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Utility of Phylloplanins as Antibiotics, Selective Fungicides and for Enhancing Microbial Resistance in Crop Plants

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(54) **UTILITY OF PHYLLOPLANINS AS ANTIBIOTICS, SELECTIVE FUNGICIDES AND FOR ENHANCING MICROBIAL RESISTANCE IN CROP PLANTS**

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A61K 38/00 (2006.01)
C07K 14/00 (2006.01)

(52) **U.S. Cl.** **530/350**; 514/2

(58) **Field of Classification Search** None
See application file for complete search history.

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(74) Attorney, Agent, or Firm—Crowell & Moring LLP

(57) **ABSTRACT**

The invention is directed to methods and compositions that inhibit pathogen proliferation. More particularly, these methods and compositions involve the use of phylloplane proteins as antibiotics against human and animal pathogens.

6 Claims, 10 Drawing Sheets

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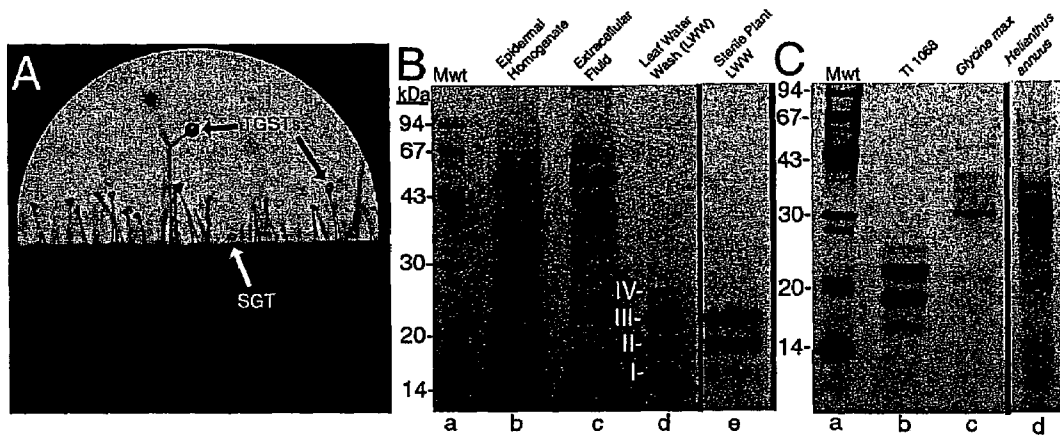


Figure 1. Proteins are present on plant leaf surfaces.

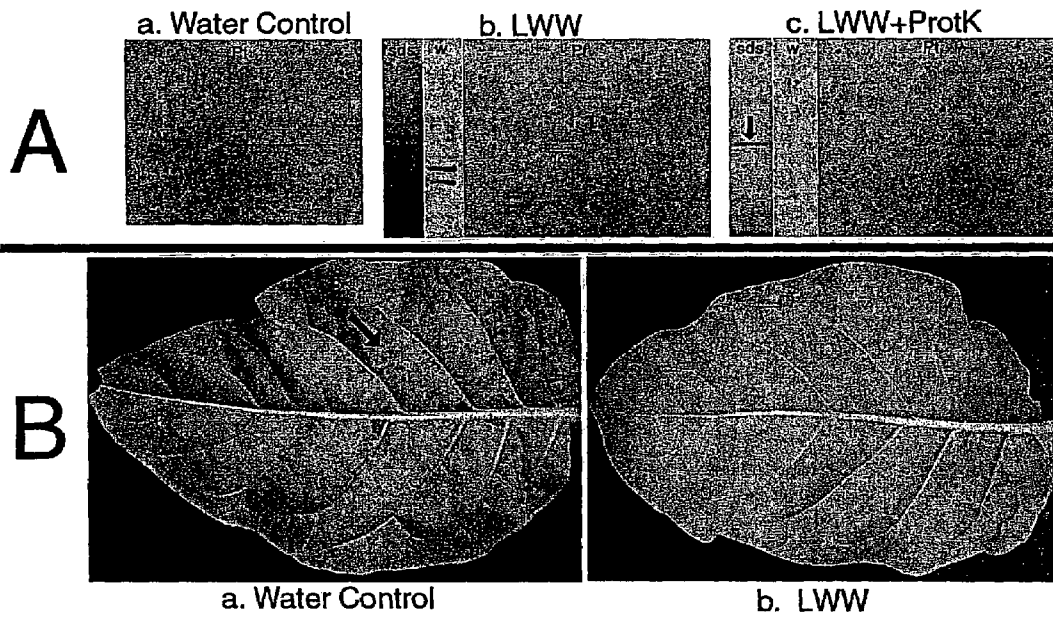


Figure 2. Proteins in T1 1068 LWW inhibit *P. tabacina* spore germination and leaf infection.

AACACAATTTCTTACAGCAATAACTATCACATATAACAATAACTGCC	M A S	ATG GCT TCA	56
A K I F L I F L L A A L I A T			
GCA AAA ATT TTC TTG ATT TTC CTT TTG GCT GCA TTA ATC GCA ACC			101
		aa-N1	
P A A F A I L V P T L V S T H			
CCC GCT GCA TTT GCC ATA CTT GTT CCA ACA CTT GTT TCA ACA CAT			146
I S G L V F C S V N G N L D V			
ATA AGT GGG CTT GTA TTT TGC AGC GTT AAC GGC AAT TTA GAT GTC			191
		aa-T1	
I N G L S P Q V F P N A S V Q			
ATC AAC GGA CTC AGT CCC CAA GTT TTT CCT AAT GCA TCA GTG CAA			236
		aa-T2	
L R C G A T N V I S S T I T N			
TTG CGG TGT GGA GCA ACA AAT GTG ATA TCA AGT ACA ATA ACA AAT			281
G S G A F S L A V N T F P L L			
GGA TCG GGA GCA TTT TCC TTG GCG GTG AAT ACT TTC CCA CTG CTA			326
		aa-T3	
N C N L V V A T P L S T C N A			
AAC TGC AAT TTA GTG GTT GCA ACT CCA CTA TCA ACA TGT AAC GCG			371
T L Q S V G R L A S S L R L V			
ACC TTA CAA TCG GTT GGG CGT TTG GCG TCA TCC TTG AGA CTT GTA			416
		aa-P1	
N I T L G S G T G L I R V G L			
AAT ATC ACT CTT GGC AGT GGC ACC GGT CTT ATT AGA GTC GGT TTA			461
		aa-T4	
A P T G F I L N L N I N			
GCT CCT ACT GGT TTT ATA CTT AAT CTT AAC ATC AAT TAA TATTGAAC		*(SEQ ID NO:18)	508
GAGCTAGCCTGCTGGTTCTTAATTAGTACTACTACTATGCATCAGCTAGTTAACTTTCTT			568
GGCCAGCTGCTTACTGCAAGAATAAGGACTGTTGTTCCACTAGTGAATAAAGTGCAAT			628
CATATTTGCAAGTCTAAAAAAAAAAAAAAAAAAAAAAAAAAAA		(SEQ ID NO:17)	666

Figure 3. Nucleotide and predicted amino acid sequence of TI 1068 *Phylloplanin* cDNA.

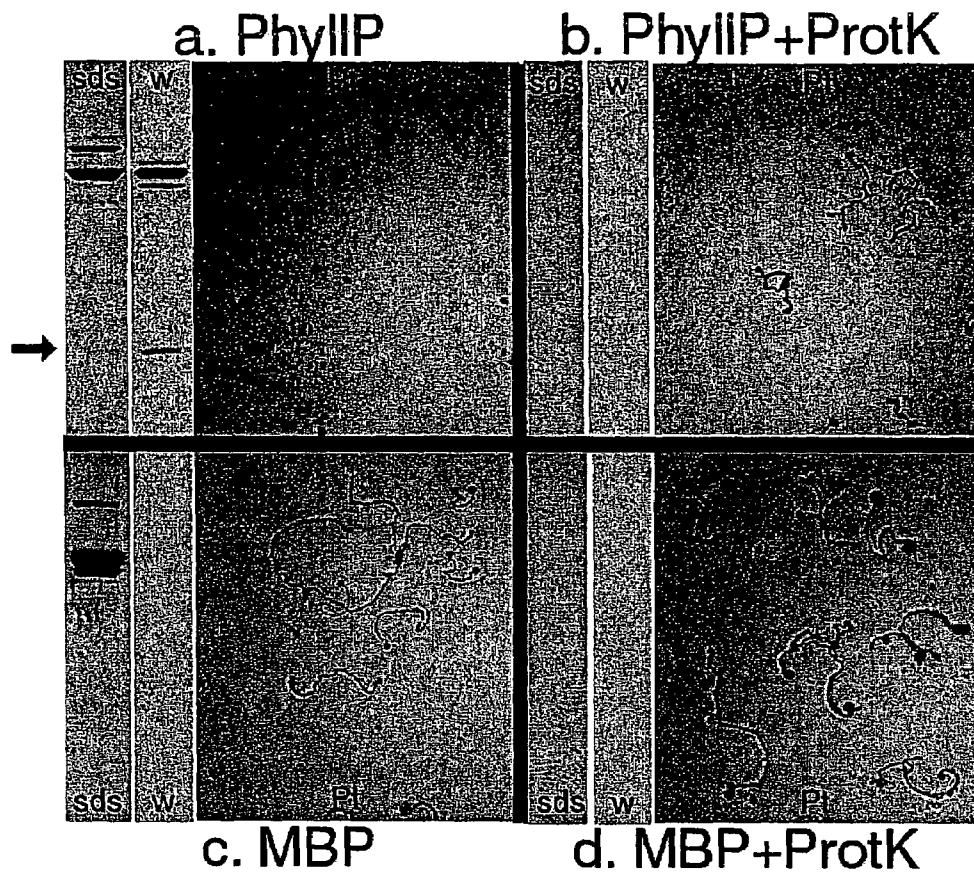


Figure 4. *E. coli*-expressed TI 1068 phylloplanin inhibits *P. tabacina* spore germination.

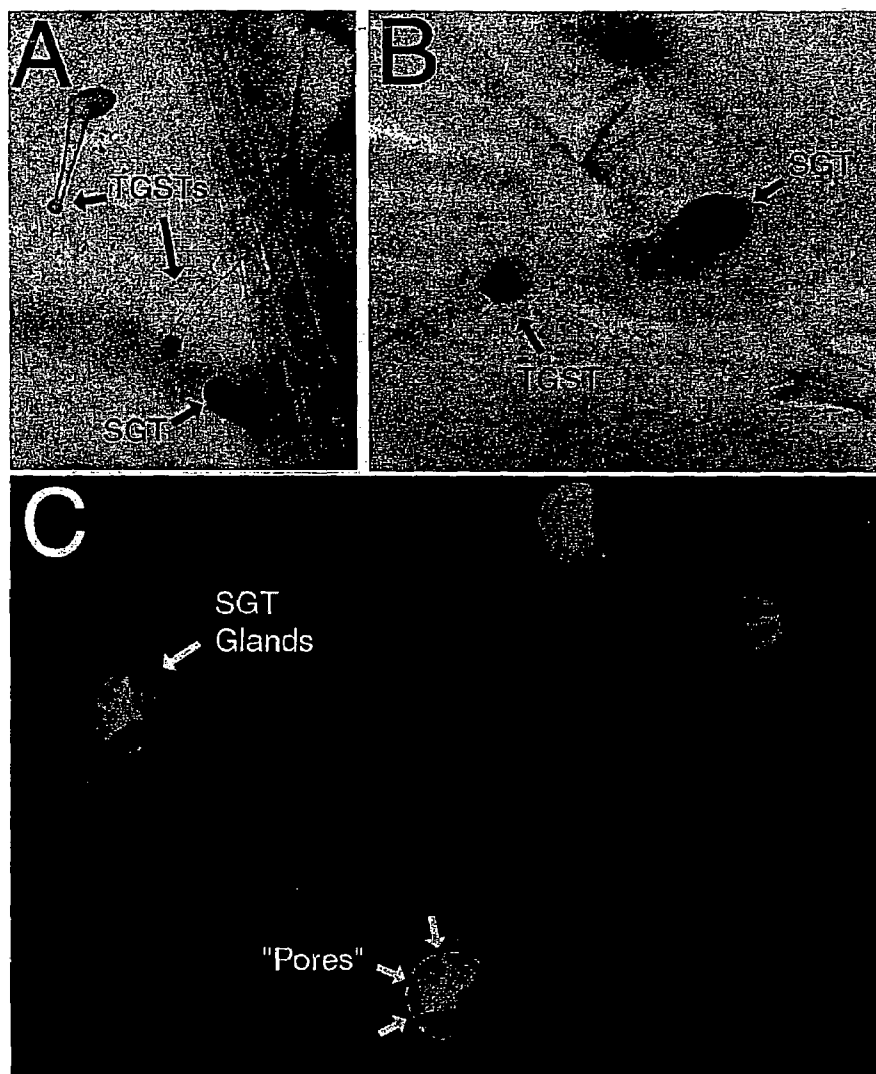


Figure 5. The phylloplanin promoter directs protein expression only in short glandular trichomes (SGTs).

