of this description, the phrase "CaMV35S" promoter thus includes variations of CaMV35S promoter, e.g., promoters derived by means of ligation with operator regions, random or controlled mutagenesis, etc. Furthermore, the promoters may be altered to contain multiple "enhancer sequences" to assist in elevating gene expression. Examples of such enhancer sequences have been reported by Kay et al. (1987).

An enhanced CaMV35S promoter has been constructed as follows. A fragment of the CaMV35S promoter extending between position -343 and +9 was previously constructed in pUC13 [Odell et al., 1985]. This segment contains a region identified as being necessary for maximal expression of the CaMV35S promoter. It was excised as a ClaI-HindIII fragment, made blunt ended with DNA polymerase I (Klenow fragment) and inserted into the HincII site of pUC18. This upstream region of the 35S promoter was excised from this plasmid as a HindIII-EcoRV fragment (extending from -343 to -90) and inserted into the same plasmid between the HindIII and PstI sites. The enhanced CaMV35S

promoter (hereafter "CaMV E35S") thus contains a duplication of sequences between -343 and -90 [Kay et al., 1987].

The RNA produced by a DNA construct of the present invention also contains a 5' non-translated leader sequence. This sequence can be derived from the promoter selected to express the gene, and can be specifically modified so as to increase translation of the mRNA. The 5' non-translated regions can also be obtained from viral RNA's, from suitable eukaryotic genes, or from a synthetic gene sequence. The present invention is not limited to constructs wherein the non-translated region is derived from the 5' non-translated sequence that accompanies the promoter sequence. Rather, the non-translated leader sequence can be derived from an unrelated promoter or coding sequence. For example, the petunia heat shock protein 70 (Hsp70) contains such a leader. [Winter]

As noted above, the 3' non-translated region of the chimeric plant genes of the present invention contains a polyadenylation signal which functions in plants to cause the addition of adenylate nucleotides to the 3' end of the RNA. Examples of preferred 3' regions are (1) the 3' transcribed, non-translated regions containing the polyadenylate signal of *Agrobacterium* tumor-inducing (Ti) plasmid genes, such as the nopaline synthase (NOS) gene and (2) plant genes like the soybean 7s storage protein genes and the pea ssRUBISCO E9 gene. [Fischhoff, et al.]

Plant Transformation and Expression

A chimeric plant gene containing a structural coding sequence of the present invention can be inserted into the genome of a plant by any suitable method. Suitable plant transformation vectors include those derived from a Ti plasmid of *Agrobacterium tumefaciens*, as well as those disclosed, e.g., by Herrera-Estrella (1983), Bevan (1983), Klee (1985) and EPO publication 0 120 516 (Schilperoort et al.). In addition to plant transformation vectors derived from the Ti or root-inducing (Ri) plasmids of *Agrobacterium*, alternative methods can be used to insert the DNA constructs of this invention into plant cells. Such methods may involve, for example, the use of liposomes, electroporation, chemicals that increase

free DNA uptake, free DNA delivery via microprojectile bombardment, and transformation using viruses or pollen.

A *E. coli* plasmid cassette vector, designated pMON8998, was preliminarily constructed for transformation of dicotyledonous plants. The BglII-BglII fragment containing the full length protein coding sequence was inserted into a previously constructed vector containing an FMV 35S promoter with a petunia Hsp70 leader sequence. The 3' nontranslated polyadenylation sequence of the NOS gene was also provided as the terminator. The vector also contained a multilinker site between the leader and the terminator sequences, NotI sites before and after the promoter and the terminator sequences, and an ampicillin resistance site.

The NotI-NotI 1.7 kb fragment from pMON8998, containing the FMV 35S promoter, the Hsp70 leader, the full length P139 gene (SEQ ID NO:1), and the NOS 3' terminator, was inserted in both orientations into the NotI site of pMON17227, a Ti plasmid vector disclosed and described by Barry et al. in WO 92/04449 (1991), incorporated herein by reference, to produce pMON8993 and pMON8994. This vector contained the glyphosate resistance gene described by Barry for future selection of transformed plants.

An additional plant vector was constructed by inserting the BglII-BglII fragment containing the full length protein coding sequence into a BglII site of a previously constructed plasmid so as to place the P139 gene under the control of the CaMV E35S promoter, discussed above. A NotI-NotI fragment from this resulting plasmid, containing the CaMV E35S promoter, the full length P139 gene, and the 3' terminator from the pea ssRUBISCO E9 gene, was inserted into the NotI site of pMON17227, described above, to make pMON22511.

Transient Expression of Acidic Osmotin-like Protein in Plant Protoplasts

Transient expression of the P139 gene in plant protoplasts was tested using the vectors constructed as described above. For transient expression analysis, DNA of pMON8998, pMON8993, pMON8994 and pMON22511 was purified and introduced into tobacco protoplasts by elec-

troporation. Protoplasts containing the plasmid DNA were incubated for 24 to 48 hours and then the cells and the incubation medium were analyzed immunologically for the presence of the P139 protein to demonstrate expression of the gene in plant cells. Western blot analysis showed that P139 protein is expressed from all four constructs tested. Protein was in both the media supernatant and the intact protoplasts. Higher levels were found to be in the supernatant indicating that the P139 is being excreted from the plant protoplasts.

pMON8998 is a vector for expression of the P139 gene in plant cells, but this vector lacks appropriate sequences for use in *Agrobacterium*-mediated plant transformation. However, this vector can be used for either transient expression of protein in plant cells, or it can be used to generate stably transformed plants via free DNA delivery such as biolistic bombardment of potato meristems.

Stable Transformation of Potato Plants with an Acidic Osmotin-like Protein Gene

Vectors pMON8993, 8994, and 22511 were introduced into disarmed *Agrobacterium* ABI and used to transform potato explants in tissue culture. After selection for glyphosate resistance and plant regeneration, whole potato plants containing the P139 coding sequences were recovered. Potato plants containing the P139 gene were confirmed to be expressing the gene by both disease resistance assay and immunoassay.

Antibodies to a synthetic peptide identical in sequence to the first twelve amino acids of the P139 protein were raised. These antibodies did not cross-react with the endogenous potato osmotins. The western blot analysis showed that the transgenic plants made with vectors pMON8993, 8994, and 22511, were producing a cross-reactive protein of the predicted size. Further analysis to show extracellular targeting of P139 in potato was performed. Extracellular fluid was collected from leaves of F1 transgenics and non transgenic control plants. After the isolation of the extracellular fluid, proteins were extracted from the remaining leaf material. Western blots using the P139 specific antibody

show that detectable levels of P139 are found only in the extracellular fluid indicating that P139 is indeed targeted into the extracellular space.

To assess the fungal resistance to *P. infestans* of whole potato plants transformed with pMON8993 and pMON8994, 20 plants that grew on selective media are sprayed with a suspension of 1 x 10^5 sporangia/ml of the fungus until wet. As a control 20 nontransformed plants and 20 plants transformed with a vector without the P139 gene were sprayed with an equal amount of sporangia. The plants were grown in a growth chamber at 19 °C and air humidity of 90-95%. The progress of disease was evaluated at day 7 after spraying by determining the leaf area and stem area that is affected by the fungus. The plants are rated on a 0 to 4 scale with 0 being low or no disease and 4 being highest disease levels. Five clonal plantlets of each putative transformation event are scored and an average composite score is assigned. The score for that transformation event is then assigned to a group. In the Table 1 below the percentage of transformation events falling into each disease grouping is shown. As can be seen, the plants transformed with pMON8993 and pMON8994 exhibit little or no disease after exposure to Phytophthora infestans, whereas the controls all show at least 25% disease with most plants showing greater than 50% infection.

TABLE 1

	Disease Score*				
	0	1	2	3	4
Non-transgenic	-	-	17%	50%	33%
vector control	-	-	11%	33%	56%
pMON8993	25%	15%	15%	30%	15%
pMON8994	5%	16%	21%	21%	37%

0=0-10% infection, 1=10-25% infection, 2=25-50% infection, 3=50-85% infection, 4=85-100% infection.

Two tests, which were similar to those described for pMON8993 and pMON8994, were conducted on plantlets transformed with pMON22511. A shift in the population of putative transformants toward less disease development versus the controls was not observed in either

test with construct pMON22511, however plantlets from one transformation event with that vector, pMON22511-12, had the least amount of late blight development in both tests.