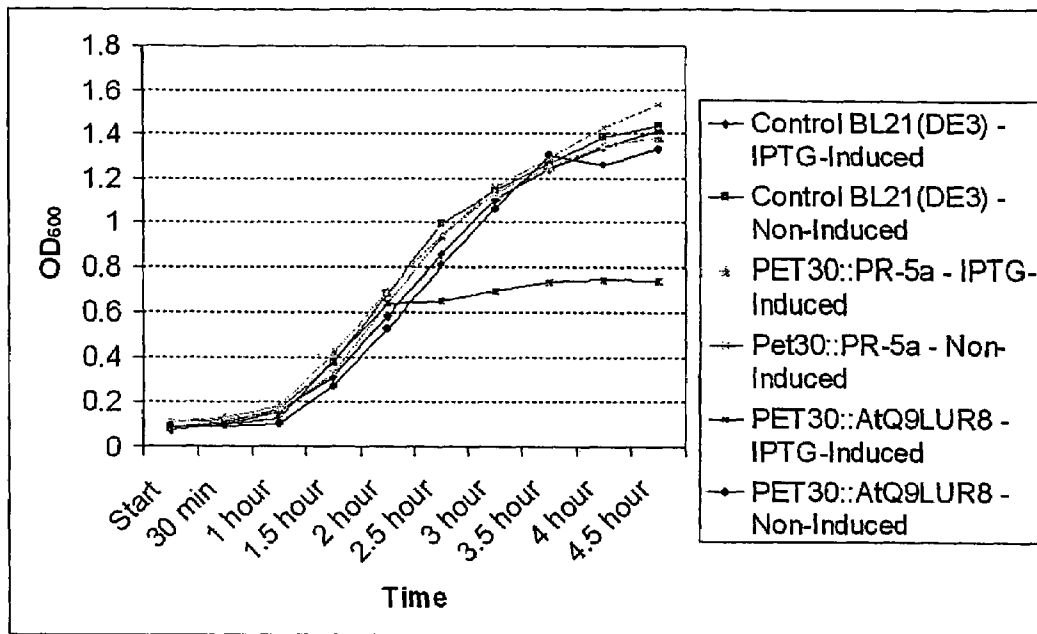


FIGURE 6

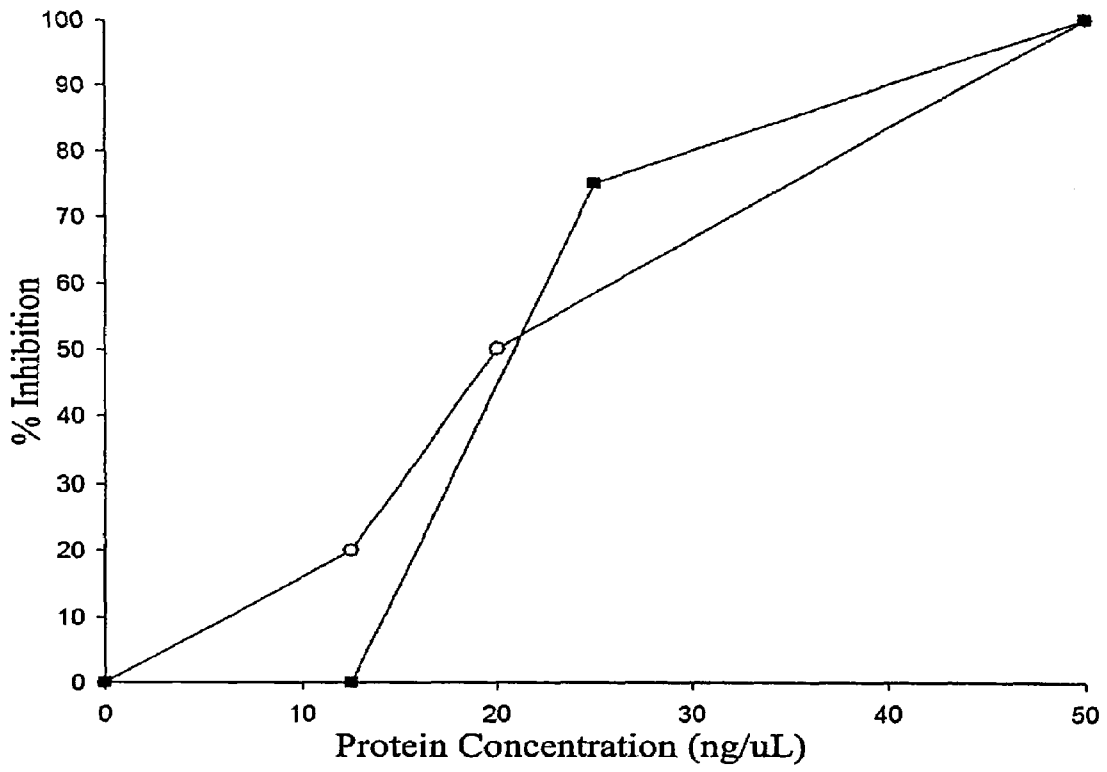


FIGURE 7

1	-----IIVPILV-----STH--ISGLVFCSSWGNLDVINGLSPOVFPNHSVQL	Nt	AY705384
1	QILPPPILPPTIIRP-----P---PIIPIVLPPIVLPVFN--VIGIUSCSVMATVNT---TIAPFPNACVQL	Sr	BG525459
1	---QLG-GLGGGLGG-----LG---MLLGGITN-----IFN--IIGLLLCSVIGIVSTINNAIAVEPFPNAGIVE	Br	CV545024
1	---VLIARQADE-----A---QGLPPITA-----AVN--ISGLVFCSSWGNLSAN-----APPFANALVQL	Ha	CD847345
1	---QLGLGGSGGLGGLIGGLVGGGLVGGVLSGILN-----LVN--INEVVFCSLNGCAPS---ETSTFANAGVQL	At	AAP75801
1	---QSGLGG-----IN-----VPI--INCVLICTINGCAPLN---ETPAFANAVVQL	At	AAN28743
1	-----APPAQPPR-----IQADVV-----VM---GYVPCNNGTSMK---SGSAGFPNAAVQL	Os	XP_479490
1	-----KLERIV-----VI---GVVPCNTGSLID---IATSEAFPNADVQL	Os	XP_479489
1	---QLGG-----ILLGILG-----PIS--IDGVLFCSLNGKIDVLNCAITTEPFPNHSVQL	St	CV502724
1	---QLGG-----ILLGILA-----PTS--IDGVLFCSLNGKIDVLNCAITTEPFPNHSVQL	Le	BP903020
1	-----CTPNCNIG--VMGTSTVEFPNAAVQL	Am	AJ789767
1	---APVAEALG-----LIEGILG-----LIR--IIGTILECTADGNIG--ANGTATVEFPNALVQL	Pt	BU829351
1	---QLG-----IUSGLIG-----SVSNIGCTIVECTSKDNMG--VKCASVEVFPNACVQL	Gm	BMS27339
1	-----KLERIV-----VS---GVVPCNTGSLID---IATSEAFPNADVQL	Hv	BM371086
42	RCCAGN-VIS---STIIRGSGCAFSLAVNIETP-----ILN--DNLVVAIPLSTCNPTICSVERLASSRLVNIILGSGIG	Nt	AY705384
64	RCCG-L-VVG---AATIRQSCAENIVVNIFLSIVANLLS---DRVVTIPLATCNVTLPSTETILOAPLQIVE---N--I	Sr	BG525459
56	QCTEQM-VS---STIIRANCVESIPTIIGLPFSPSTLLSGORLVVITPLATCNVSLAAEELDMAFSLVGTAGD--G	Br	CV545024
47	SCGG-N-VIA---SEWIRACQVENIIVNILLVITENLLS---ORLIIVATPLSNCHATEIACILOSALQVAETIFIRG--I	Ha	CD847345
66	QCCRQRVVS---TATTRAAGLFSLPDSIQMLSTLLS---DORVVVITPLSTCNANQPSVENLVERLAMIENSLTG--L	At	AAP75801
41	QCCNLRVVA---ETIITDLAGLFTFSTNGIQISLPTLLN---DORIVVITPERSCDATLPSTEQILISQDNLVGSIVSG--L	At	AAN28743
43	QDAG-DAAARVAAGSATIDGKQERMMAMNITA-ALSSVAS---GCSLVVITPLATCNADALAEATEITLQSGDR---LLVS	Os	XP_479490
35	RCAG-KLVAG---AATIRNCSFAMEADLTS-GLAMTIG---GOKLVVITPLIKCDANLEAAESTVSYLQGPLTRLIG---	Os	XP_479489
46	RCCAGN-VVS---STIIRGSGCAFSLVINEVONILSSLLS---NONIVVITPLSTCNASLPSVEVLOAPLQIVEKITTG---	St	CV502724
46	RCCAGN-VVS---STIIRGSGCAFSLVTSVQSILSSLLS---DONIVVITPLSTCNATLPSVEVLOAPLQIVEKITAGGGL-	Le	BP903020
25	QCGG-I-VVS---TITIRGLQCFMSLLDELNFVLSLVS---GORDAVITPLATCNASLPSAEGLISTLQFVGSVTLG--L	Am	AJ789767
50	QCGG-N-VVS---TSTIRGSEMESILLDELSYLESSILS---DONIKVITPLISCHSSLEAVEGGLSPDRFIENTALCA-V	Pt	BU829351
45	VCGG-K-ELS---NAKTRDDCTESMAMDELILLDASLLS---GONLVVATPLSNCHAKLPSTEGLISTINFAEITSVG--I	Gm	BMS27339
35	RCAG-QVVAG---AATIRNCSFAMEADLTS-ALAAFIG---RCSLVVITPLIKCDALQLEPAERIVSYLQGPLTRLIG---	Hv	BM371086
110	LIRVGLAETCFILNIN	Nt	AY705384
130	LNIILFAISGQQLYLQV	Sr	BG525459
128	LNIIFSLVESAGGLVG	Br	CV545024
118	LNVNIVSIRR-LVV	Ha	CD847345
139	LNIISIIFAGGLLN	At	AAP75801
114	LNIIVAILTGSPTI	At	AAN28743
113	-MVF--FPRGFSYV	Os	XP_479490
104	-GIFRIFRAGSFHAH	Os	XP_479489
117	--LVNIVTGVQLIPLLN	St	CV502724
119	LNIIVAVTGAQLIN	Le	BP903020
96	LNVGNIIISGRNFSANMLN	Am	AJ789767
122	LSVANIIFAGRFVPSN	Pt	BU829351
116	QTMANIIISGRHELPSI	Gm	BMS27339
104	-GIFRIFRAGSFHSR	Hv	BM371086

FIGURE 8A

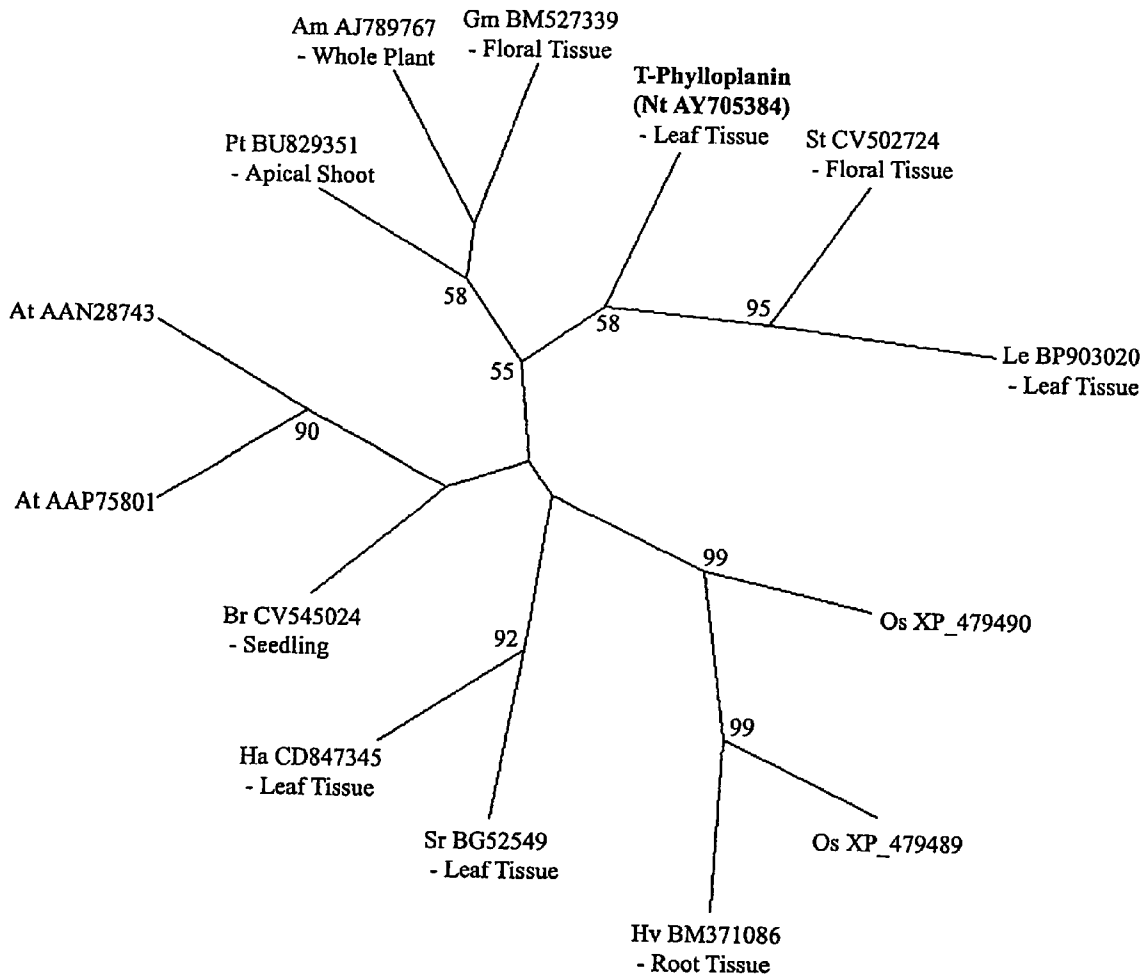


FIGURE 8B

-1157 cccattccac tatgaacttc ccggaattca attctgacta tgcgtacaag tcataatgaa
-1097 gctgcacata gccttcatat cgctaaacga cgtgctaggt ctcaaacga cctgtcgggg
-1037 tcgttacatt agaggtgatt aacttcgtgt atacttgtgc aagtgttcta taacaatttc
-977 aggccaacct agtaagagta gaaatagtga atggcacata acaaacgac accacgaaat
-917 gtacatgata taactcacac aaggtaggca cgctactaga caattaccaa taacaacaat
-857 gcctaggaca tcacaagata tgaaaaatca atccttacta tcacggttga gttgtaacgt
-797 gtaagaatat ttcacacttt ttagggcact aagatcactc caccaacatt tcaagagaat
-737 cactggcact gccaaaaagc cctctacact gtagtgaatt tttgttagtt atctaaagtt
-677 aattattcac ttagtattct ttacattagg ttccccctt ctaggctctg cacgtaacta
-617 gattgaatgg attggtccac tctattatta cagagtaatt attaaatttt tatttgacta
-557 ggcaacacta attgcactat caacaaagta ttagttctag ctttctgggt acttcatacc
-497 tatgcaaatg ataattttat ttaaaacaat agatgtacat ggatataaat acctatgaaa
-437 attaaataaa atataactaa gaaaaaaaaat ttaaagttca ctctaagat atcgggttat
-377 tacatgacca aacacaattt gtttatcaaa tactttcaaa agaatttgtc aaacgtaaat
-317 tatttttctc caaagtgact tatgaattac tatgttgata aaatactttt caaagtaact
-257 aatgtttaga agtcaaggat gggcttcttt tgattattga agttttagc aattgtatgt
-197 agttatagtc agggtgacca ccagcatctc atatagcaat acacaagtggt gttagcgtat
-137 ttgaaatttc aattagttca ttcaaatata cacgtaatag cattataagc cactttcaca
-77 acagatagat taggggtttt aaaatttcaa ccaatgatat ttactataaa ttgatcatgc
-17 acaaacctta attgagcac acaatttctt acagcaataa ctatcacata taacaataac
M A S A K I F L I F L L A A L ... (SEQ ID NO:49)
+44 tgccatggct tcagcaaaaa ttttcttgat tttccttttg gctgcatta (SEQ ID NO:50)

FIGURE 9

1

**UTILITY OF PHYLLOPLANINS AS
ANTIBIOTICS, SELECTIVE FUNGICIDES
AND FOR ENHANCING MICROBIAL
RESISTANCE IN CROP PLANTS**

CROSS-REFERENCE TO RELATED
APPLICATIONS

This patent application claims priority under 35 U.S.C. § 119 to U.S. Provisional Application Ser. No. 60/682,814, filed May 20, 2005, the disclosure of which is incorporated herein by reference.

STATEMENT REGARDING FEDERALLY
SPONSORED RESEARCH

The invention was supported by Grant 5-41170 from the Kentucky Tobacco Research And Development Center, and therefore the government may have rights in the invention.

TECHNICAL FIELD

Methods of producing and using compositions useful as antimicrobial agents are provided. More particularly, these compositions involve the use of phylloplanin proteins.

BACKGROUND

The need for discovery of new anti-fungal and anti-bacterial agents for use in preventing human and animal disease is growing because resistance to existing antibiotics is increasing. In agriculture, available approaches for preventing crop loss due to particularly fungal diseases are inadequate in many cases. Oomycete fungi are significant causes of disease in many plant crops, including tobacco, and it is important that we explore alternate approaches to microbial disease control in plants effected by these fungi. In 1979, an epidemic of blue mold (caused by the oomycete *Peronospora tabacina*) caused an estimated \$ 1.1 billion loss of tobacco crops in Ontario. In the future, outbreaks such as this may be just as damaging to tobacco grown for molecular farming purposes as for conventional tobacco production.

SUMMARY

The invention relates to an isolated nucleic acid molecule comprising the polynucleotide sequence set forth in SEQ ID NO: 17 or a homolog thereof with >80% identity. The invention further relates to a polypeptide encoded by the polynucleotide sequence of SEQ ID NO: 17 or a homolog thereof with >80% identity, such as SEQ ID NO:18 which is a disease- or pest-resistance conferring protein, more specifically a phylloplanin protein. The invention further relates to a method of inhibiting microbial proliferation in or on a plant comprising overexpression of phylloplanin genes in the plant, or contacting an infected plant with a phylloplanin protein.

The invention further provides a novel promoter sequence that is useful for expression of a protein of interest. In accordance with the invention, a nucleic acid construct is provided comprising a non-coding regulatory domain isolated upstream from a Phylloplanin gene, wherein said non-coding regulatory domain is operably associated with a nucleic acid which expresses a protein of interest, wherein said nucleic acid is heterologous to said non-coding domain, and wherein the non-coding regulatory domain comprises a sequence at least 80% identical to the sequence set forth in SEQ ID

2

NO:34. The construct comprises a transcriptional and translational initiation region and translational termination region functional in plants.

The invention further relates to plants selected from the group consisting of corn, soybean, tobacco, potato, tomato, pepper, *datura*, alfalfa, cucumber, and *medicago*, genetically modified by a polynucleotide of the invention.

The invention further relates to a method of inhibiting microbial proliferation in an organism comprising administering a therapeutically effective amount of a phylloplanin polypeptide.

The invention further relates to a method of screening endogenous proteins having antimicrobial properties on a plant leaf surface comprising a) washing the plant leaf surface with an aqueous solution; b) collecting the aqueous solution after washing; and c) analyzing the solution for proteins having antimicrobial properties.

The invention further relates to methods of screening endogenous proteins from plants obtained from a plant of a species selected from the group consisting of *Medicago* sp., *Trifolium* sp., *Ulmus* sp., *Pyrus malus*, *Prunus armeniaca*, *Cynara acolymus*, *Asparagus officinale*, *Hordeum* sp., *Galium* sp., *Beta vulgaris*, *Prunus serotina*, *Vigna sinensis*, *Nyssa sylvatica*, *Quercus* sp., *Artocarpus altilis*, *Brassica* sp., *Andropogon scoparius*, *Fagopyrum sagittatum*, *Manihot esculenta*, *Apium graveolens*, *Agropyron desertorum*, *Cornus florida*, *Phaseolus* sp., *Triticum* sp., *Oenothera caespitosa*, *Carya* sp., *Lactuca* sp., *Impatiens* sp., *Helianthus* sp., *Ledum decumbens*, *Astragalus pattersoni*, *Setaria italica*, *Vaccinium myrtilloides*, *Avena sativa*, *Petroselinum crispum*, *Pastinaca sativa*, *Pisum* sp., *Prunus* sp., *Pyrus communis*, *Musa paradisiaca*, *Astragalus preussii*, *Raphanus sativus*, *Secale cereale*, *Sassafras albidum*, *Atriplex confertifolia*, *Tillandsia usneoides*, *Spinacia oleracea*, *Liquidambar styraciflua*, *Linaria triphylla*, *Liriodendron tulipifera*, *Vicia* sp., *Citrullus vulgaris*, *Melilotus* sp., *Salix* sp., *Rhus copallina*, *Nicotiana* sp., *Vitis* sp., *Datura* sp., *Medicago* sp., *Lycopersicon* sp., *Solanum* sp., *Capsicum* sp., *Cucumis* sp., *Fragaria* sp., *Petunia* sp., *Geranium* sp., *Coleus* sp., *Stevia* sp., *Oryza* sp., *Nepeta* sp., *Zea mays*, *Glycine max*.

The invention provides phylloplanin proteins, methods and compositions, as an avenue for enhancing fungal resistance in plants, using tobacco and blue mold as the model. In addition, the invention demonstrates that phylloplanin is an antimicrobial protein useful in molecular farming products. Plant phylloplanins have the potential to be used as antibiotics against human and animal microorganisms. Overexpression of phylloplanin genes in crop plants such as corn, soybean, tobacco, tomato, potato, pepper, *datura*, alfalfa, cucumber, *medicago* and the like enhances crop resistance to fungal and bacterial microorganisms. In addition, phylloplanins can be used as a topical fungicide.

Other aspects of the invention are described throughout the specification.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A-C depicts data demonstrating proteins present on plant leaf surfaces. A: 40× magnification of TI 1068 phylloplane with tall glandular secreting trichomes (TGSTs) and

short glandular trichome (SGTs) identified. B: Coomassie-stained SDS-PAGE of TI 1068-derived samples. Phylloplanins I-IV are identified. Loaded volumes of LWW (B,d) and sterile-grown plant LWW (B,e) represent 25 cm² leaf surface area. Mwt (B,a) denotes protein standards. C: Silver-stained SDS-PAGE of LWWs from field-grown TI 1068 (C,b; 10 cm²), *G. max* (C,c; 30 cm²), and *H. annuus* (C,d; 6 cm²). Mwt (C,a) denotes protein standards.

FIGS. 2A-B depicts proteins in TI 1068 LWW that inhibit *P. tabacina* spore germination and leaf infection. A: *P. tabacina* spore germination assays (Pt), Coomassie-stained SDS-PAGE (sds), and western blots with 1:10,000 phylloplanin antiserum (w). a, water+spores. b, TI 1068 LWW (diluted to 100 ng/μL total protein)+spores. c, TI 1068 LWW (100 ng/μL total protein) digested with Proteinase K (ProtK)+spores. Arrow marks residual, soluble ProtK. B: *P. tabacina* leaf infection assay of Petite Havana. a, water+spores (10⁴ spores/mL). A sporulating lesion is indicated with arrow. b, TI 1068 LWW (diluted to 50 ng/μL)+spores (10⁴ spores/mL).

FIG. 3 provides a nucleotide (SEQ ID NO: 17) and predicted amino acid sequence (SEQ ID NO:18) of TI 1068 Phylloplanin. Nucleotides are numbered on right. Start and stop codons are underlined, and the signal sequence is bold-faced. Segments corresponding to peptides amino acid-N1, amino acid-T1, amino acid-T2, amino acid-T3, amino acid-T4, and amino acid-P1 are marked by lines above the amino acid sequence and labelled.

FIGS. 4A-D depicts *E. coli*-expressed TI 1068 phylloplanin inhibits *P. tabacina* spore germination. Coomassie-stained SDS-PAGE (sds), western blots with 1:10,000 phylloplanin antiserum (w), and *P. tabacina* spore germination assays (Pt). a: *E. coli* expressed MBP-PhyllP (M-P; 160 ng/μL total protein) treated with Factor-Xa. Arrow indicates released PhyllP. b: *E. coli* expressed MBP-PhyllP (160 ng/μL total protein) treated with Factor-Xa and Proteinase-K (ProtK). Volume used was equivalent to (a). c: *E. coli* expressed MBP (M; 200 ng/μL total protein) treated with Factor-Xa. d: *E. coli* expressed MBP (200 ng/μL, total protein) treated with Factor-Xa and ProtK. Volume used was equivalent to (c).

FIGS. 5A-C shows that the phylloplanin promoter directs protein expression in short glandular trichomes (SGTs). A: Magnification of X-gluc stained plantlet leaf from TI 1068 with GUS under control of phylloplanin promoter. Tall glandular secreting trichomes (TSGTs) are also indicated. B: X-gluc stained SGT on TI 1068 plantlet expressing GUS under control of phylloplanin promoter. Surface structures are indicated. C: Fluorescent magnification/detection of TI 1068 plantlet with GFP under control of phylloplanin promoter. GFP was present only in SGT gland cells. The yellow arrows indicate constrictions between gland cells that may be "pores" to release protein to the leaf surface.