Plant lines transformed to express P139 which showed little or no late blight disease development in initial testing were evaluated more rigorously. Experiments were conducted using a randomized complete block design with 12 to 24 replicates (three clonal plantlets per replicate). Nontransformed and vector only plantlets were included as controls. Plantlets were inoculated with a suspension of 5 x 10^4 sporangia/mL to uniform wetness, and incubated at 19 °C. Plantlets were scored for late blight development seven, eight, and nine days post inoculation. The amount of infected tissue was scored using a 0 - 9 rating scale (Cruickshank et. al., *Potato Res.* 25 (1982) 213-214), where 9 = 0% disease, 8 ≤ 10% disease, 7 = 11-25% disease, 6 = 26-40% disease, 5 = 41-60% disease, 4 = 61-70% disease, 3 = 71-80% disease, 2 = 81-90% disease, 1 > 90% disease.

The results of this experiment, shown in Table 2 below, indicated that transformed plantlets containing constructs pMON8994-14, pMON8994-1, and pMON8994-11 had less late blight development (i.e., higher disease evaluation scores) than the nontransgenic or vector controls. The difference between pMON8994-14 and the controls was statistically significant at seven and eight days post inoculation.

TABLE 2

0 - 9 Late Blight Evaluation Score‡

Line	N	Day 7	Day 8	Day 9
pMON8994-14	12	6.67 a	5.83 a	4.50 a
pMON8994-1	12	5.83 abc	4.92 ab	4.33 a
pMON8994-11	12	6.33 ab	4.75 abc	4.08ab
Non Transgenic	12	5.50 bc	4.42 bc	3.58ab
Vector Control	12	5.75 abc	4.08 bc	3.17ab
pMON8994-17	12	4.91 c	3.58 c	2.83 b
F Value		2.81	2.92	1.93
MSE		1.62	2.44	2.77

† Disease evaluation scores followed by the same letter are not significantly different by LSD, α=0.05.

In a separate study of tranformants made with pMON22511, the results, reported in Table 3, similarly indicated that transformed plantlets containing construct pMON22511-12 had less late blight development than the controls and the difference between pMON22511-12 and the vector control was statistically significant seven and eight days post inoculation.

TABLE 3

0 - 9 Late Blight Evaluation Score†

Line	N	Day 7	Day 8
pMON22511-12	12	6.50 a	3.50 a
Non Transgenic	12	5.83 ab	2.75 ab
Vector Control	12	4.83 b	2.00 b
F Value		3.85	3.04
MSE		2.19	2.21

† Disease evaluation scores followed by the same letter are not significantly different by LSD, α=0.05.

Transformed plantlets for which results are given above were reevaluated and the results given in Table 4 below. The number of replicates was increased to 24 to increase the power to discern differences between lines. Results confirmed previous experiments showing pMON8994-14 and pMON22511-12 to have less disease than the controls. The difference between the controls and pMON8994-14 was significant at all evaluation times, and the difference between the controls and pMON22511-12 was significant nine days post inoculation.

TABLE 4

0 - 9 Late Blight Evaluation Score†

Line	N	Day 7	Day 8	Day 9
pMON8994-14	24	4.38 a	2.80 a	1.75 a
pMON22511-12	24	3.58 b	2.50 ab	1.67 a
pMON8994-1	24	2.96 bc	1.96 bc	1.38 ab
Non Transgenic	19	2.89 bc	1.89 bc	1.16 b

Vector Control	24	3.37	bc	1.88	bc	1.04 b
pMON8994-11	24	3.33	bc	1.83	c	1.08 b
pMON8994-17	24	2.71	c	1.58	c	1.25 b
F Value		3.89		3.55		4.19
MSE		1.88		1.20		0.44

† Disease evaluation scores followed by the same letter are not significantly different by LSD, α=0.05.

Tomato explants may also be transformed with pMON8993, 8994, or 22511 using disarmed *Agrobacterium* hosts such as ABI or ACO. Such tomato plants will express P139 which may be confirmed by immunoassay and disease resistance assays.

That the wild type P139 protein is secreted extracellularly is suggested by the fact that the deduced amino acid sequence of the P139 protein lacks the carboxy-terminal propeptide (CTPP) that is found in the vacuolar thaumatin-like proteins. As noted above, the expression of the P139 protein in transformed plant cells also led to the secretion of the mature protein further supporting the extracellular targeting of the mature P139 protein.

However, it might be advantageous to target the acidic osmotin-like protein to the vacuole. It might be possible to target the protein to the vacuole by attaching the carboxy-terminal propeptide sequence of a vacuolar thaumatin-like or osmotin-like protein from another plant to the protein sequence of an acidic osmotin-like protein gene of the present invention at its carboxy-terminal end. The carboxy-terminal propeptide sequences of the vacuolar proteins such as barley and rice lectins, wheat germ agglutinin, tobacco ß-1,3-glucanase and chitinase could also be attached to the carboxy-terminus of the acidic osmotin-like protein in order to localize the protein in the vacuole.

In another embodiment of the invention, amino-terminal propeptide sequences of some plant vacuolar proteins could also be used to localize the acidic osmotin-like protein in the vacuole. Such amino-terminal propeptide sequences could be derived from the vacuolar proteins such as sporamin, a storage protein from sweet potato or 22 kd protein

and cathepsin D inhibitor from potato or a thiol protease from barley. The amino-terminal propeptide sequences of these proteins could be inserted between the signal peptide sequence and the mature protein sequence of the P139 protein by site-directed mutagenesis.

All publications and patents mentioned in this specification are herein incorporated by reference as if each individual publication or patent was specifically and individually stated to be incorporated by reference.

From the foregoing, it will be seen that this invention is one well adapted to attain all the ends and objects hereinabove set forth together with advantages which are obvious and which are inherent to the invention.

It will be understood that certain features and subcombinations are of utility and may be employed without reference to other features and subcombinations. This is contemplated by and is within the scope of the claims.

Since many possible embodiments may be made of the invention without departing from the scope thereof, it is to be understood that all matter herein set forth or shown in the accompanying drawings is to be interpreted as illustrative and not in a limiting sense.

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SEQUENCE LISTING

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Shah, Dilipkumar M.
Vu, Linh V.

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(iii) NUMBER OF SEQUENCES: 6

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
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(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/953495
(B) FILING DATE: 28-SEP-1992

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(B) REGISTRATION NUMBER: 32,963
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(B) TELEFAX: (314)537-6047

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 693 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..693

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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192
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    50                  55                  60

GGC ACC GTC CAA GCC CGC ATC TGG GGA CGC ACC ACC TGC AAC TTC GAC
240
Gly Thr Val Gln Ala Arg Ile Trp Gly Arg Thr Thr Cys Asn Phe Asp
65                  70                  75                  80

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Gly Ser Gly His Gly Ser Cys Gln Ser Gly Asp Cys Asn Gly Leu Leu
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GAA TGC AAA GGC TAC GGC AGC CCG CCC AAC ACC CTC GCC GAG TTC GCC
336
Glu Cys Lys Gly Tyr Gly Ser Pro Pro Asn Thr Leu Ala Glu Phe Ala
            100                 105                 110

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 230 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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            180                 185                 190

Leu Ser Lys Phe Phe Lys Glu Arg Cys Pro Asp Ala Tyr Ser Tyr Pro
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Gln Asp Asp Pro Thr Arg Leu Phe Thr Cys Pro Ala Gly Thr Asn Tyr
    210                 215                 220

Lys Val Ile Phe Cys Pro
225                 230
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 627 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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AGCCCGCCCA ACACCCTCGC CGAGTTCGCC CTCAACCAAC CTAACAACGT CGACTTCGTC300

GACATCTCCA ACGTTGACGG ATTCAACATC CCGATGGAAT TCAGCCCCCT CAACGCCGGC360

ACCTGCAAGG ATCTCAAGTG CACCGCCGAC ATTGTCCGGC AGTGCCCGGC GGAGCTGAAG420

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GACGGGCCGG GGAGCTGCTC GGAGACGCCT TTGTCGAAGT TTTTCAAGGA ACGGTGTCCA540

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCCGTGAAGC TTATGAAGAT CTTCTCCATC G 31

WHAT IS CLAIMED IS:

1. A method of controlling fungal damage to plants comprising providing to the plant locus an acidic osmotin-like protein which inhibits fungal growth.