FIG. 6 depicts growth curves of *E. coli* BL21 cultures expressing *N. tabacum* PR-5a and *A. thaliana* AAP75801 with the pET-30 system. Induction with IPTG, if performed, occurred at 2.25 hrs. The IPTG-induced PR-5a expressing culture continued logarithmic growth along with control BL21 cells and non-IPTG-induced cultures. The IPTG-induced AAP75801 expressing culture seemed to stop growing. This suggests that the induced AAP75801 protein product was toxic towards the culture.

FIG. 7 depicts inhibition of *P. tabacina* Spore Germination and Leaf Infection by T-Phylloplanins in LWW. For both assays, the results of a single experiment that is representative of three separate experiments are shown. Open circles, spore germination; closed squares, leaf infection.

FIGS. 8A-B depicts multiple alignment and phylogenetic analysis of T-phylloplanin and similar sequences in other plants (SEQ ID NOS: 18 and 36-48, respectively). A: The amino acid sequence of T-phylloplanin was aligned against sequences giving significant BLAST similarity scores using the ClustalW algorithm of DNASTAR (Lasergene Software, Madison, Wis.). Amino acids conserved between any six sequences are indicated in reverse contrast. B: Unrooted phylogenetic tree showing the evolutionary relationships between the sequences in (A). Bootstrap values of >50% are given on the respective branches. The first two letters of the acronyms indicate the species (Am, *Antirrhinum majus*; At, *Arabidopsis thaliana*; Br, *Brassica rapa*; Gm, *Glycine max*; Ha, *Helianthus annuus*; Le, *Lycopersicon eschulentum*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; Pt, *Populus tremuloides*; Sr, *Stevia rebaudiana*; St, *Stevia tuberosum*). The GenBank accession numbers of the sequences follow the species identifiers. Tissue localizations of ESTs and cDNAs are indicated beneath the acronyms.

FIG. 9 depicts the Promoter sequence of the gene Phylloplanin (SEQ ID NOS:49 and 50). A putative TATA box (-33 to -30) and a putative CAAT box (-47 to -43) are bold-faced. The phylloplanin transcription start site (+1) is bold-faced and underlined. The phylloplanin start codon (+48) is underlined. A portion of the phylloplanin amino acid sequence is indicated.

DETAILED DESCRIPTION

The invention is directed to methods and compositions useful as antimicrobial and antifungal agents. More particularly, these methods and compositions involve the use of phylloplanin proteins as antimicrobial and antifungal agents in plants, humans and animals.

DEFINITIONS

To facilitate understanding of the invention set forth in the disclosure that follows, a number of terms are defined below.

The term "innate immunity" refers to a defense system that inhibits growth of microorganisms at their first point of contact.

The term "phylloplane" refers to a plant's epidermal layer that contributes to the innate pest/disease resistance of the plant.

The term "phylloplanins" refers to proteins produced by a plant's epidermal layer that contribute to the innate pest/disease resistance of the plant.

The term "TGSTs" refers to the tall glandular secreting trichome.

The term "SGTs" refers to the short glandular trichome.

The meaning of other terminology used herein should be easily understood by someone of ordinary skill in the art.

Fungicides are commonly used to inhibit fungal disease in plants, and are commercially available and used for preventing crop loss. Examples of such fungicides are listed in the following table.

FRAC CODE ¹	TARGET SITE ²	GROUP NAME ³	CHEMICAL GROUP ⁴	COMMON NAME ⁵	COMMENTS ⁶
1	mitosis: β-tubuline assembly	MBC - fungicides (Methyl Benzimidazole Carbamates)	benzimidazoles benomyl carbendazim thiophanates	Fuberidazole thiabendazole thiophanate thiophanate-methyl	Resistance common in many fungal species. Several target site mutations, mostly E198A/G/K, F200Y Positive cross resistance between the group members. Negative cross resistance to N- Phenylcarbamates High risk. See FRAC Benzimidazole Guidelines for resistance management.
2	NADH cytochrome c reductase in lipid peroxidation (proposed)	dicarboximides		chlozolinate iprodone procymidone vinclozolin	Resistance common in <i>Botrytis cinerea</i> and found in some other fungal species. Several mutations found in OS1 histidine kinase (Daf 1), mostly I365S Cross resistance common between the group members. Medium to high risk. See FRAC Dicarboximide Guidelines for resistance management.
3	C14- demethylation in sterol biosynthesis	DMI-fungicides (DeMethylation Inhibitors) (SBI: Class I)	imidazoles piperazines pyridines pyrimidines triazoles	imazalil pefurazoate prochloraz triflumizole triforine pyrifenox fenarimol nuarimol azaconazole bitertanol bromuconazole cyproconazole difenoconazole diniconazole epoxiconazole fenbuconazole fluquinconazole flusilazole flutriafol hexaconazole imibenconazole ipconazole metconazole myclobutanil penconazole propiconazole prothioconazole simeconazole tebuconazole tetraconazole triadimefon triadimenol triticonazole	There are great differences in the activity spectra of the different DMI fungicides. Resistance is known in various fungal species. Several resistance mechanisms known incl. target site mutation Y136F, ABC transporters and others. Generally wise to accept that cross resistance is present between fungicides active against the same fungus. DMI fungicides are Sterol Biosynthesis Inhibitors (SBI's) but show no cross resistance to other SBI classes. Medium risk. See FRAC SBI Guidelines for resistance management.

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FRAC CODE ¹	TARGET SITE ²	GROUP NAME ³	CHEMICAL GROUP ⁴	COMMON NAME ⁵	COMMENTS ⁶
4	RNA polymerase I	PA - fungicides (PhenylAmides)	acylalanines oxazolidinones butyrolactones	benalaxyl furalaxyl metalaxyl metalaxyl-M (=mefenoxam) oxadixyl ofurace	Resistance and cross resistance well known in various Oomycetes but mechanism unknown. High risk. See FRAC Phenylamide Guidelines
5	Δ^{14} -reductase and $\Delta^8 \rightarrow \Delta^7$ isomerase in sterol biosynthesis	Amines ("Morpholines") (SBI: Class II)	morpholines piperidines spiroketalamines	Aldimorph dodemorph fenpropimorph tridemorph fenpropidin piperalin spiroxamine	Decreased sensitivity described for powdery mildews. Cross resistance within the group generally found but not to other SBI classes. Low to medium risk. See FRAC SBI Guidelines for resistance management.
6	phospholipid biosynthesis, methyltransferase	phosphorothiolates dithiolanes		edifenphos iprobenfos (IBP) pyrazophos isoprothiolane	Resistance known for specific fungi. Low to medium risk. Resistance management required if used for risky pathogens.
7	complex II in fungal respiration (succinate-dehydrogenase)	carboxamides		benodanil boscalid carboxin fenfuram flutolanil furametpyr mepronil oxycarboxin thifluzamide	Resistance known for specific fungi. Target site mutation H257L. Medium risk. Resistance management required if used for risky pathogens.
8	adenosin-deaminase	hydroxy-(2-amino-)pyrimidines		bupirimate dimethirimol thirimol	Medium risk. Resistance and cross resistance known in powdery mildews. Resistance management required.
9	methionine biosynthesis (proposed)	AP - fungicides (Anilino-Pyrimidines)		cyprodinil mepanipyrim pyrimethanil	Resistance known in <i>Botrytis and</i> sporadically in <i>Venturia</i> , mechanism speculative (CGS). Medium risk. See FRAC Anilinopyrimidine Guidelines for resistance management.
10	mitosis: β -tubulin assembly	N-phenyl carbamates		diethofencarb	Resistance known. Target site mutation E198K. Negative cross resistance to benzimidazoles. High risk. Resistance management required.

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FRAC CODE ¹	TARGET SITE ²	GROUP NAME ³	CHEMICAL GROUP ⁴	COMMON NAME ⁵	COMMENTS ⁶
11	complex III of fungal respiration: ubiquinol oxidase, Qo site	Qol-fungicides (Quinone outside Inhibitors)	methoxy-acrylates Methoxy-carbamates Oximino acetates Oximino-acetamides Oxazolidine-diones dihydro-dioxazines imidazolinones	Azoxystrobin picoxystrobin Pyraclostrobin Kresoxim-methyl trifloxystrobin metominostrobin Famoxadone fluoxastrobin fenamidone	Resistance known in various fungal species. Target site mutations G143A, F129L and additional mechanisms. Cross resistance shown between all members of the Qol group. High risk. See FRAC Qol Guidelines for resistance management.
12	MAP protein kinase in osmotic signal transduction	PP-fungicides (PhenylPyrroles)		fenpiclonil fluidioxonil	Resistance found sporadically, mechanism speculative (OS-2 kinase). Low to medium risk. Resistance management required.
13	G-proteins in early cell signalling (proposed)	quinolines		quinoxifen	Resistance known. Medium risk. Resistance management required.
14	lipid peroxidation (proposed)	AH-fungicides (Aromatic Hydrocarbons) (chlorophenyls, nitroanilines)		biphenyl chloroneb dicloran quintozene (PCNB) tecnazene (TCNB) tolclofos-methyl etridiazole	Resistance known to some fungi. Low to medium risk. Cross resistance patterns complex due to different activity spectra.
15	cell wall synthesis (proposed)	heteroaromatics cinnamic acids	1,2,4-thiadiazoles	dimethomorph	Low to medium risk. Resistance management required.
16.1	reductase in melanin biosynthesis	MBI-R (Melanin Biosynthesis Inhibitors - Reductase	isobenzofuranone pyrroloquinolinone triazolobenzothiazole	fthalide pyroquilon tricyclazole	Resistance not known
16.2	dehydratase in melanin biosynthesis	MBI-D (Melanin Biosynthesis Inhibitors - Dehydratase	cyclopropane-carboxamide carboxamide propionamide	carpropamid diclocyme fenoxani	Resistance known. Medium risk. Resistance management required.
17	3-keto reductase during C4 demethylation in sterol biosynthesis	hydroxyanilides (SBI: Class III)		fenhexamid	Low to medium risk. Resistance management required.
18	squalene epoxidase in sterol biosynthesis	(SBI: class IV)	thiocarbamates allylamines	pyributicarb naftifine terbinafine	Resistance not known. Herbicide and fungicide. Medical fungicides
19	chitin synthase	polyoxins	peptidyl pyrimidine nucleoside	polyoxin	Resistance known. Medium risk. Resistance management required.
20	cell division (proposed)	phenylureas		pencycuron	Resistance not known
21	complex III of fungal respiration: ubiquinone reductase, Qi site	QiI - fungicides (Quinone inside Inhibitors)	cyanoimidazole	cyazofamid	Resistance risk unknown but assumed to be medium to high (mutations at target site known in model organisms). Resistance management required.

-continued

FRAC CODE ¹	TARGET SITE ²	GROUP NAME ³	CHEMICAL GROUP ⁴	COMMON NAME ⁵	COMMENTS ⁶
22	mitosis β-tubulin assembly	benzamides		zoxamide	Low to medium risk. Resistance management required.
23	protein synthesis	enopyranuronic acid antibiotic		blasticidin-S	Low to medium risk. Resistance management required.
24	protein synthesis	hexopyranosyl antibiotic		kasugamycin	Medium risk. Resistance known. Resistance management required.
25	protein synthesis	glucopyranosyl antibiotic		streptomycin	Bactericide. Resistance known. High risk. Resistance management required.
26	trehalase and/or inositol-biosynthesis	glucopyranosyl antibiotic		validamycin	Resistance not known
27	unknown	cyanoacetamide-oximes		cymoxanil	Resistance claims described. Low to medium risk. Resistance management required.
28	cell membrane permeability, fatty acids (proposed)	carbamates		iodocarb propamocarb prothiocarb	Low to medium risk. Resistance management required.
29	uncoupler of oxidative phosphorylation		dinitrophenyl crotonates pyrimidinone-hydrazones 2,6-dinitro-anilines	binapacryl dinocap ferimzone fluazinam	Resistance not known Resistance not known Low risk. However, resistant isolates of <i>Botrytis</i> claimed to exist in Japan in 2000
30	inhibitors of oxidative phosphorylation, ATP synthases	organo tin compounds	tri phenyl tin compounds	fentin acetate fentin chloride fentin hydroxide	Some resistance cases known. Low to medium risk
31	DNA topoisomerase type II (gyrase)	carboxylic acids		oxolinic acid	Bactericide. Resistance known. Risk unknown. Resistance management required.
32	DNA/RNA synthesis (proposed)	heteroaromatics	isoxazoles isothiazolones	hymexazole octhilineone	Resistance not known
33	unknown	phosphonates	ethyl phosphonates	fosetyl-Al phosphorous acid	Miscellaneous compounds, mode of action generally not known.
34	unknown	phthalamic acids		teclofthalam (Bactericide)	
35	unknown	benzotriazines		triazoxide	Use over several years has not revealed major resistance problems.
36	unknown	benzene-sulfonamides		flusulfamide	Risk assumed to be low. No cross resistance between group members
37	unknown	pyridazinones		diclomezine	Resistance not known
P	host plant defence induction	P1 salicylic acid pathway P2	benzo-thiadiazole BTH benzisothiazole	acibenzolar-S-methyl probenazole (also antibacterial and antifungal activity)	

-continued

FRAC CODE ¹	TARGET SITE ²	GROUP NAME ³	CHEMICAL GROUP ⁴	COMMON NAME ⁵	COMMENTS ⁶
U	cell wall synthesis (proposed)	U1	amino acid amide carbamates	benthiavdicarb iprovaldicarb	Mode of action and resistance risk unknown.
	ATP production in respiration (proposed)	U2	thiophene-carboxamides	siltiofam	No long term experience available.
	complex I of respiration (proposed)	U3	pyrimidinamines	Diflumetorim	Resistance management required if high risk
	unknown	U4	thiocarbamate	methasulfocarb	organisms are targeted.
	Unknown	U5	thiazole-carboxamides	ethaboxam	No cross resistance between group members
M	multi-site contact activity	M1	inorganics	Copper (different salts) Sulphur	Generally considered a low risk group with no signs of resistance developing to the majority of fungicides. No cross resistance between Group members.
		M2	dithio-carbamates and relatives	ferbam mancozeb maneb metiram propineb thiram zineb ziram	
		M3	phthalimides	captan captafol folpet	
		M4	chloronitriles (phthalonitriles)	chlorothalonil	
		M5	sulphamides	dichlofuanid tolylfuanid	
		M6	guanidines	dodine guazatine iminocadine	
		M7	triazines	anilazine	
		M8	quinones (anthraquinones)	dithianon	
		M9	inorganics	Copper (different salts) Sulphur	

Source: www.frac.info/publications/frac_list01

¹FRAC Code. Numbers and letters are used to distinguish the fungicide groups. The numbers were assigned primarily according to the time of product introduction to the market. The letters refer to P = host plant defence inducers, M = multi-site inhibitors, and U = recent molecules with unknown mode of action.

²Target Site of Action. If available the biochemical mode of action is given. In many cases the precise target site is not known. However, a grouping can be made due to cross resistance profiles within a group or in relation to other groups.

³Group Name. The Group Names listed are widely accepted in literature. They are based on different sources (mode of action, first important representative, chemical group).

⁴Chemical Group. Sub-grouping due to chemical considerations.

⁵Common name. Accepted (or proposed) common name for an individual active ingredient expected to appear on the product label as definition of the product.

⁶Comments on Resistance. If field resistance is known to one member of the Group, it is most likely but not exclusively valid that cross resistance to other Group members will be present. There is increasing evidence that cross resistance may not be clearly visible between Group members and that the degree of the effect can differ both between group members and fungal species or even within species. The intrinsic risk for resistance evolution to a given fungicide group is estimated to be low, medium or high according to the principles described in FRAC Monographs 1 and 2. A similar classification list of fungicides was published recently also by T. Locke on behalf of FRAC - UK (Fungicide Resistance, August 2001) and by P. Leroux (Classification des fongicides agricoles et resistance, Phytoma, La Defense des Vegetaux, No. 554, 43-51, November 2002).

Phylloplanins

Phylloplanin polypeptides are expressed in crop plants such as corn, soybean, tobacco, tomato, potato, pepper, datura, alfalfa, cucumber, medicago, and the like. Typical biological activities or functions associated with this family of polypeptides include inhibition of fungal spore germination. In one aspect, phylloplanin polypeptides of the invention further include oligomers or fusion polypeptides comprising at least one domain portion of one or more phylloplanin polypeptides, and fragments of any of these polypeptides that are capable of inhibiting fungal germination or which have antimicrobial activity.

This invention provides a family of polypeptides, termed phylloplanins, and the utility of these polypeptides and homologous polypeptides (>80% homology, commonly >90% homology, more typically >95% homology) from other species as antimicrobials (e.g., antifungals and antibacterials) against human and animal pathogens. A phylloplanin of the invention was first identified by washing *Nicotiana tabacum* leaves with water, lyophilizing the wash solution, resuspending the lyophilized material in water to give a leaf water wash (LWW) sample, and analyzing LWW by SDS-PAGE (FIG. 1).

A phylloplanin polypeptide of the invention includes a polypeptide that shares a sufficient degree of amino acid identity or similarity to a polypeptide having a sequence as set forth in SEQ ID NO: 18, to be identified by those of skill in the art as a polypeptide likely to share particular structural domains, have biological activities in common with phylloplanin polypeptides, and/or bind to antibodies that also specifically bind to phylloplanins. The phylloplanin polypeptides of the invention may be isolated from naturally occurring sources. Alternatively, the phylloplanin polypeptides may be recombinantly produced and have the same structure as naturally occurring phylloplanin polypeptides, or may be produced to have structures that differ from naturally occurring phylloplanin polypeptides. Polypeptides derived from any phylloplanin polypeptide of the invention by any type of alteration (for example, but not limited to, insertions, deletions, or substitutions of amino acids, changes in glycosylation of the polypeptide, refolding or isomerization to change its three-dimensional structure or self-association state, and changes to its association with other polypeptides or molecules) are also phylloplanin polypeptides for the purposes of the invention. Therefore, the polypeptides provided by the invention include polypeptides characterized by amino acid sequences similar to those of the phylloplanin polypeptides or similar to phylloplanin polypeptides described herein, but into which modifications are naturally provided or deliberately engineered. A polypeptide that shares biological activities in common with members of the phylloplanin polypeptide family is a polypeptide having antimicrobial activity (e.g., antifungal activity)

Amino acid substitutions and other alterations (deletions, insertions, and the like) to the phylloplanin amino acid sequences (e.g., SEQ ID NO:18) are predicted to be more likely to alter or disrupt phylloplanin polypeptide activities if they result in changes to the consensus residues of the amino acid sequences, see FIGS. 8A and B, and particularly if those changes do not substitute an amino acid of similar structure (e.g., such as substitution of any one of the aliphatic residues -Ala, Gly, Leu, Ile, or Val- for another aliphatic residue), or a residue present in other phylloplanin polypeptides at that conserved position. Conversely, if a change is made to a phylloplanin polypeptide resulting in substitution of a residue at a position in the alignment that is not conserved from one of the other phylloplanin and phylloplanin-like sequences, it

is less likely that such an alteration will affect the function of the altered phylloplanin polypeptide. The invention provides phylloplanin polypeptides and fragments of phylloplanin polypeptides, comprising altered amino acid sequences. Altered phylloplanin polypeptide sequences share at least 75% to 80%, or typically at least 85% to 90%, or more commonly at least 95% to 97.5%, or most commonly at least 99%, or most commonly at least 99.5% amino acid identity with the phylloplanin amino acid sequences provided herein.

The invention provides both full-length and mature forms of phylloplanin polypeptides. "Full-length" polypeptides are those having the complete primary amino acid sequence of the polypeptide as initially translated (see e.g., SEQ ID NO:17 and 18). The amino acid sequences of full-length polypeptides can be obtained, for example, by translation of the complete open reading frame ("ORF") of a cDNA molecule (e.g., SEQ ID NO:17). Several full-length polypeptides may be encoded by a single genetic locus if multiple mRNA forms are produced from that locus by alternative splicing or by the use of multiple translation initiation sites. An example of a full length polypeptide of the invention includes the sequence as set forth in SEQ ID NO:18, from amino acid 1 to amino acid 150. The "mature form" of a polypeptide refers to a polypeptide that has undergone post-translational processing steps such as cleavage of the signal sequence or proteolytic cleavage to remove a prodomain. Multiple mature forms of a particular full-length polypeptide may be produced, for example by cleavage of the signal sequence at multiple sites, or by differential regulation of proteases that cleave the polypeptide. The mature form(s) of such polypeptide may be obtained by expression, in a suitable plant cell or other host cell, of a polynucleotide that encodes the full-length polypeptide. The sequence of the mature form of the polypeptide may also be determinable from the amino acid sequence of the full-length form, through identification of signal sequences or protease cleavage sites. An example of a mature form of the polypeptide of the invention is SEQ ID NO: 18, from amino acid residue x_1 to amino acid residue 150, wherein x_1 is an amino acid between and including residues 22, 23 and 24 (e.g., amino acid 24-150 of SEQ ID NO: 18). The phylloplanin polypeptides of the invention also include those that result from post-transcriptional or post-translational processing events such as alternate mRNA processing which can yield a truncated but biologically active polypeptide. Also encompassed within the invention are variations attributable to proteolysis such as differences in the N- or C-termini upon expression in different types of host cells, due to proteolytic removal of one or more terminal amino acids from the polypeptide (generally from about 1 to 5 terminal amino acids).

The invention further includes phylloplanin polypeptides with or without associated native-pattern glycosylation. Polypeptides expressed in yeast or plant expression systems (e.g., COS-1 or CHO cells) can be similar to or significantly different from a native polypeptide in molecular weight and glycosylation pattern, depending upon the choice of expression system. Expression of polypeptides of the invention in bacterial expression systems, such as *E. coli*, typically provides non-glycosylated molecules. Further, a given preparation can include multiple differentially glycosylated species of the polypeptide. Glycosyl groups can be removed through conventional methods, in particular those utilizing glycopeptidase (Boehringer Mannheim).

Species homologues of phylloplanin polypeptides and polynucleotides are also provided by the invention. As used herein, a "species homologue" is a polypeptide or polynucleotide with a different species of origin from that of a given

polypeptide or polynucleotide, but with significant sequence similarity to the given polypeptide or polynucleotide. Species homologues may be isolated and identified by making suitable probes or primers from polynucleotides encoding the phylloplanin polypeptides provided herein and screening a suitable nucleic acid source from the desired species. Alternatively, homologues may be identified by screening a genome database containing sequences from one or more species utilizing a sequence (e.g., nucleic acid or amino acid) of a phylloplanin molecule of the invention. Such genome databases are readily available for a number of species (e.g., on the world wide web (www) at tigr.org/tdb; genetics.wisc.edu; stanford.edu/about.ball; hiv-web.lanl.gov; ncbi.nlm.nih.gov; and ebi.ac.uk; pasteur.fr/other/biology). Computer algorithms, which connects two proteins through one or more intermediate sequences, can be used to identify closely related as well as distant homologs. For example, an algorithm that repetitively uses the results of the previous query as new search seeds such as Saturated BLAST can be used. Starting with a protein sequence, Saturated BLAST runs a BLAST search and identifies representative sequences for the next generation of searches. The procedure is run until convergence or until some predefined criteria are met. Saturated BLAST is available on the world wide web (www) at: bioinformatics.burnham-inst.org/xblast (see also, Li et al. *Bioinformatics* 16(12):1105-1110, 2000).

The invention also encompasses allelic variants of phylloplanin polypeptides and polynucleotides; that is, naturally-occurring forms of such polypeptides and polynucleotides in which differences in amino acid or nucleotide sequence are attributable to genetic polymorphism.

Fragments of the phylloplanin polypeptides of the invention are encompassed by the invention and may be in linear form or cyclized using known methods, for example, as described in H. U. Saragovi, et al., *Bio/Technology* 10:773-778 (1992) and in R. S. McDowell, et al., *J. Amer. Chem. Soc.* 114:9245-9253 (1992). Phylloplanin polypeptides and fragments thereof, and the polynucleotides encoding them, include amino acid or nucleotide sequence lengths that are at least 25% (typically at least 50%, 60%, 70%, and most commonly at least 80%) of the length of a phylloplanin polypeptide or polynucleotide and have at least 60% sequence identity (typically at least 70%, 75%, 80%, 85%, 90%, 95%, 97.5%, or at least 99%, and, most commonly at least 99.5%) with that phylloplanin polypeptide or polynucleotide, where sequence identity is determined by comparing the amino acid or nucleotide sequences when aligned so as to maximize overlap and identity while minimizing sequence gaps. Also included in the invention are polypeptides and fragments, and polynucleotides encoding them, that contain or encode a segment comprising at least 8, or at least 10, or at least 15, or typically at least 20, or still more typically at least 30, or most commonly at least 40 contiguous amino acids. Such polypeptides and fragments may also contain a segment that shares at least 70% sequence identity (typically at least 75%, 80%, 85%, 90%, 95%, 97.5%, or at least 99%, and most commonly at least 99.5%) with any such segment of any of the phylloplanin polypeptides or polynucleotides, where sequence identity is determined by comparing the sequences of the polypeptide or polynucleotide when aligned so as to maximize overlap and identity while minimizing sequence gaps.

The percent identity can be determined by visual inspection and mathematical calculation. The percent identity of two amino acid sequences or two polynucleotide sequences can be determined by comparing sequence information using the GAP computer program, version 6.0 described by Devereux et al. (*Nucl. Acids Res.* 12:387, 1984) and available from the University of Wisconsin Genetics Computer Group.

The default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess, *Nucl. Acids Res.* 14:6745, 1986, as described by Schwartz and Dayhoff, eds., *Atlas of Polypeptide Sequence and Structure*, National Biomedical Research Foundation, pp. 353-358, 1979; (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps. Other programs used by those skilled in the art of sequence comparison may also be used, such as, for example, the BLASTN program version 2.0.9, available for use via the National Library of Medicine website, or the UW-BLAST 2.0 algorithm. Standard default parameter settings for UW-BLAST 2.0 are described at the following Internet webpage: blast.wustl.edu/blast/README.html#References. In addition, the BLAST algorithm typically uses the BLOSUM62 amino acid scoring matrix, and optional parameters that may be used are as follows: (A) inclusion of a filter to mask segments of the query sequence that have low compositional complexity (as determined by the SEG program of Wootton & Federhen (*Computers and Chemistry*, 1993); also see Wootton and Federhen, *Methods Enzymol.* 266:554-71, 1996) or segments consisting of short-periodicity internal repeats (as determined by the XNU program of Claverie & States, *Computers and Chemistry*, 1993), and (B) a statistical significance threshold for reporting matches against database sequences, or E-score (the expected probability of matches being found merely by chance, according to the stochastic model of Karlin and Altschul (1990); if the statistical significance ascribed to a match is greater than this E-score threshold, the match will not be reported.); preferred E-score threshold values are 0.5, or in order of increasing preference, 0.25, 0.1, 0.05, 0.01, 0.001, 0.0001, 10^{-5} , 10^{-10} , 10^{-15} , 10^{-20} , 10^{-25} , 10^{-30} , 10^{-40} , 10^{-50} , 10^{-75} , 10^{-100} .

The invention also provides for soluble forms of phylloplanin polypeptides comprising certain fragments or domains of these polypeptides, so long as the fragment retains a phylloplanin polypeptide activity. Soluble polypeptides are polypeptides that are capable of being secreted from the cells in which they are expressed. Soluble phylloplanin also include those polypeptides which include part of the trans-membrane region, provided that the soluble phylloplanin polypeptide is capable of being secreted from a cell, and typically retains phylloplanin polypeptide activity. Soluble phylloplanin polypeptides further include oligomers or fusion polypeptides comprising at least one phylloplanin polypeptide and fragments of any of these polypeptides that have phylloplanin polypeptide activity. A secreted soluble polypeptide may be identified (and distinguished from its non-soluble membrane-bound counterparts) by separating intact cells which express the desired polypeptide from the culture medium, e.g., by centrifugation, and assaying the medium (supernatant) for the presence of the desired polypeptide. The presence of the desired polypeptide in the medium indicates that the polypeptide was secreted from the cells and thus is a soluble form of the polypeptide. The use of soluble phylloplanin polypeptides are advantageous for many applications. Purification of the polypeptides from recombinant host cells is facilitated, since the soluble polypeptides are secreted from the cells. Moreover, soluble polypeptides are generally more suitable than membrane-bound forms for parenteral administration and for many enzymatic procedures.

In another aspect, the invention provides polypeptides comprising various combinations of polypeptide domains

from different phylloplanin polypeptides. In one embodiment, a fusion construct comprising at least one phylloplanin domain are linked via a peptide linker.

Additional variants within the scope of the invention include polypeptides that can be modified to create derivatives thereof by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives can be prepared by linking the chemical moieties to functional groups on amino acid side chains or at the N-terminus or C-terminus of a polypeptide. Conjugates comprising diagnostic (e.g., detectable) or therapeutic agents attached thereto are contemplated herein. Typically, such alteration, substitution, replacement, insertion or deletion retains the desired activity of the polypeptide or a substantial equivalent thereof.

Other derivatives include covalent or aggregative conjugates of the polypeptides with other polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusion polypeptides. Examples of fusion polypeptides are discussed herein in connection with oligomers. Further, fusion polypeptides can comprise peptides added to facilitate purification and identification. Such peptides include, for example, poly-His or the antigenic identification peptides described in U.S. Pat. No. 5,011,912 and in Hopp et al., *Bio/Technology* 6:1204, 1988. One such peptide is the FLAG® peptide, which is highly antigenic and provides an epitope reversibly bound by a specific monoclonal antibody, enabling rapid assay and facile purification of expressed recombinant polypeptide. A murine hybridoma designated 4E11 produces a monoclonal antibody that binds the FLAG® peptide in the presence of certain divalent metal cations, as described in U.S. Pat. No. 5,011,912. The 4E11 hybridoma cell line is available from the American Type Culture Collection under accession no. HB9259. Monoclonal antibodies that bind the FLAG® peptide are available from Eastman Kodak Co., Scientific Imaging Systems Division, New Haven, Conn.

As used herein, a “chimeric polypeptide” or “fusion polypeptide” comprises a phylloplanin (including fragments) amino acid sequence of the invention operatively linked to a second polypeptide. The second polypeptide can be any polypeptide of interest having an activity or function independent of or related to the function of a phylloplanin polypeptide. For example, the second polypeptide can have a related activity to a phylloplanin polypeptide and can be a domain of a related but distinct member of the phylloplanin family of proteins such as, for example, cytoplasmic or transmembrane domain of a related phylloplanin polypeptide. Within the fusion polypeptide, the term “operatively linked” is intended to indicate that a phylloplanin polypeptide and the second polypeptide are fused in-frame to each other. The second polypeptide can be fused to the N-terminus or C-terminus of a phylloplanin of the invention. Additional examples of polypeptides of interest include peptide linkers, Fc polypeptides, leucine zipper polypeptides, and the like.

Encompassed by the invention are oligomers or fusion polypeptides that contain a phylloplanin polypeptide, one or more fragments of phylloplanin polypeptides, or any of the derivative or variant forms thereof as disclosed herein. In particular embodiments, the oligomers comprise soluble phylloplanin polypeptides. Oligomers can be in the form of covalently linked or non-covalently-linked multimers, including dimers, trimers, or higher oligomers. Leucine zippers and polypeptides derived from antibodies are among the peptides that can promote oligomerization of the polypeptides attached thereto.

Preparation of Fusion Polypeptides Comprising Certain Heterologous Polypeptides Fused to various portions of antibody-derived polypeptides (including the Fc domain) has been described (see, e.g., by Ashkenazi et al. *PNAS USA* 88:10535, 1991; Byrn et al. *Nature* 344:677, 1990; and Hollenbaugh and Aruffo, “Construction of Immunoglobulin Fusion Polypeptides”, in *Current Protocols in Immunology*, Suppl. 4, pages 10.19.1-10.19.11, 1992).

In another aspect, a fusion polypeptide comprising multiple phylloplanin polypeptides, with or without peptide linkers (spacer peptides) is provided. Among the suitable peptide linkers are those described in U.S. Pat. Nos. 4,751,180 and 4,935,233. In some embodiments, a linker moiety separates the phylloplanin polypeptide domain and the second polypeptide domain in a fusion polypeptide. Such linkers are operatively linked to the C- and the N-terminal amino acids, respectively, of the two polypeptides. Typically a linker will be a peptide linker moiety. The length of the linker moiety is chosen to optimize the biological activity of the soluble phylloplanin and can be determined empirically without undue experimentation. The linker moiety should be long enough and flexible enough to allow a phylloplanin moiety to freely interact with a substrate or ligand. The linker moiety is a peptide between about one and 30 amino acid residues in length, typically between about two and 15 amino acid residues. One linker moiety is a -Gly-Gly- linker. The linker moiety can include flexible spacer amino acid sequences, such as those known in single-chain antibody research. Linking moieties are described, for example, in Huston, J. S., et al., *PNAS* 85:5879-5883 (1988), Whitlow, M., et al., *Protein Engineering* 6:989-995 (1993), and Newton, D. L., et al., *Biochemistry* 35:545-553 (1996). Other suitable peptide linkers are those described in U.S. Pat. Nos. 4,751,180 and 4,935,233, which are hereby incorporated by reference. A DNA sequence encoding a desired peptide linker can be inserted between, and in the same reading frame as, the heterologous sequences (e.g., a phylloplanin encoding nucleic acid) and a second polypeptide encoding nucleic acid, using any suitable conventional technique. For example, a chemically synthesized oligonucleotide encoding the linker can be ligated between the sequences encoding a phylloplanin polypeptide and a second polypeptide of interest. In particular embodiments, a fusion polypeptide comprises from two to four soluble phylloplanin polypeptides separated by peptide linkers.

A polypeptide of the invention may be prepared by culturing transformed and/or recombinant host cells under culture conditions suitable to express the recombinant polypeptide. The resulting expressed polypeptide may then be purified from such culture (i.e., from culture medium or cell extracts) using known purification processes, such as gel filtration and ion exchange chromatography. The purification of the polypeptide may also include an affinity column containing agents which will bind to the polypeptide; one or more column steps over such affinity resins as concanavalin A-agarose, heparin-toyopearl® or Cibacrom blue 3GA Sepharose®; one or more steps involving hydrophobic interaction chromatography using such resins as phenyl ether, butyl ether, or propyl ether; or immunoaffinity chromatography. Alternatively, the polypeptide of the invention may be expressed in a form that will facilitate purification. For example, it may be expressed as a fusion polypeptide comprising, for example, maltose binding polypeptide (MBP), glutathione-S-transferase (GST) or thioredoxin (TRX). Kits for expression and purification of such fusion polypeptides are commercially available from New England BioLab (Beverly, Mass.), Pharmacia (Piscataway, N.J.) and InVitrogen, respectively. The

polypeptide can also be tagged with an epitope and subsequently purified by using a specific antibody directed to such epitope. One such epitope ("FLAG®") is commercially available from Kodak (New Haven, Conn.). Finally, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify the polypeptide. Some or all of the foregoing purification steps, in various combinations, can be employed to provide a substantially purified homogeneous recombinant polypeptide. A phylloplanin polypeptide thus purified is substantially free of other polypeptides and is defined in accordance with the invention as a "substantially purified polypeptide"; such purified polypeptides of the invention include purified antibodies that bind to a phylloplanin polypeptide, fragment, variant, binding partner and the like. A phylloplanin polypeptide of the invention may also be expressed as a product of transgenic animals or plants, e.g., as a component of the milk of transgenic cows, goats, pigs, or sheep which are characterized by somatic or germ cells containing a polynucleotide encoding the phylloplanin polypeptide of the invention.

It is also possible to utilize an affinity column comprising a polypeptide that binds a phylloplanin polypeptide of the invention, such as a monoclonal antibody generated against a phylloplanin polypeptide, to affinity-purify expressed polypeptides. Polypeptides can be removed from an affinity column using conventional techniques, e.g., in a high salt elution buffer and then dialyzed into a lower salt buffer or by changing pH or other components depending on the affinity matrix utilized, or be competitively removed using the naturally occurring substrate of the affinity moiety, such as a polypeptide derived from the invention. In this aspect of the invention, phylloplanin-binding polypeptides, such as the anti-phylloplanin antibodies of the invention or other polypeptides that can interact with a phylloplanin polypeptide of the invention, can be bound to a solid phase support such as a column chromatography matrix or a similar substrate suitable for identifying, separating, or purifying expressed polypeptides of the invention. Adherence of binding polypeptides (e.g., antibodies) to a solid phase contacting surface can be accomplished by any means; for example, magnetic microspheres can be coated with these binding polypeptides and held in the incubation vessel through a magnetic field.

A phylloplanin polypeptide may also be produced by known conventional chemical synthesis. Methods for constructing polypeptides by synthetic means are known in the art. The synthetically-constructed polypeptide, by virtue of sharing primary, secondary or tertiary structural and/or conformational characteristics with phylloplanin polypeptides, may possess biological properties in common therewith, including antimicrobial activity. Thus, they may be employed as biologically active or immunological substitutes for natural, purified polypeptides in screening assays, the development of antibodies, and in treating microbial infections.

The desired degree of purity depends on the intended use of the polypeptide. A relatively high degree of purity is desired when the polypeptide is to be administered *in vivo*, for example. In such a case, the polypeptides are purified such that no polypeptide bands corresponding to other polypeptides are detectable upon analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). It will be recognized by one skilled in the art that multiple bands corresponding to the polypeptide can be visualized by SDS-PAGE, due to differential glycosylation, differential post-translational processing, and the like. In one aspect, the polypeptide of the inven-

tion is purified to substantial homogeneity, as indicated by a single polypeptide band upon analysis by SDS-PAGE. The polypeptide band can be visualized by silver staining, Coomassie blue staining, or by autoradiography.

Antibodies that are immunoreactive with a phylloplanin polypeptide are provided herein. Such antibodies specifically bind to the polypeptide (e.g., a polypeptide consisting of SEQ ID NO: 18 or fragment thereof) via the antigen-binding site of the antibody (as opposed to non-specific binding). In the invention, specifically binding phylloplanin antibodies are those that will specifically recognize and bind with phylloplanin polypeptides, homologues, and variants, but not with other molecules. Similarly, specifically binding anti-phylloplanin antibodies are those that will specifically recognize and bind with phylloplanin polypeptides, homologues, and variants, but not with other molecules. In one embodiment, the antibodies are specific for a phylloplanin polypeptide consisting of SEQ ID NO: 18 or fragment thereof, and do not cross-react with other polypeptides including related phylloplanin. In this manner, the phylloplanin polypeptides, fragments, variants, fusion polypeptides, and the like, as set forth above can be employed as "immunogens" in producing antibodies immunoreactive therewith.