2. The method of Claim 1 wherein the fungus is *Phytophthora* sp.

3. The method of Claim 1 wherein said protein is provided by plant-colonizing microorganisms which produce an acidic osmotin-like protein which inhibits fungal growth.

4. The method of Claim 1 wherein said protein is provided by expression of a gene for the protein incorporated in the plant by previous genetic transformation of a parent cell of the plant.

5. The method of Claim 1 wherein said protein has the amino acid sequence SEQ ID NO:2.

6. The method of Claim 1 wherein said protein is P139.

7. A gene for an acidic osmotin-like protein comprising SEQ ID NO:1.

8. A recombinant, double-stranded DNA molecule comprising in operative sequence:

a) a promoter which functions in plant cells to cause the production of an RNA sequence;

b) a structural coding sequence that encodes for production of an acidic osmotin-like protein which inhibits fungal growth; and

c) a 3' non-translated region which functions in plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the RNA sequence,

said promoter being heterologous with respect to the structural coding sequence.

9. The DNA molecule of Claim 8 wherein said structural DNA sequence is SEQ ID NO:1.

10. The DNA molecule of Claim 8 wherein said promoter is selected from FMV35S and CaMV35S promoters.

11. A method of producing genetically transformed plants which express a fungal inhibitory-effective amount of an acidic osmotin-like protein, comprising the steps of:

a) inserting into the genome of a plant cell a recombinant, double-stranded DNA molecule comprising

(i) a promoter which functions in plant cells to cause the production of an RNA sequence;

(ii) a structural coding sequence that causes the production of an acidic osmotin-like protein which inhibits fungal growth;

(iii) a 3' non-translated region which functions in said plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the RNA sequence,

said promoter being heterologous with respect to the structural coding sequence;

b) obtaining transformed plant cells; and

c) regenerating from the transformed plant cells genetically transformed plants with express a fungal inhibitory-effective amount of an acidic osmotin-like protein.

12. The method of Claim 11 wherein said structural DNA sequence is SEQ ID NO:1.

13. The method of Claim 11 wherein said promoter is selected from FMV35S and CaMV35S promoters.

14. A plant produced by the method of Claim 11.

15. The plant of Claim 14 wherein additional antifungal protein genes have been inserted into the genome of the plant.

16. The plant of Claim 14 also containing one or more genes expressing *B.t.* endotoxins.

17. The plant of Claim 14 which is a potato plant.

18. A potato seedpiece produced by a plant of Claim 17.

INTERNATIONAL SEARCH REPORT

Inter ıal Application No
PCT/US 93/07882

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/29 C12N15/82 A01N65/00 A01H5/00

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)
IPC 5 C12N A01N A01H

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 practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE PLANT CELL vol. 1, no. 3 , March 1989 pages 285 - 291 LINTHORST, H.J.M., ET AL. 'Constitutive expression of pathogenesis-related proteins PR-1, GRP, and PR-S in tobacco has no effect on virus infection' see the whole document --- -/--	8,10,11, 13,14

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"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

"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

"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

"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.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 December 1993	18 -01- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Maddox, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIOLOGICAL ABSTRACTS, xol. 94, no. 4 15 August 1992, Philadelphia, PA, US; abstract no. 44165, VIGERS, A.J., ET AL. 'Thaumatin-like pathogenesis-related proteins are antifungal' see abstract & PLANT SCI.(LIMERICK) vol. 83, no. 2 , 1992 pages 155 - 161	1-4,8, 10,11, 13-15, 17,18
Y	EP,A,0 460 753 (MOGEN) 11 December 1991 cited in the application see the whole document	1-4,8, 10,11, 13-15, 17,18
Y	EP,A,0 392 225 (CIBA-GEIGY) 17 October 1990 cited in the application see example 16	1-4,8, 10,11, 13-15, 17,18
A	PLANT MOLECULAR BIOLOGY vol. 11 , 1988 pages 223 - 224 PAYNE, G., ET AL. 'Isolation and nucleotide sequence of a novel cDNA clone encoding the major form of pathogenesis-related protein R' see the whole document	1-18
A	NATURE vol. 321 , 1986 pages 531 - 532 CORNELISSEN, B.J.C., ET AL. 'A tobacco mosaic virus-induced protein is homologous to the sweet-tasting protein thaumatin' see the whole document	1-18
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