The antigenic determinants or epitopes of phylloplanins used for immunization can be either linear or conformational (discontinuous). Linear epitopes are composed of a single section of amino acids of the polypeptide, while conformational or discontinuous epitopes are composed of amino acids sections from different regions of the polypeptide chain that are brought into close proximity upon polypeptide folding (Janeway et al., *Immunobiology* 3:9 (Garland Publishing Inc., 2nd ed. 1996)). Because folded polypeptides have complex surfaces, the number of epitopes available is quite numerous; however, due to the conformation of the polypeptide and steric hinderances, the number of antibodies that actually bind to the epitopes is less than the number of available epitopes (Janeway et al., *supra*). Epitopes can be identified by methods known in the art. Thus, one aspect of the invention relates to the antigenic epitopes of phylloplanin polypeptides. Such epitopes are useful for raising antibodies, in particular monoclonal antibodies, as described in more detail below. Additionally, epitopes from the polypeptides of the invention can be used as research reagents, in assays, and to purify specific binding antibodies from substances such as polyclonal sera or supernatants from cultured hybridomas. Such epitopes or variants thereof can be produced using techniques known in the art such as solid-phase synthesis, chemical or enzymatic cleavage of a polypeptide, or using recombinant DNA technology.

Antigen-binding antibody fragments that recognize specific epitopes may be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')₂ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the (ab')₂ fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, *Science*, 246:1275) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity. Techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778; Bird, 1988, *Science* 242:423; Huston et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:5879; and Ward et al., 1989, *Nature* 334:544) can also be adapted to produce single chain antibodies against phylloplanin gene products. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge.

The terms "polynucleotide" as used herein, refers to a polymeric form of nucleotides of at least 10 bases in length (smaller nucleotide sequences are typically referred to as oligonucleotides). The nucleotides can be ribonucleotides, deoxyribonucleotides, or modified forms of either type of nucleotide. The term includes single and double stranded forms of DNA or RNA. DNA includes, for example, cDNA, genomic DNA, chemically synthesized DNA, DNA amplified by PCR, and combinations thereof. The polynucleotides of the invention include full-length genes or cDNA molecules as well as a combination of fragments thereof.

By "isolated polynucleotide" is meant a polynucleotide that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. The term therefore includes, for example, a recombinant polynucleotide molecule, which is incorporated into a vector, e.g., an expression vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA) independent of other sequences.

A phylloplanin polynucleotide of the invention comprises: (a) a polynucleotide that encodes a polypeptide comprising a sequence set forth in SEQ ID NO:18 from amino acid 1 to amino acid 150; (b) a polynucleotide encoding a polypeptide comprising a sequence as set forth in SEQ ID NO:18 from amino acid residue x_1 to amino acid residue 150, wherein x_1 is an amino acid between and including residues 22, 23 and 24; (c) a sequence as set forth in SEQ ID NO:17 from nucleotide residue 1 to nucleotide residue 666, or a fragment thereof; (d) sequences complementary to a sequence as set forth in SEQ ID NO:17; (e) fragments of SEQ ID NO: 17 from nucleotide residue x_1 to 666, wherein x_1 is a nucleotide residue between and including residues 66-72, or their complements that specifically hybridize to the polynucleotide consisting of SEQ ID NO:17, under moderate to highly stringent conditions; and any of the foregoing wherein T can also be U (e.g., RNA sequences). Also encompassed by the invention are homologues of a phylloplanin polynucleotide of the invention. Polynucleotide homologues can be identified in several ways, including isolation of genomic or cDNA molecules from a suitable source, or computer searches of available DNA sequence databases.

Polynucleotides encoding a polypeptide or fragment thereof of the invention described herein, can be used as probes or primers for the isolation of nucleic acids or as query sequences for database searches. Such probes or primers can be obtained by "back-translation" from the amino acid sequences, or by identification of regions of amino acid identity with polypeptides for which the coding DNA sequence has been identified. The polymerase chain reaction (PCR) procedure can be employed to isolate and amplify a polynucleotide encoding a phylloplanin polypeptide or a desired combination of phylloplanin polypeptide fragments. Oligonucleotides that define the desired termini of a combination of DNA fragments are employed as 5' and 3' primers. The oligonucleotides can additionally contain recognition sites for restriction endonucleases to facilitate insertion of the amplified DNA fragments into an expression vector. PCR techniques are described in Saiki et al., *Science* 239:487 (1988); *Recombinant DNA Methodology*, Wu et al., eds., Academic Press, Inc., San Diego (1989), pp. 189-196; and *PCR Protocols: A Guide to Methods and Applications*, Innis et al., eds., Academic Press, Inc. (1990). The primer and probe sequences are presented in SEQ ID NOS:1-16 and 32-35.

Among the uses of the disclosed phylloplanin polynucleotides, and combinations of fragments thereof, is the use of fragments as probes or primers. Such fragments generally comprise at least about 17 contiguous nucleotides of a DNA sequence. In other embodiments, a DNA fragment comprises at least 30, or at least 60, contiguous nucleotides of a DNA sequence. The basic parameters affecting the choice of hybridization conditions and guidance for devising suitable conditions are set forth by Sambrook et al., 1989 and are described herein. Using knowledge of the genetic code in combination with the amino acid sequences set forth above, sets of degenerate oligonucleotides can be prepared. Such oligonucleotides are useful as primers, e.g., in polymerase chain reactions (PCR). In certain embodiments, degenerate primers can be used as probes for non-human genetic libraries. Such libraries include, but are not limited to, cDNA libraries, genomic libraries, and even electronic EST (express sequence tag) or DNA libraries. Homologous sequences identified by this method would then be used as probes to identify phylloplanin homologues.

The invention also includes polynucleotides that hybridize under moderately stringent conditions or highly stringent conditions, to polynucleotides encoding phylloplanin polypeptides described herein. The basic parameters affecting the choice of hybridization conditions and guidance for devising suitable conditions are set forth by Sambrook et al. (1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., chapters 9 and 11; and *Current Protocols in Molecular Biology*, 1995, Ausubel et al., eds., John Wiley & Sons, Inc., sections 2.10 and 6.3-6.4), and can be readily determined by those having ordinary skill in the art based on, for example, the length and/or base composition of the DNA. One way of achieving moderately stringent conditions involves the use of a prewashing solution containing 5×SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0), hybridization buffer of about 50% formamide, 6×SSC, and a hybridization temperature of about 55° C. (or other similar hybridization solutions, such as one containing about 50% formamide, with a hybridization temperature of about 42° C.), and washing conditions of about 60° C., in 0.5×SSC, 0.1% SDS. Generally, highly stringent conditions are defined as hybridization conditions as above, but with washing at approximately 68° C., 0.2×SSC, 0.1% SDS. SSPE (1×SSPE is 0.15M NaCl, 10 mM NaH₂PO₄, and 1.25 mM EDTA, pH 7.4) can be substituted for SSC (1×SSC is 0.15M NaCl and 15 mM sodium citrate) in the hybridization and wash buffers; washes are performed for 15 minutes after hybridization is complete. The wash temperature and wash salt concentration can be adjusted as necessary to achieve a desired degree of stringency by applying the basic principles that govern hybridization reactions and duplex stability, as known to those skilled in the art and described further below (see, e.g., Sambrook et al; 1989). When hybridizing a nucleic acid to a target nucleic acid of unknown sequence, the hybrid length is assumed to be that of the hybridizing nucleic acid. When nucleic acid of known sequences are hybridized, the hybrid length can be determined by aligning the sequences of the nucleic acids and identifying the region or regions of optimal sequence complementarity. The hybridization temperature for hybrids anticipated to be less than 50 base pairs in length should be 5 to 10° C. less than the melting temperature (T_m) of the hybrid, where T_m is determined according to the following equations. For hybrids less than 18 base pairs in length, T_m (° C.)=2(# of A+T bases)+4(# of G+C bases). For hybrids above 18 base pairs in length, T_m (° C.)=81.5+16.6 (log₁₀[Na⁺])+0.41(% G+C)-(600/N), where N is the number of bases in the hybrid, and [Na⁺] is the concentration of

sodium ions in the hybridization buffer ($[Na^+]$ for $1 \times SSC = 0.165M$). Each such hybridizing nucleic acid molecule has a length that is at least 15 nucleotides (or typically at least 18 to about 20 nucleotides, or at least 25 to about 30 nucleotides, or at least 40 nucleotides, or more commonly at least 50 nucleotides), or at least 25% (e.g., at least 50%, or at least 60%, or at least 70%, and most typically at least 80%) of the length of a polynucleotide of the invention to which it hybridizes, and has at least 60% sequence identity (e.g., at least 70% to about 75%, at least 80% to about 85%, at least 90% to about 95%, at least 97.5%, or at least 99%, and most commonly at least 99.5%) with a polynucleotide of the invention to which it hybridizes, where sequence identity is determined by comparing the sequences of the hybridizing nucleic acids when aligned so as to maximize overlap and identity while minimizing sequence gaps as described above.

The invention also provides genes corresponding to the polynucleotides disclosed herein. "Corresponding genes" are the regions of the genome that are transcribed to produce the mRNAs from which cDNA molecules are derived and may include contiguous regions of the genome necessary for the regulated expression of such genes. Corresponding genes may therefore include but are not limited to coding sequences, 5' and 3' untranslated regions, alternatively spliced exons, introns, promoters, enhancers, and silencer or suppressor elements. The corresponding genes can be isolated in accordance with known methods using the sequence information disclosed herein. Such methods include the preparation of probes or primers from the disclosed sequence information for identification and/or amplification of genes in appropriate genomic libraries or other sources of genomic materials. An "isolated gene" is a gene that has been separated from the adjacent coding sequences, if any, present in the genome of the organism from which the gene was isolated and includes both coding and non-coding regions.

Methods for making phylloplanin polypeptides are described below. Expression, isolation, and purification of the polypeptides and fragments of the invention can be accomplished by any suitable technique, including but not limited to the following methods.

An isolated polynucleotide of the invention may be operably linked to an expression control sequence such as the pDC412 or pDC314 vectors (Microbix Biosystems Inc., Toronto, Canada), or the pMT2 or pED expression vectors disclosed in Kaufman et al., *Nucleic Acids Res.* 19:4485-4490 (1991); and Pouwels et al. *Cloning Vectors: A Laboratory Manual*, Elsevier, N.Y., (1985), in order to produce a phylloplanin polypeptide recombinantly. Many suitable expression control sequences are known in the art. General methods of expressing recombinant polypeptides are also known and are exemplified in R. Kaufman, *Methods in Enzymology* 185, 537-566 (1990). As used herein "operably linked" means that a polynucleotide of the invention and an expression control sequence are situated within a construct, vector, or cell in such a way that the polypeptide encoded by a polynucleotide is expressed when appropriate molecules (such as polymerases) are present. In one embodiment, at least one expression control sequence is operably linked to a phylloplanin polynucleotide of the invention in a recombinant host cell or progeny thereof, the polynucleotide and/or expression control sequence having been introduced into the host cell by transformation or transfection, for example, or by any other suitable method. In another embodiment, at least one expression control sequence is integrated into the genome of a recombinant host cell such that it is operably linked to a polynucleotide encoding a phylloplanin polypeptide. In one embodiment of the invention, at least one expression control

sequence is operably linked to a polynucleotide of the invention through the action of a trans-acting factor such as a transcription factor, either in vitro or in a recombinant host cell.

In addition, a polynucleotide encoding an appropriate signal peptide (native or heterologous) can be incorporated into expression vectors. The choice of signal sequence can depend on factors such as the type of host cells in which the recombinant polypeptide is to be produced. A DNA sequence for a signal sequence (secretory leader) can be fused in frame to a polynucleotide of the invention so that the DNA is initially transcribed, and the mRNA translated, into a fusion polypeptide comprising the signal peptide. A signal peptide that is functional in the intended host cells promotes secretion of the polypeptide. The signal peptide is cleaved from the polypeptide upon secretion of polypeptide from the cell. The skilled artisan will also recognize that the position(s) at which the signal peptide is cleaved can differ from that predicted by computer program, and can vary according to such factors as the type of host cells employed in expressing a recombinant polypeptide. A polypeptide preparation can include a mixture of polypeptide molecules having different N-terminal amino acids, resulting from cleavage of the signal peptide at more than one site. A phylloplanin polypeptide of the invention comprises a signal peptide from amino acid 1-23. This can be substituted by heterogenous signal peptides using known recombinant DNA techniques.

Established methods for introducing DNA into cells have been described (Kaufman, *Large Scale Mammalian Cell Culture*, 1990, pp. 15-69). Additional protocols using commercially available reagents, such as Lipofectamine lipid reagent (Gibco/BRL) or Lipofectamine-Plus lipid reagent, can be used to transfect cells (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7417, 1987). Selection of stable transformants can be performed using methods known in the art such as, for example, resistance to cytotoxic drugs. Kaufman et al., *Meth. in Enzymology* 185:487-511, 1990, describes several selection schemes, such as dihydrofolate reductase (DHFR) resistance. A suitable strain for DH selection can be CHO strain DX-B11, which is deficient in DHFR (Urlaub et al., *Proc. Natl. Acad. Sci. USA* 77:4216-4220, 1980). A plasmid expressing the DHFR cDNA can be introduced into strain DX-B11, and only cells that contain the plasmid can grow in the appropriate selective media. Examples of selectable markers that can be incorporated into expression vectors include cDNAs conferring resistance to antibiotics, such as G418 and hygromycin B. Cells having the vector can be selected based on resistance to such compounds.

Alternatively, gene products can be obtained via homologous recombination, or "gene targeting" techniques. Such techniques employ the introduction of exogenous transcription control elements (such as the CMV promoter or the like) in a particular predetermined site on the genome, to induce expression of an endogenous phylloplanin of the invention. The location of integration into a host chromosome or genome can be determined by one of skill in the art, given the known location and sequence of the gene. In one embodiment, the invention contemplates the introduction of exogenous transcriptional control elements in conjunction with an amplifiable gene, to produce increased amounts of the gene product. The practice of homologous recombination or gene targeting is explained by Chappel in U.S. Pat. No. 5,272,071 (see also Schimke, et al. "Amplification of Genes in Somatic Mammalian cells," *Methods in Enzymology* 151:85 (1987), and by Capecchi, et al., "The New Mouse Genetics: Altering the Genome by Gene Targeting," *TIG* 5:70 (1989)).

A number of cell types may act as suitable host cells for expression of a polypeptide of the invention. It may be possible to produce the polypeptide in lower eukaryotes such as yeast or in prokaryotes such as bacteria, and in plant cells. Potentially suitable yeast strains include *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces* strains, *Candida*, or any yeast strain capable of expressing heterologous polypeptides. Potentially suitable bacterial strains include *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhimurium*, or any bacterial strain capable of expressing heterologous polypeptides. If the polypeptide is made in yeast or bacteria, it may be necessary to modify the polypeptide produced therein, for example by phosphorylation or glycosylation of the appropriate sites, in order to obtain the functional polypeptide. Such covalent attachments may be accomplished using known chemical or enzymatic methods. The polypeptides may also be produced by operably linking an isolated polynucleotide of the invention to suitable control sequences in one or more insect expression vectors, and employing an insect expression system. Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, e.g., Invitrogen, San Diego, Calif., U.S.A. (the MaxBac® kit), or as described in Summers and Smith, Texas Agricultural Experiment Station Bulletin No. 1555 (1987), and Luckow and Summers, *Bio/Technology* 6:47 (1988). As used herein, a host cell capable of expressing a polynucleotide of the invention is "transformed." Cell-free translation systems could also be employed to produce polypeptides using RNAs derived from polynucleotide constructs disclosed herein. A host cell that comprises an isolated polynucleotide of the invention, typically operably linked to at least one expression control sequence, is a "recombinant host cell".

The polynucleotides encoding the phylloplanin polypeptides of the invention can be used for numerous diagnostic or other useful purposes. The polynucleotides of the invention can be used to express recombinant polypeptides for analysis, characterization or therapeutic uses; as markers for tissues in which the corresponding polypeptide is preferentially expressed (either constitutively or at a particular stage of tissue differentiation or development or in disease states); as molecular weight markers on Southern gels; as chromosome markers or tags (when labeled) to identify chromosomes or to map related gene positions; to compare with endogenous DNA sequences in subjects to identify potential genetic disorders; as probes to hybridize and thus discover novel, related nucleic acid molecules; as a source of information to derive PCR primers for genetic fingerprinting; as a probe to "subtract-out" known sequences in the process of discovering other novel nucleic acids; for selecting and making oligomers for attachment to a "gene chip" or other support, including for examination of expression patterns; to raise anti-polypeptide antibodies using DNA immunization techniques; as an antigen to raise anti-DNA antibodies or elicit another immune response, and for gene therapy. Any or all polynucleotides suitable for these uses are capable of being developed into reagent grade or kit format for commercialization as products. For example, a kit of the invention will include one or more containers being compartmentalized and designed to hold primers and/or probes (e.g., SEQ ID Nos:1-16 and 32-35 to amplify phylloplanin), antibodies, polypeptides and related reagents (e.g., Taq polymerase and the like). Methods for performing these uses are known in the art. References disclosing such methods include, without limitation, "Molecular Cloning: A Laboratory Manual", 2d ed., Cold Spring Harbor Laboratory Press, Sambrook et al. eds., 1989, and "Methods in Enzymology: Guide to Molecular Cloning

Techniques", Academic Press, Berger and Kimmel eds., 1987. The phylloplanin polypeptides can be employed in inhibiting antimicrobial infections *in vitro* or *in vivo*.

The polynucleotides encoding phylloplanin polypeptides, and the disclosed fragments and combinations of these polynucleotides can be used by one skilled in the art using known techniques to analyze abnormalities associated with the genes corresponding to these molecules. This enables one to distinguish conditions in which this marker is rearranged or deleted. In addition, polynucleotides of the invention or a fragment thereof can be used as a positional marker to map other genes of unknown location. The DNA can be used in developing treatments for any disorder mediated (directly or indirectly) by defective, or insufficient amounts of, the genes corresponding to the polynucleotides of the invention. The polynucleotides disclosed herein permit the detection of defective genes, and the replacement thereof with normal genes. Defective genes can be detected in *in vitro* diagnostic assays, and by comparison of a native nucleotide sequence disclosed herein with that of a gene derived from a person suspected of harboring a defect in this gene.

The phylloplanin polypeptides, fragments (including soluble fragments), variants, antibodies, and binding partners of the invention are useful to improve the disease-resistance or disease-tolerance of crops either during the life of the plant or for post-harvest crop protection. Pathogens exposed to the peptides are inhibited. The antifungal properties of a phylloplanin can eradicate a pathogen already established on the plant or may protect the plant from future pathogen attack. The eradicator effect of the polypeptide is particularly advantageous.

Exposure of a plant pathogen to a phylloplanin polypeptide can be achieved in various ways, for example: (a) The isolated phylloplanin polypeptide may be applied to plant parts or to the soil or other growth medium surrounding the roots of the plants or to the seed of the plant before it is sown using standard agricultural techniques (such as spraying). The phylloplanin polypeptide may have been extracted from plant tissue or chemically synthesized or extracted from micro-organisms genetically modified to express the peptide. The phylloplanin polypeptide may be applied to plants or to the plant growth medium in the form of a composition comprising the phylloplanin polypeptide in admixture with a solid or liquid diluent and optionally various adjuvants such as surface-active agents. Solid compositions may be in the form of dispersible powders, granules, or grains. (b) A composition comprising a micro-organism genetically modified to express a phylloplanin polypeptide may be applied to a plant or the soil in which a plant grows. (c) An endophyte genetically modified to express the phylloplanin polypeptide may be introduced into the plant tissue (for example, via a seed treatment process). An endophyte is defined as a micro-organism having the ability to enter into non-pathogenic endosymbiotic relationships with a plant host. A method of endophyte-enhanced protection of plants has been described in a series of patent applications by Crop Genetics International Corporation (for example, International Application Publication Number WO90/13224, European Patent Publication Number EP-125468-B1, International Application Publication Number WO91/10363, International Application Publication Number WO87/03303). The endophyte may be genetically modified to produce agricultural chemicals. International Patent Application Publication Number WO94/16076 (ZEN-ECA Limited) describes the use of endophytes which have been genetically modified to express a plant-derived antifungal peptide. (d) DNA encoding a phylloplanin polypeptide may be introduced into the plant genome so that the polypep-

tide is expressed within the plant body (the DNA may be cDNA, genomic DNA or DNA manufactured using a standard nucleic acid synthesizer).

In practicing a method of treatment or use of the invention, a therapeutically effective amount of a therapeutic agent of the invention is contacted with a plant, subject or surface to treat or ameliorate a microbial (e.g., a fungal) infection. "Therapeutic agent" includes without limitation any of the phylloplanin polypeptides, fragments, and variants; phylloplanin polynucleotides, fragments, and variants; soluble forms of the phylloplanin polypeptides; antibodies to a phylloplanin polypeptide or fragment; phylloplanin polypeptide binding partners; complexes formed from the phylloplanin polypeptides, fragments, variants, and binding partners, and the like. As used herein, the term "therapeutically effective amount" means the total amount of each therapeutic agent or other active component of the pharmaceutical composition or method that is sufficient to show a meaningful benefit, e.g., treatment, healing, prevention or amelioration of microbial contamination or infection, or an increase in rate of treatment, healing, prevention or amelioration of such contamination and infections. Contacting a therapeutic agent with a subject, organism or surface can be done *in vitro* or *in vivo* for an amount and for a time sufficient to reduce microbial infection or presence.

Compositions comprising an effective amount of a phylloplanin polypeptide or fragment, or oligomer thereof, of the invention (from whatever source derived, e.g., recombinant and non-recombinant sources), in combination with other components such as a physiologically acceptable diluent, carrier, or excipient, are provided herein. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredient(s).

A phylloplanin polypeptide of the invention (including fragments) may be active in multimers (e.g., heterodimers or homodimers) or complexes with itself or other polypeptides. As a result, pharmaceutical compositions of the invention may comprise a polypeptide of the invention in such multimeric or complexed form. Such compositions contemplate the preparation of fragments of phylloplanin in any combination thereof as oligomers.

The invention further relates to gene promoter sequences isolated from a plant and their use to regulate chimeric gene expression in plants. In particular, the invention describes the isolation and use of DNA sequences which permit a high level of expression of foreign genes in transgenic plants.

The expression of genes in plants is controlled by a number of regulatory components, including nucleic acid and protein elements. Where the plant gene exists as double stranded DNA, the primary steps of expression involve the production of a messenger RNA by a polymerase enzyme. The initiation of this part of the expression process is controlled by a region commonly referred to as the "promoter". The promoter lies upstream (5') of the protein encoding region and may be constitutive or tissue-specific, developmentally-regulated and/or inducible.

Manipulation of crop plants to alter and/or improve phenotypic characteristics (such as productivity, quality, pathogen resistance) uses the expression of heterologous genes in plant tissues. Such genetic manipulation therefore relies on the availability of means to drive and to control gene expression as required; for example, on the availability and use of suitable promoters which are effective in plants and which regulate gene expression so as to give the desired effect(s) in the transgenic plant. It is advantageous to have the choice of a variety of different promoters so that the most suitable

promoter may be selected for a particular gene, construct, cell, tissue, plant or environment.

Promoters (and other regulatory components) from bacteria, viruses, fungi and plants have been used to control gene expression in plant cells. Numerous plant transformation experiments using DNA constructs comprising various promoter sequences fused to various foreign genes (for example, bacterial marker genes) have led to the identification of useful promoter sequences. It has been demonstrated that sequences up to 500-1000 bases in most instances are sufficient to allow for the regulated expression of foreign genes. However, it has also been shown that sequences much longer than 1 kb may have useful features which permit high levels of gene expression in transgenic plants. A range of naturally-occurring promoters are known to be operative in plants and have been used to drive the expression of heterologous (both foreign and endogenous) genes in plants: for example, the constitutive 35S cauliflower mosaic virus promoter, the ripening-enhanced tomato polygalacturonase promoter (Bird et al, 1988, *Plant Molecular Biology*, 11:651-662), the E8 promoter (Diekman & Fischer, 1988, *EMBO*, 7:3315-3320) and the fruit specific 2A11 promoter (Pear et al, 1989, *Plant Molecular Biology*, 13:639-651) and many others.

As stated above, successful genetic manipulation relies on the availability of means to control plant gene expression as required. A scientist uses a suitable expression cassette (incorporating one or more promoters and other components) to regulate gene expression in the desired manner (for example, by enhancing or reducing expression in certain tissues or at certain developmental stages). The ability to choose a suitable promoter from a range of promoters having differing activity profiles is thus important.

In the invention, phylloplanin gene promoter has been isolated and fully sequenced from the surface of plant leaves. The phylloplanin promoter essentially controls the production of the protein known as "phylloplanin" in plants. This phylloplanin protein is associated with the plant epidermal layer.

According to the invention, there is provided a polynucleotide phylloplanin promoter capable of driving gene expression in plants. Such polynucleotide may contain a segment that shares at least 70% sequence identity (typically at least 75%, 80%, 85%, 90%, 95%, 97.5%, or at least 99%, and most commonly at least 99.5%) with any such segment of any of the polynucleotide phylloplanin promoter sequence, where sequence identity is determined by comparing the sequences of the polynucleotide when aligned so as to maximize overlap and identity while minimizing sequence gaps. An example of a polynucleotide promoter of the invention includes the sequence as set forth in SEQ ID NO:34.

"Active variants" are DNA sequences partially homologous to SEQ ID NO:34 which retain promoter activity. It may be possible to alter the level or type of activity of these promoters by manipulating their sequences: for example, by altering the nucleotide sequence in key regulatory regions, by truncating the sequence or by deleting parts within the sequence.

The promoter of the invention is suitable for incorporation into polynucleotide constructs encoding any target gene or transcribable polynucleotide region so that the target gene or polynucleotide is expressed when the construct is transformed into a plant. The construct will typically contain a transcription termination signal.

The phylloplanin promoter may be synthesised *ab initio* using the sequence shown in SEQ ID NO:34 as a guide.

Alternatively, the promoters may be isolated from plant genomic libraries using suitable probes derived from the sequences or the promoter may be isolated using a PCR approach.

In practice the promoter of the invention may be inserted as a promoter in a recombinant polynucleotide construct designed for use in a plant. The construct is then inserted into the plant by transformation. Any plant species may be transformed with the construct, and any suitable transformation method may be employed.

In another embodiment, the fusion construct comprises (a) a polynucleotide comprising a sequence that is at least 80% identity (typically at least 90%, 95%, or at least 98%) with SEQ ID NO:34; and (b) a polynucleotide comprising a coding region of a polypeptide of interest, wherein the polynucleotide of (a) is operably linked to the polynucleotide of (b).

In yet another aspect, the invention provides methods for producing a transgenic plant which expresses a nucleic acid segment encoding the phylloplanin protein of the invention. The process of producing transgenic plants is well-known in the art. In general, the method comprises transforming a suitable host cell with a DNA segment which contains a promoter operatively linked to a coding region that encodes a phylloplanin protein. Such a coding region is generally operatively linked to a transcription-terminating region, whereby the promoter is capable of driving the transcription of the coding region in the cell, and hence providing the cell the ability to produce the recombinant protein *in vivo*. Alternatively, in instances where it is desirable to control, regulate, or decrease the amount of a particular recombinant phylloplanin protein expressed in a particular transgenic cell, the invention also provides for the expression of phylloplanin protein antisense mRNA. The use of antisense mRNA as a means of controlling or decreasing the amount of a given protein of interest in a cell is well-known in the art.

The invention further provides a transgenic plant, seed, cell, or any other form of regenerant, comprising a heterologous polynucleotide (>80% homology, commonly >90% homology, more typically >95% homology) selected from the group consisting of a) a polynucleotide comprising SEQ ID NO:17; b) a polynucleotide comprising a sequence selected from the group consisting of: from about nucleotide x_1 to 666 of SEQ ID NO: 17 wherein x_1 is a nucleotide between residues 66-72; c) a polynucleotide that hybridizes under moderate to highly stringent conditions to a polynucleotide comprising the sequence of SEQ ID NO: 17 and encoding a polypeptide that is a disease- or pest-resistant conferring protein; d) a nucleotide sequence complementary to a sequence of SEQ ID NO: 17; and e) any of the nucleotide sequences of a) to d) wherein T can also be U.

In yet another aspect, the invention provides a transgenic plant comprising a heterologous promoter of a fusion construct comprising (a) a polynucleotide comprising a sequence that is at least 80% identical to SEQ ID NO:34; and (b) a polynucleotide comprising a coding region of a polypeptide of interest, wherein the polynucleotide of (a) is operably linked to the polynucleotide of (b).

EXAMPLES

SDS-PAGE analyses of LWW from greenhouse-grown TI 1068 leaves indicated the presence of four bands with molecular masses of 16 (I), 19 (II), 21 (III), and 25 (IV) kDa (FIG. 1B, lane d), which are collectively termed phylloplanins. Phylloplanins in LWW were relatively pure and abundant, compared to proteins present in leaf epidermal cells (FIG. 1B, lane b) or leaf extracellular fluid (FIG. 1B, lane c), suggesting selective deployment on the phylloplane.

Sterile-grown TI 1068 LWW contained phylloplanins (FIG. 1B, lane e), indicating these proteins were not from leaf surface microbes and were not induced by pathogen attack. From measurement of the protein concentration in LWW (BCA assay), it was estimated that the phylloplane of greenhouse-grown TI 1068 leaves contains 100-200 ng protein/square-cm leaf surface. Field-grown TI 1068 LWW also contained phylloplanins, indicating that leaf surface proteins are present under natural conditions (FIG. 1C, lane a), and phylloplanins were renewed after washing. *N. tabacum* cultivars TI 1112 and TI 1406 that lack TGSTs, or secretion, respectively, produce substantial phylloplanins, so diterpene/sugar ester producing TGSTs are not the site of phylloplanin biosynthesis. Field-grown soybean and sunflower LWWs contained varying amounts of proteins (FIG. 1C, lanes c-d), as did greenhouse-grown corn, tomato, soybean, and potato, but these proteins were not further characterized.

T-Phylloplanins Inhibit *P. tabacina* Spore Germination and Leaf Infection

P. tabacina is an oomycete pathogen that reproduces via airborne spores (Lucas, G. B. 1975), and initial host contact and spore deposition commences at the phylloplane (Svircev, A. M et al. 1989). LWW from greenhouse-grown TI 1068 plants inhibited *P. tabacina* spore germination (FIG. 2A, b; LD₅₀ ~15-20 ng/μL [50 spores/μL]), as did LWW from sterile-grown plants. Protein digestion by immobilized Proteinase K relieved inhibition of spore germination (FIG. 2A,c), indicating that proteins were necessary for inhibition. Spore germination was not affected by water incubated with immobilized Proteinase K. Once spore germination was initiated, addition of LWW (100 ng/μL total protein) immediately arrested germination tube growth and development. Using GC, the levels of residual exudate diterpenes found in LWW were <1/10 of the LD₅₀ reported to inhibit *P. tabacina* germination (Kennedy, B. S. et al. 1992), and nicotine was not detected.

Intact *N. tabacum* Petite Havana SR1 plants, considered susceptible to *P. tabacina*, were infected by applying spores (50 spores/μL in 4 μL water) to the leaf surface. After 5 days, sporulating lesions developed at sites of application (FIG. 2B,a). Phylloplanins in TI 1068 LWW, when mixed with spores at total protein concentrations of 50 ng/μL or higher, inhibited leaf infection by *P. tabacina* (FIG. 2B,b). At 25 ng/μL total protein, 75% inhibition was observed, and no inhibition occurred with titrations below 12.5 ng/μL total protein. Similar results were observed in three independent experiments and in identical experiments using the susceptible cultivar KY 14. Like TI 1068, LWWs of Petite Havana and KY 14 contain a similar phylloplanin pattern, but unlike TI 1068, they produce much less trichome exudate. Other surface chemicals (e.g., surface lipids or trichome exudate components) may influence or accentuate phylloplanin activity, dispersion, or longevity, by acting as adducts or as solubilizing agents. Although it is difficult to estimate the role of a single component, such as the presence of phylloplanins, in blue mold susceptibility or resistance, outside the experimental conditions used here, phylloplanins appear to be a key component.

Isolation of the Novel T-Phylloplanin Gene

N. tabacum phylloplanins I, II, III, and IV share an identical N-terminal amino acid sequence (Table 1). Internal amino acid sequences were elucidated from peptides generated by trypsin digestion of Phylloplanins II and IV, and pepsin digestion of total LWW (Table 1). Degenerate, deoxyinosine-containing primers were synthesized and used in RT-PCR with cDNA generated from *N. tabacum* total leaf RNA as a tem-

plate, and a 332 bp fragment was amplified. RLM-RACE was used to recover a full-length, *N. tabacum* Phylloplanin cDNA sequence (FIG. 3; Accession No. Genbank AY705384 (SEQ ID NO: 17)) of 666 bp in length, encoding a hydrophobic, basic (50% hydrophobicity, estimated pI 9.3, Vector NTI) 15.4 kDa protein containing 150 amino acids (SEQ ID NO: 18). Based on the N-terminus recovered from the mature phylloplanin (Ile-24) the first 23 amino acids comprise a signal sequence that targets the protein to the secretory pathway [TargetP version 1.0 (Emanuelsson, O. et al. 2000)]. The molecular mass of the mature protein is estimated to be ~13 kDa. The protein of this mass from the leaf surface is not recovered, but instead recover four apparent bands of higher molecular masses. It is speculated that the molecular masses of native phylloplanins I-IV are increased due to the occurrence of complexes with cuticular lipids, or trichome exudate diterpenes or sugar esters. These complexes could serve to increase phylloplanin solubility in TGST exudate (diterpenes and sugar esters) and their subsequent dispersion on the leaf surface. Amphiphathic sugar esters are known to solubilize largely hydrophobic diterpenes of TGST exudate. It is noted that highly hydrophobic, basic saposin-like proteins of animals (see below) also display anomalous migration in SDS-PAGE (Curstedt, T. et al. 1987), and it is suggested that phylloplanins may behave similarly. Putative sequences from *Arabidopsis thaliana* (Accession BAB02757) (SEQ ID NO:19) and from *Oryza sativa* (Accession BAC83536) (SEQ ID NO:20) have significant percent similarity to the Phylloplanin cDNA, as do unamiotated ESTs from *N. sylvestris*, *Solanum tuberosum*, and *Lycopersicon esculentum*. The genomic structure of gene Phylloplanin was elucidated from *N. tabacum* genomic DNA using a Genomewalker kit. The gene contains two exons (1: 175 bp; 2: 278 bp) that are separated by a 508 bp intron.

fusion protein (MBP-PhyllIP) was purified on an amylose column, cut with the protease Factor Xa to release PhyllIP, and desalted on a 3 kDa centrifugal filter. Both MBP-PhyllIP and PhyllIP reacted with the phylloplanin-specific antibody (FIG. 4). The sample containing PhyllIP inhibited *P. tabacina* spore germination at total protein concentrations greater than 160 ng/ μ L (FIG. 4a). Protease digestion relieved PhyllIP inhibition of spore germination (FIG. 4b). A control sample containing MBP alone, produced by an empty pMal-c2x vector and treated exactly as the PhyllIP sample, had no effect on spore germination (FIG. 4c), nor did protease-treated MBP (FIG. 4d), at total protein concentrations \leq 500 ng/ μ L. We note that no inhibition of spore germination was observed with MBP-PhyllIP fusion protein not treated with Factor Xa. It was concluded that released PhyllIP is responsible for the observed inhibition, and since it is evident (FIG. 4a, SDS gel) that released PhyllIP is a minor component of the sample, the inhibitory concentration of PhyllIP is considered \ll 160 ng/ μ L. PhyllIP was lost when purification from MBP and Factor Xa was attempted.

In leaf infection assays performed with KY 14 plants, PhyllIP did not totally inhibit infection, but it greatly reduced necrotic leaf damage. MBP and uncut MBP-PhyllIP fusion samples allowed successful infections. Lack of total inhibition with PhyllIP may be due to insufficient protein concentration, or alternatively, adducts with lipids or trichome exudate components are essential for a native-protein like response.

The T-Phylloplanin Promoter Region Directs Expression in Small Glandular Trichomes

1.8 kb of genomic DNA sequence was elucidated upstream from the Phylloplanin transcription start site. A 1.1 kb region of this DNA, as well as the 5'UTR and the Phylloplanin signal sequence, was fused in-frame with the reporter genes P-glu-

TABLE 1

Amino acid sequences recovered from phylloplanin N-terminal analyses, trypsin digestion, and pepsin digestion.				
Method	Peak (min)	Phylloplanin	Amino Acid Sequence	Name
N-terminus	N/A	I	ILVPTLVST	(SEQ ID NO:21)
	N/A	II	ILVPTLVSTHISGLVFCSV	(SEQ ID NO:22) aa-N1
	N/A	III	ILVPTLVSTHISGLVFCSV	(SEQ ID NO:23) aa-N1
	N/A	IV	ILVPTLVSTHISGLVFCSV (major)	(SEQ ID NO:24) aa-N1
Trypsin	36.2	I	ASVQLR	(SEQ ID NO:25) aa-T1
	59.8	I	ILNLNI (major)	(SEQ ID NO:26) aa-T4
			CGATNVISSTIT (minor)	(SEQ ID NO:27) aa-T2
	56.7	III	LVVATPLSTC \times ATL \times SVG	(SEQ ID NO:28) aa-T3
	58.7	III	ILNLNI (major)	(SEQ ID NO:29) aa-T4
CGAT \times V \times SSTIT (minor)			(SEQ ID NO:30) aa-T2	
Pepsin	1	I, II, III, IV	IRVGLAPTG	(SEQ ID NO:31) aa-P1

Table 1. Amino acid sequences recovered from phylloplanin N-terminal analyses, trypsin digestion, and pepsin digestion.

Escherichia coli-Expressed T-Phylloplanin

A 10.3 kDa portion of the Phylloplanin gene (PhyllIP) was expressed in *E. coli* as a fusion protein with MBP. Soluble

65 curonidase (GUS) and Green Fluorescent Protein (GFP) and introduced into TI 1068 plants using *Agrobacterium* mediated plant transformation. GUS and GFP were expressed only in SGTs (FIG. 5), indicating activity of a SGT-specific promoter. TI 1068 SGTs are uniformly distributed over the leaf

surface and protrude over surrounding epidermal cells (FIG. 1A). It appears that phylloplanins are biosynthesized locally in SGTs and are secreted to the leaf surface where, because of their hydrophobicity and basicity, phylloplanins dissolve in TGST exudate and are dispersed widely on the leaf surface during exudate flow, as exudate can reach ~17% of leaf dry weight in TI 1068. Certain animal saposin proteins are also hydrophobic and basic, are secreted by epithelial cells, and operate as components of innate immunity at the pulmonary air:water interface (Weaver, T. E. and Conkright, J. J. 2001).

Ultrastructural studies of Akers et al. (Akers, C. P. et al. 1978) defined the structures of *N. tabacum* L. cv Xanthi SGTs and TGSTs. Glands of SGTs were observed to have about four cells separated by large intracellular spaces that contained substantial OsO₄ stained material, apparently destined for secretion outside the gland. The nature of the accumulated substance was not defined, and it is now concluded that this substance is phylloplanins, as phylloplanins have been found on all tobaccos during this investigation. The pattern of intracellular space disposition observed in that study is very similar to that observed here using the phylloplanin-promoter-GFP construct (FIG. 5C). It was concluded that phylloplanins are produced exclusively in SGT gland cells, secreted to gland extracellular spaces, and then transferred outside the glands through constrictions at termini of intracellular spaces which is presumed to be "secretory pores" (yellow arrows, FIG. 5C) of unknown structure.

The majority of plant pathogens are fungi. When airborne spores land on a leaf surface, germination is the initial step leading to host colonization. It is hypothesized that by rapidly inhibiting spore germination at the leaf surface, preformed plant proteins may suppress pathogen infection before induced defenses become functional, in a manner analogous to secreted surface proteins of animals. This hypothesis is supported by the observations that surface-accumulated *N. tabacum* phylloplanins I-IV and *E. coli*-expressed PhyllP inhibit *P. tabacina* spore germination in vitro and limit leaf infection in situ. This hypothesis is also supported by the observation that the phylloplanin promoter directs expression specifically in SGTs, and from this we propose that phylloplanins are secreted to the leaf surface. Three observations link the gene Phylloplanin to phylloplanin I-IV proteins collected from the leaf surface. First, all amino acid sequences recovered from leaf surface phylloplanins I-IV are present in the predicted protein sequence from Phylloplanin, representing 54% of the mature protein open reading frame. Secondly, a functional link is provided between the gene and the proteins by replicating LWW blue mold inhibition with *E. coli*-expressed PhyllP. The phylloplanin promoter is a third, critical link between the gene and the proteins, and implicates SGTs as the sites of phylloplanin biosynthesis and delivery to the surface.

Secreted phylloplanins represent a novel leaf surface defense in the plant kingdom, wherein protein biosynthesis in SGTs allows deposition and dispersion of phylloplanins on leaf aerial surfaces to deter pathogen establishment. Further study is needed to identify the post-translational modifications or biochemical adducts thought to be present in phylloplanins I-IV, and to elucidate the mechanisms of phylloplanin-mediated fungal inhibition. It is emphasized that this work shows that SGTs must be regarded as specialized biosynthetic structures akin to TGSTs. Further study is needed to understand details of how SGTs deliver phylloplanins to the leaf surface, and whether these surface-disposed "protein factories" can be utilized for molecular farming.

Biological material and growth conditions. Greenhouse plants (*Nicotiana tabacum* L. tobacco introduction (TI) 1068,

TI 1112, TI 1406; cultivars KY 14 and Petite Havana SR1 [hereafter referred to by TI number or cultivar name]) were germinated and grown in soil under natural light at 22-24° C. with weekly fertilization (20-20-20, N-P-K). Plants were transplanted into 15-cm pots and treated with the insecticide Marathon (Olympic Horticultural Products, Mainland, Pa.) at 3-4 wk post-emergence. Field plants (TI 1068, *Glycine max*, *Helianthus annuus*) were grown at a farm near Lexington, Ky. during the 2002 growing season.

To grow sterile TI 1068 plants, seeds were immersed in 10% (v/v) sodium hypochlorite for 10 min, rinsed briefly in 70% (v/v) ethanol, washed 4 times in sterile water, and germinated on Murashige-Skoog (MS) medium (Murashige, T. and Skoog, F. 1962) containing B5 vitamins (100 mg/L myo-inositol, 10 mg/L thiamine-HCl, and 1 mg/L each pyridoxine-HCl and nicotinic acid) in a 22° C. growth chamber under fluorescent illumination (light/dark 16/8 h daily). Individual plants were transferred to PlantCons (ICN Biomedicals, Aurora, Ohio) containing MS agar at 3 wks post-emergence.

E. coli strain ER2508 (New England Biolabs, Beverly, Mass.) was stored and propagated as described by the supplier. Spores of *Peronospora tabacina* (isolate KY-79) were harvested from sporulating lesions on KY 14 plants as described (Reuveni, M. et al. 1986).

Phylloplanin collection and SDS-PAGE. Water-soluble phylloplane components were collected from mature, fully-expanded leaves of all greenhouse-grown and field-grown plants by washing freshly-detached leaves in 200 mL nanopure water (NANOpure water system D4751, Barnstead/Thermolyne, Dubuque, Iowa) for 15 s. Cut petioles or cut leaf surfaces were not exposed to wash solutions.

Water washes were filtered (No. 1 filter paper, Whatman, Clifton, N.J.), lyophilized to dryness, resuspended in 3 mL sterile water, and centrifuged at 12,000×g for 5 min at 21° C. The supernatants were filtered (13 mm/0.45-µm syringe filter, Corning Glass Works, Corning, N.Y.) to exclude bacteria and fungi, and are hereafter referred to as leaf water washes (LWW).

Proteins were separated by SDS/12%/glycine-PAGE (Laemmli, U. K. 1970) or SDS/15%/tricine-PAGE (Judd, R. C. 1994) using a Mini-Protean II electrophoresis system (Bio-Rad, Hercules, Calif.), according to the manufacturer's instructions, and visualized with Coomassie blue or silver staining.

Protein concentration was estimated using the bicinchoninic acid assay (Pierce Chemical, Rockford, Ill.) with BSA as a standard. Leaf surface areas were estimated by tracing leaves onto uniform-weight paper and weighing the cutouts.

Collection of epidermal peels and extracellular fluid (EF). Epidermal peels were prepared from greenhouse-grown TI 1068 plants as described (Kandra, L. et al. 1990), pulverized with liquid N₂, and proteins were analyzed by SDS-PAGE. EF was collected using a vacuum infiltration method (Terry, M. E. and Bonner, B. A. 1980) and analyzed by SDS-PAGE.

GC analysis. Trichome exudate was collected from greenhouse-grown TI 1068 by immersing unwashed leaves for 15 s in 200 mL acetonitrile. The wash solutions were filtered (No. 1 filter paper, Whatman), dried, and trichome exudate was resuspended in 5 mL acetonitrile and quantified by GC (flame ionization detection) as trimethylsilyl derivatives prepared in dimethylformamide, as previously described (Wang, E. et al. 2001). To determine the amounts of trichome exudate biochemicals occurring in LWW, volumes equivalent to 200 cm² leaf surface areas were transferred to glass GC vials and dried in a vacuum oven (37° C.) overnight. Trichome exudate biochemicals were extracted at 21° C. with methylene chloride, dried, solubilized, derivatized, and analyzed by GC. The

amount of residual trichome exudate biochemicals in LWW was assessed relative to total trichome exudate on an equivalent surface area basis.

Phylloplanin Amino Acid Sequencing. Proteins in greenhouse-grown TI 1068 LWW were separated by SDS-PAGE, transferred to polyvinylidene difluoride (Immobilon-PSQ, Millipore, Bedford, Mass.) using a Mini-Protean II electroblot apparatus (Bio-Rad), and visualized with Coomassie blue. Phylloplanin bands were subjected to N-terminal sequencing using automated Edman degradation (Matsudaira, P. 1987) at the University of Kentucky Macromolecular Structure Analysis Facility (Lexington, Ky.). To recover internal aa sequence information, LWW from greenhouse-grown TI 1068 was separated by SDS-PAGE, stained with Coomassie, and 21 kDa and 19 kDa bands were excised and digested with trypsin. Total proteins in TI 1068 LWW were also digested with pepsin. Resulting tryptic or peptic peptides were separated by reversed-phase HPLC (Aquapore RP-300 7 μ m particle size octyl reversed-phase column [Applied Biosystems, San Jose, Calif.]), manually collected based on absorbance at 214 nm, and samples were reduced in volume under vacuum to ~50 μ L. Amino acid sequence analyses of tryptic peptides were performed as above. For peptic peptides, similar analyses were performed at The Protein Facility of Iowa State University (Ames, Iowa).

A. thaliana AAP75801 cloning and expression in *E. coli* Total RNA was isolated from *A. thaliana* leaf tissue (100 mg FW) using an RNaqueous isolation kit. cDNA was synthesized from 5 μ g total RNA using a Qiagen Omniscript RT kit, according to the manufacturer's protocol. The PCR amplification reaction (50 μ L volume) was performed using PCR master mix (Promega), 2 μ L cDNA template and 0.2 μ M each of a primer pair designed from the GenBank *A. thaliana* AAP75801 mRNA sequence, RWS102-s (5'-ATGGGGGGAAGACACTTTATCACATCCAAAAC-3') (SEQ ID NO:32) and RWS102-as (5'-TCAGTTAAGAAGACCAAAGCCGGCAGG-3') (SEQ ID NO:33). The PCR product was size-fractionated by electrophoresis in a 1.0% (w/v) agarose gel, isolated using a gel extraction kit (Qiagen Qiaex II), cloned into a pGem-T vector (Promega), and the purified plasmid (pGemT::AAP75801) was sequenced.

To overexpress AAP75801 in *E. coli*, the coding sequence was amplified from pGemT::AAP75801 using the primers RWS109-s (5'-GACGACGACAAGATGGGGGAAGACACTTTATCACACA-3') (SEQ ID NO:34); the underlined section is for ligation independent cloning [LIC], the bold text is the start codon and RWS109-as (5'-GAGGAGAAGCCCGGTCAGTTAAGAAGACCAAAGCC-3') (SEQ ID NO:35); the underlined section is for LIC, the bold text is the stop codon. Amplification was performed for 32 cycles using the following thermal profile: 95° C. for 45 s, 60° C. for 45 s, 72° C. for 45 s, followed by a final 5 min extension at 72° C. The PCR product was size-fractionated by electrophoresis in a 1.0% (w/v) agarose gel, and isolated using a gel extraction kit. The product was then annealed with the pET-30Ek/LIC vector (Novagen), according to the manufacturer's ligation independent protocol, and cloned into *E. coli* BL21 (DE3) competent cells. Cultures (ranging in volumes from 100 mL to 1000 mL) were incubated at 37° C. (or 28° C. for low-temperature experiments) with shaking (250 rpm). When OD₆₀₀ reached 0.6, isopropyl-beta-D-thiogalactoside (IPTG) was added to a final concentration of 0.1 mM, and the culture was incubated with shaking for another 2 hours. Immediately after IPTG induction of protein expression, the Pet30: AAP75801 culture stopped growing (FIG. X). We were unable to prepare any heterologous AAP75801 from induced

cultures, even using 1 L culture volumes and His-tag binding resin mediated column chromatography. These results indicate that the AAP75801 gene product is antibacterial.

Degenerate RT-PCR, RLM-RACE, and Elucidation of Genomic Structure. Total RNA was extracted from TI 1068 leaf tissue (100 mg fresh weight [FW]) with an RNeasy kit (Qiagen, Chatsworth, Calif.), and cDNA was synthesized from 5 μ g total RNA using an Omniscript RT kit (Qiagen). PCR was performed using PCR master mix (Promega, Madison, Wis.) containing 3 μ L cDNA template and 4 μ M of each primer in a 50 μ L volume. Successful amplification of a PCR product occurred with the primers 5'-ACWTTTGTITCI-ACWCATATYTCIGGICTIGTYTTTTG-3' (SEQ ID NO:1) and 5'-AARAAICCGTTGGIGCIARICCIACYCTAAT-3' (SEQ ID NO:2) where I=Inosine, W=A or T, Y=C or T, and R=A or G. Amplification was for 46 cycles using the following thermal profile: 95° C. for 45 s, 50° C. for 45 s, 72° C. for 1 min, followed by a final 4 min extension at 72° C. The PCR product was size-fractionated by electrophoresis in a 1% (w/v) agarose gel, extracted using a Qiaex II kit (Qiagen), cloned into a pGem-T vector (Promega), and sequenced.

For RNA ligase mediated rapid amplification of cDNA ends (RLM-RACE), total RNA was extracted from TI 1068 leaf tissue, as above. A GeneRacer kit (Invitrogen, Grand Island, N.Y.) containing SuperScript III was used to generate cDNAs, according to the manufacturer's instructions. Successful amplification of a 3'RACE product occurred with the GeneRacer 3'Primer and the gene-specific primer 5'-CTCAGTCCCCAAGTTTTTCCTAATGCATCAG-3' (SEQ ID NO:3). Successful amplification of a 5'RACE product occurred with the GeneRacer 5'Primer and the gene-specific primer 5'-GGCCAAGAAAGTTAACTAGCTGATGCATA-3' (SEQ ID NO:4). PCR cycling parameters were according to the GeneRacer protocol.

Phylloplanin genomic structure was elucidated using a GenomeWalker kit (Clontech, Palo Alto, Calif.), according to the manufacturer's protocol, using genomic DNA isolated from TI 1068 leaf tissue (100 mg FW) with a DNeasy plant kit (Qiagen). Primary PCR reactions were performed with a sense outer adaptor primer AP1, provided in the kit, and the antisense Phylloplanin-specific primer (5'-TGGAACAAGTATGGCAAATGCAGCGGGG-3') (SEQ ID NO:5). Primary PCR cycling parameters were 7 cycles of 25 s at 94° C. and 3 min at 72° C., followed by 32 cycles of 25 s at 94° C. and 3 min at 67° C., with a final extension of 7 min at 67° C. Products of primary PCR were diluted 1:25 and 1 μ L was used in nested PCR reactions with a sense inner adaptor primer (AP2), provided in the kit, and a nested antisense Phylloplanin-specific primer (5'-GGGGGTTGCGATTAATGCAGC-CAAAAGGAAAA-3') (SEQ ID NO:6). Nested PCR cycling parameters were 5 cycles of 25 s at 94° C. and 3 min at 72° C., followed by 20 cycles of 25 s at 94° C. and 3 min at 67° C., with a final extension of 7 min at 67° C. Amplified PCR products were amplified, size fractionated by gel electrophoresis, gel-extracted, cloned into pGem-T, and sequenced.

Expression vector construction and fusion protein purification. To overexpress the Phylloplanin gene in *E. coli*, a 10.3 kDa portion of the coding sequence (His33-Gly142, termed PhyllP) and the full-length mature protein coding sequence (Ile24-Asn150) were amplified incorporating XbaI and PstI restriction sites (PhyllP-sense: 5'-AGCT TCTAGACATATTTCGGGGCTGGTTTT (SEQ ID NO:7); PhyllP-antisense: 5'-AGCT CTGCAGTTAGCCGGTGGGGCGAGGCC-3' (SEQ ID NO:8); Full-sense: 5'-AGCT TCTAGAATACTTGTTCCAACACT-3' (SEQ ID NO:9); Full-antisense: 5'-AGCT

CTGCAGTAAATTGATGTTAAGA-3' (SEQ ID NO:10); restriction sites underlined). The PCR products were digested with XbaI and PstI and cloned into the pMal-c2x expression vector (New England Biolabs) to create a translation fusion between the gene inserts and malE (which encodes Maltose Binding Protein [MBP]). Protein expression was induced at 0.5 OD₆₀₀ by the addition of 0.1 mM isopropyl-beta-D-thiogalactoside. Cells were harvested and resuspended in column binding buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA) containing 1 mg/mL lysozyme. Cell lysate was centrifuged at 10,000×g for 10 min and the resulting supernatant was collected. Fusion protein was purified using amylose-mediated column chromatography (New England Biolabs) according to the manufacturer's instructions and examined by SDS-PAGE. Fractions containing purified fusion protein were pooled and concentrated to ~1 mg/mL using a 3 kDa centrifugal filter (Microsep 3K Omega, Pall Laboratories, Ft. Myers, Fla.). Factor Xa (New England Biolabs) was added and samples were incubated for 48 h at 21° C. Salts and buffer components were removed using a 3 kDa centrifugal filter, and protein concentration was adjusted to 1 mg/mL with the addition of sterile water.

Phylloplanin antibody and western blots. TI 1068 LWW was separated by SDS-PAGE and stained with Coomassie Blue. Phylloplanin III was excised and used to generate a rabbit polyclonal antibody (Strategic Biosolutions, Newark, Del.). Immunodetection was performed using a 1:10,000 dilution of phylloplanin antiserum and a 1:10,000 dilution of horseradish peroxidase-coupled anti-rabbit secondary antibody (Sigma, St. Louis, Mo.).

Protease treatment. Insoluble Proteinase K (ProtK) affixed to acrylic beads (100 mg; P0803, Sigma) was placed into mini-spin filters (732-6027, Bio-Rad). The filters containing beads were placed into empty 1.5 mL Eppendorf tubes, and the filters were washed with sterile water (700 µL; 2600×g for 1 min). The flow-through was discarded, and washing was repeated five times. The spin filters were transferred to empty 1.5 mL Eppendorf tubes. Samples were added to filters containing protease beads and incubated at 37° C. for 4 h, with periodic inversion to mix. The tubes were then centrifuged at 2600×g for 10 min, and the flow-through from each was collected and analyzed by SDS-PAGE or used in blue mold assays.

Peronospora tabacina spore germination and leaf infection assays. Freshly-collected *P. tabacina* spores were mixed with various concentrations of TI 1068 LWW, ProtK-treated TI 1068 LWW, or water incubated with ProtK, and germinated for 16 h in dark, humidified chambers as water drops (4 µL drops; 50 spores/µL) on microscope slides. The spores were then inspected visually at 100× magnification for germination. The absence of a germination tube after 16 h indicated inhibition. Similar experiments were performed with PhyllIP, MBP, ProtK-treated PhyllIP, and ProtK-treated MBP. To assess the immediacy of germination tube arrest by LWW, spores were observed after 3 h.

For the leaf infection assay, 6-wk-old, greenhouse-grown Petite Havana SR1 plants were pre-conditioned by incubation in a 21° C. growth room (14 h light) for 5 days. Dilution series (1, 5, 12.5, 25, 50, 75, 100 ng protein/µL) of TI 1068 LWW were prepared and mixed with freshly-collected *P. tabacina* spores immediately before inoculation. For each LWW dilution, 8-10 drops (4 µL drops; 100 spores/µL) were applied to one leaf of pre-conditioned plants. Plants were placed in dark, humidified chambers for 16 h to provide optimal conditions for infection, and then returned to the growth room. Treated leaves were excised 5 days after inoculation, placed in dark, humid chambers for 16 h, and then inspected for sporulation.

The formation of *P. tabacina* sporulating lesions indicated successful leaf infection.

Elucidation of phylloplanin promoter sequence and activity. Genomic DNA was isolated from TI 1068 leaf tissue (100 mg FW) using a DNeasy plant mini kit (Qiagen). The DNA sequence upstream of the Phylloplanin gene was recovered using a GenomeWalker kit (Clontech), according to the manufacturer's protocol. Briefly, ~4 µg genomic DNA was digested to completion (36 h) in four separate reactions with restriction enzymes that generated blunt ends (Dra I, EcoR V, Pvu II, Stu II). The resulting 'libraries' were purified by phenol/chloroform extraction and precipitation. Digested genomic DNA in each library were then ligated to 5'GenomeWalker Adaptor molecules and purified again. A primary PCR reaction for each library was performed with a sense outer adaptor primer AP1, provided in the kit, and the anti-sense Phylloplanin-specific primer (5'-TGGAACAAGTATGGCAAATGCAGCGGGG-3') (SEQ ID NO:5). Primary PCR cycling parameters were seven cycles of 25 s at 94° C. and 3 min at 72° C., followed by 32 cycles of 25 s at 94° C. and 3 min at 67° C., with a final extension of 7 min at 67° C. Products of primary PCR were diluted 1:25 and 1 µL was used in nested PCR reactions with a sense inner adaptor primer AP2, provided in the kit, and a nested antisense Phylloplanin-specific primer (5'-GGGGTTGCGATTAATGCAGCAAAAAGGAAAA-3') (SEQ ID NO:6). Nested PCR cycling parameters were five cycles of 25 s at 94° C. and 3 min at 72° C., followed by 20 cycles of 25 s at 94° C. and 3 min at 67° C., with a final extension of 7 min at 67° C. A 1.8 kB product was amplified from the Stu II-based library, and gel-extracted, cloned into pGem-T, and sequenced.

PCR using a Phylloplanin promoter-specific sense primer (5'-TGCTCCCACCACTAGAATCACCA-3') (SEQ ID NO:11) and a Phylloplanin-specific antisense primer with an Xba I cut site (5'-AGCT TCTAGATGTTGGAACAAGTATGG-3' (SEQ ID NO:12); Xba I site underlined) was then used to amplify the region of *N. tabacum* genomic DNA that included the first 25 amino acids of the phylloplanin protein (which included the signal sequence), the 5' UTR, and a further 1.1 kB upstream. The PCR product was then cut with Xba I and Hind III (at a restriction site endogenous to the promoter) and cloned into the Hind III/Xba I-sites of pBIMC (kindly provided by D. Falcone, pBIMC is a variant of pBI121 modified to include a polylinker in place of the GUS gene) to replace the CaMV-35S promoter and create the vector pBI-PhylloProm. To analyze the spatial expression of the promoter, the reporter genes GUS and sGFP (kindly provided by D. Falcone) were PCR-amplified with primers that incorporated Xba I and Xho I restriction sites (GUS-sense: 5'-AGCT TCTAGAATGTTACGTCCTGTAGAAACCCCA-3' (SEQ ID NO:13); GUS-antisense: 5'-AGCT CTCGAGTCATTGTTTGCCTCCCTGCT-3' (SEQ ID NO:14); sGFP-sense: 5'-AGCT TCTAGAATGGTGAGCAAGGGCGAGGA-3' (SEQ ID NO:15); sGFP-antisense: 5'-AGCT CTCGAGGCTTTACTTGTACAGCTCGT-3' (SEQ ID NO:16); restriction sites underlined). The PCR products were gel-extracted, cut with Xba I and Xho I, and ligated between Xba I/Xho I sites in the polylinker of pBI-PhylloProm to create in-frame fusions with the Phylloplanin start codon and signal sequence. These constructs were transformed into *Agrobacterium tumefaciens* GV3101 by triparental mating, and introduced into TI 1068 using the leaf disk method (Horsch, R. B. et al. 1985). Kanamycin-resistant plantlets were derived from kanamycin-resistant callus tissue and

transferred to soil. Leaf disks from pBI-PhylloProm:GUS explants were stained for GUS activity by incubation with 0.1% X-gluc (Jefferson, R. A. 1987) and photographed. Leaf disks from pBI-PhylloProm:GFP explants were magnified and photographed using a Zeiss Axioplan-2 imaging system.

Sequence data have been deposited with the EMBL/GenBank data libraries under accession number AY705384.

Bioinformatic Analysis

Homologous open reading frames of selected cDNA or EST sequences giving significant (e-value cutoff 104) BLASTn, BLASTp, and tBLASTx (Altschul et al., 1990) scores against T-Phylloplanin nucleotide and amino acid sequences were first analyzed for the presence of signal peptides using TargetP. A multiple alignment of protein sequences with the predicted signal peptides removed was performed using the CLUSTALW algorithm (DNASTAR Lasergene Software, Madison, Wis. (See FIG. 8A)).

The identity of the sequences compared to that of TI 1068 Phylloplanan cDNA (Genbank Accession No. AY705384) are as follows:

Genbank Accession No.	Species
BG525459	<i>Stevia rebaudiana</i> (SEQ ID NO: 36)
CV545024	<i>Brassica rapa</i> (subspecies <i>pekinensis</i>) (SEQ ID NO: 37)
CD847345	<i>Helianthus annuus</i> (SEQ ID NO: 38)
AAP75801	<i>Arabidopsis thaliana</i> (SEQ ID NO: 39)
AAN28743	<i>Arabidopsis thaliana</i> (subspecies <i>thalecress</i>) (SEQ ID NO: 40)
XP479490	<i>Oryza sativa</i> (SEQ ID NO: 41)
XP479489	<i>Oryza sativa</i> (SEQ ID NO: 42)

-continued

Genbank Accession No.	Species
CV502724	<i>Solanum tuberosum</i> (SEQ ID NO: 43)
BP903020	<i>Lycopersicon esculentum</i> (SEQ ID NO: 44)
AJ789767	<i>Antirrhinum majus</i> (SEQ ID NO: 45)
BU829351	<i>Populustremula</i> x <i>populus tremuloides</i> (SEQ ID NO: 46)
BM527339	Soybean clone ID Gm-cl061-3715 (SEQ ID NO: 47)
BM371086	<i>Hardeum vulgare</i> (SEQ ID NO: 48)

All of the foregoing accession numbers are incorporated by reference.

An unrooted phylogenetic tree was constructed using the maximum parsimony algorithm PROTPARS in the PHYLIP version 3.63 software package (Felsenstein, 2004), and tree robustness was estimated with 1000 bootstrapped data sets. The tree was displayed with the TREEVIEW version 3.2 software (Page, 1996). [See FIG. 8B]

The examples set forth above are provided to give those of ordinary skill in the art with a complete disclosure and description of how to make and use the various embodiments of the invention, and are not intended to limit the scope of what the inventors regard as their invention. Modifications of the above-described modes for carrying out the invention that are obvious to persons of skill in the art are intended to be within the scope of the following claims. All publications, patents, and patent applications cited in this specification are incorporated herein by reference as if each such publication, patent or patent application were specifically and individually indicated to be incorporated herein by reference

SEQUENCE LISTING

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<210> SEQ ID NO 28
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: N. tabacum
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (11)..(11)
<223> OTHER INFORMATION: Xaa= any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (15)..(15)
<223> OTHER INFORMATION: Xaa= any naturally occurring amino acid

```

```

<400> SEQUENCE: 28

```

```

Leu Val Val Ala Thr Pro Leu Ser Thr Cys Xaa Ala Thr Leu Xaa Ser
1           5             10             15

```

```

Val Gly

```

```

<210> SEQ ID NO 29
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: N. tabacum

```

```

<400> SEQUENCE: 29

```

```

Ile Leu Asn Leu Asn Ile
1           5

```

```

<210> SEQ ID NO 30
<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: N. tabacum
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (5)..(5)
<223> OTHER INFORMATION: Xaa= any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa= any naturally occurring amino acid

```


-continued

<400> SEQUENCE: 30

Cys Gly Ala Thr Xaa Val Xaa Ser Ser Thr Ile Thr
 1 5 10

<210> SEQ ID NO 31

<211> LENGTH: 9

<212> TYPE: PRT

<213> ORGANISM: N. tabacum

<400> SEQUENCE: 31

Ile Arg Val Gly Leu Ala Pro Thr Gly
 1 5

<210> SEQ ID NO 32

<211> LENGTH: 31

<212> TYPE: DNA

<213> ORGANISM: Artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: primer

<400> SEQUENCE: 32

atgggggaag acactttatc acatccaaaa c 31

<210> SEQ ID NO 33

<211> LENGTH: 27

<212> TYPE: DNA

<213> ORGANISM: Artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: primer

<400> SEQUENCE: 33

tcagttaaga agaccaaagc cggcagg 27

<210> SEQ ID NO 34

<211> LENGTH: 35

<212> TYPE: DNA

<213> ORGANISM: Artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: primer

<400> SEQUENCE: 34

gacgacgaca agatggggga agacacttta tcaca 35

<210> SEQ ID NO 35

<211> LENGTH: 35

<212> TYPE: DNA

<213> ORGANISM: Artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: primer

<400> SEQUENCE: 35

gaggagaagc cgggtcagtt aagaagacca aagcc 35

<210> SEQ ID NO 36

<211> LENGTH: 145

<212> TYPE: PRT

<213> ORGANISM: S. rebaudiana

<400> SEQUENCE: 36

Gln Ile Leu Pro Pro Pro Ile Leu Pro Pro Thr Ile Ile Arg Pro Pro
 1 5 10 15

Pro Ile Leu Pro Pro Ile Val Leu Pro Pro Pro Ile Val Leu Asn Pro
 20 25 30

Val Leu Asn Val Thr Gly Ile Val Ser Cys Ser Val Asn Ala Thr Val

-continued

```

65              70              75              80
Ser Cys Arg Ile Ile Val Ala Thr Pro Leu Ser Asn Cys Asn Ala Thr
      85              90              95
Leu Pro Thr Ala Gly Thr Leu Gln Ser Ala Leu Gln Val Ala Gly Thr
      100             105             110
Phe Ile Arg Gly Ile Leu Asn Asn Val Asn Leu Val Pro Ile Arg Phe
      115             120             125
Arg Leu Val Val
      130

```

```

<210> SEQ ID NO 39
<211> LENGTH: 153
<212> TYPE: PRT
<213> ORGANISM: A. thaliana

```

```

<400> SEQUENCE: 39

```

```

Gln Leu Gly Leu Gly Gly Ser Gly Gly Leu Gly Gly Leu Ile Gly Gly
1      5      10      15
Leu Val Gly Gly Leu Gly Gly Leu Val Gly Gly Leu Val Gly Gly Ile
20     25     30
Leu Asn Leu Val Asn Ile Asn Gly Val Val Phe Cys Ser Leu Asn Gly
35     40     45
Ala Pro Ser Gly Thr Ser Thr Pro Ala Phe Ala Asn Ala Gly Val Glu
50     55     60
Leu Gln Cys Gly Arg Gln Asn Arg Val Val Ser Thr Ala Thr Thr Asn
65     70     75     80
Ala Ala Gly Leu Phe Ser Leu Pro Thr Asp Ser Ile Gln Met Leu Leu
85     90     95
Ser Thr Leu Leu Ser Asp Cys Arg Val Val Thr Thr Pro Leu Ser
100    105   110
Thr Cys Asn Ala Asn Leu Pro Ser Val Gly Asn Leu Val Ser Arg Leu
115    120   125
Ala Met Ile Gly Asn Ser Leu Thr Gly Leu Leu Asn Ile Ile Ser Ile
130    135   140
Ile Pro Ala Gly Phe Gly Leu Leu Asn
145    150

```

```

<210> SEQ ID NO 40
<211> LENGTH: 128
<212> TYPE: PRT
<213> ORGANISM: A. thaliana

```

```

<400> SEQUENCE: 40

```

```

Gln Ser Gly Leu Gly Gly Ile Asn Val Pro Ile Ile Asn Gly Val Leu
1      5      10      15
Phe Cys Thr Ile Asn Gly Ala Pro Leu Asn Gly Thr Pro Ala Pro Ala
20     25     30
Phe Ala Asn Ala Val Val Gln Leu Gln Cys Gly Asn Leu Asn Arg Val
35     40     45
Val Ala Glu Thr Ile Ile Asn Ile Ala Gly Leu Phe Thr Phe Ser Thr
50     55     60
Asn Gly Ile Gln Ile Ser Leu Pro Thr Leu Leu Asn Asp Cys Arg Ile
65     70     75     80
Val Val Pro Thr Pro Arg Ser Ser Cys Asp Ala Thr Leu Pro Ser Thr
85     90     95
Gly Gln Leu Ile Ser Gln Leu Asn Leu Val Gly Ser Ile Val Ser Gly

```

-continued

	100		105		110										
Leu	Leu	Asn	Ile	Val	Ala	Ile	Leu	Pro	Thr	Gly	Phe	Ile	Pro	Thr	Ile
	115						120					125			

<210> SEQ ID NO 41
 <211> LENGTH: 124
 <212> TYPE: PRT
 <213> ORGANISM: *O. sativa*

<400> SEQUENCE: 41

Ala	Pro	Pro	Ala	Gln	Pro	Pro	Arg	Ile	Gln	Ala	Asp	Val	Val	Val	Met
1			5					10						15	
Gly	Tyr	Val	Pro	Cys	Asn	Asn	Gly	Thr	Ser	Met	Lys	Ser	Gly	Ser	Ala
		20					25						30		
Pro	Gly	Phe	Pro	Asn	Ala	Val	Val	Gln	Leu	Gln	Cys	Ala	Gly	Asp	Ala
		35				40						45			
Val	Ala	Ala	Val	Ala	Ala	Gly	Ser	Ala	Thr	Thr	Asp	Gly	Lys	Gly	Trp
	50					55					60				
Phe	Arg	Met	Ala	Met	Asn	Thr	Thr	Ala	Ala	Leu	Ser	Ser	Val	Ala	Ser
65					70					75					80
Gly	Cys	Ser	Leu	Val	Val	Thr	Thr	Pro	Leu	Ala	Thr	Cys	Asp	Ala	Ala
			85					90						95	
Leu	Pro	Ala	Thr	Gly	Thr	Leu	Gln	Ser	Gly	Leu	Arg	Leu	Leu	Val	Ser
		100					105							110	
Met	Val	Phe	Pro	Arg	Gly	Phe	Ser	Tyr	Val	Val					
		115					120								

<210> SEQ ID NO 42
 <211> LENGTH: 118
 <212> TYPE: PRT
 <213> ORGANISM: *O. sativa*

<400> SEQUENCE: 42

Lys	Leu	Gly	Arg	Leu	Val	Val	Thr	Gly	Val	Val	Pro	Cys	Asn	Thr	Gly
1				5					10					15	
Ser	Leu	Ile	Asp	Ile	Ala	Thr	Ser	Pro	Ala	Phe	Pro	Asn	Ala	Asp	Val
			20				25						30		
Glu	Leu	Arg	Cys	Ala	Gly	Lys	Leu	Val	Ala	Gly	Ala	Thr	Thr	Asn	Ser
		35				40						45			
Asn	Gly	Ser	Phe	Ala	Met	Glu	Ala	Asp	Leu	Thr	Ser	Gly	Leu	Ala	Met
	50					55					60				
Leu	Ile	Gly	Gly	Cys	Lys	Leu	Val	Val	Asp	Thr	Pro	Leu	Ile	Lys	Cys
65					70				75					80	
Asp	Ala	Asn	Leu	Pro	Ala	Ala	Gly	Ser	Leu	Val	Ser	Tyr	Leu	Gln	Gly
			85					90						95	
Pro	Leu	Thr	Arg	Leu	Leu	Gly	Gly	Ile	Phe	Arg	Leu	Phe	Pro	Ala	Gly
		100					105						110		
Phe	Ser	Phe	His	Ala	His										
		115													

<210> SEQ ID NO 43
 <211> LENGTH: 132
 <212> TYPE: PRT
 <213> ORGANISM: *S. tuberosum*

<400> SEQUENCE: 43

Gln	Leu	Gly	Gly	Leu	Leu	Gly	Gly	Leu	Leu	Gly	Pro	Ile	Ser	Ile	Asp
1				5						10				15	

-continued

Gly Val Leu Phe Cys Ser Leu Asn Gly Lys Ile Asp Val Leu Asn Gly
 20 25 30

Ala Thr Thr Pro Ile Phe Pro Asn Ala Ser Val Gln Leu Arg Cys Gly
 35 40 45

Ala Gly Asn Val Val Ser Ser Thr Thr Thr Asn Gly Ser Gly Ala Phe
 50 55 60

Ser Leu Val Leu Asn Pro Val Gln Asn Ile Leu Ser Ser Leu Leu Ser
 65 70 75 80

Asn Cys Asn Ile Val Val Thr Thr Pro Leu Ser Thr Cys Asn Ala Ser
 85 90 95

Leu Pro Ser Val Gly Val Leu Gln Ala Pro Leu Gln Ile Val Gly Arg
 100 105 110

Thr Thr Gly Gly Leu Val Asn Leu Val Ile Gly Val Phe Gln Leu Ile
 115 120 125

Pro Leu Leu Asn
 130

<210> SEQ ID NO 44
 <211> LENGTH: 133
 <212> TYPE: PRT
 <213> ORGANISM: L. esculentum

<400> SEQUENCE: 44

Gln Leu Gly Gly Leu Leu Gly Gly Leu Leu Ala Pro Thr Ser Ile Glu
 1 5 10 15

Gly Val Leu Phe Cys Ser Leu Asn Gly Lys Ile Asp Val Leu Asn Gly
 20 25 30

Ala Thr Thr Pro Ile Phe Pro Asp Ala Ser Val Gln Leu Arg Cys Gly
 35 40 45

Ala Gly Asn Val Val Ser Ser Thr Thr Thr Asn Gly Ser Gly Ala Phe
 50 55 60

Ser Leu Val Thr Ser Pro Val Gln Ser Leu Leu Ser Ser Leu Leu Ser
 65 70 75 80

Asp Cys Asn Ile Val Val Ile Thr Pro Leu Ser Thr Cys Asn Ala Thr
 85 90 95

Leu Pro Ser Val Gly Val Leu Gln Ala Pro Leu Gln Ile Val Gly Lys
 100 105 110

Thr Ala Gly Gly Gly Leu Leu Asn Ile Val Lys Leu Val Thr Gly Ala
 115 120 125

Phe Gln Leu Ile Asn
 130

<210> SEQ ID NO 45
 <211> LENGTH: 115
 <212> TYPE: PRT
 <213> ORGANISM: A. majus

<400> SEQUENCE: 45

Cys Thr Pro Asn Gly Asn Ile Gly Val Asn Gly Thr Ser Thr Pro Val
 1 5 10 15

Phe Pro Asn Ala Ala Val Gln Leu Gln Cys Gly Gly Thr Val Val Ser
 20 25 30

Thr Thr Thr Thr Asn Gly Leu Gly Gln Phe Ser Met Leu Leu Asp Pro
 35 40 45

Leu Asn Phe Val Leu Ser Thr Leu Val Ser Gly Cys Arg Leu Ala Val
 50 55 60

-continued

Thr Thr Pro Leu Ala Thr Cys Asn Ala Ser Leu Pro Ser Ala Gly Gly
65 70 75 80

Leu Ile Ser Thr Leu Gln Phe Val Gly Ser Thr Val Leu Gly Leu Leu
85 90 95

Asn Val Gly Asn Ile Ile Pro Ser Gly Phe Asn Phe Ser Ala Asn Met
100 105 110

Asn Leu Asn
115

<210> SEQ ID NO 46
<211> LENGTH: 138
<212> TYPE: PRT
<213> ORGANISM: P. tremuloides

<400> SEQUENCE: 46

Ala Pro Val Ala Glu Ala Gln Leu Gly Leu Ile Gly Gly Leu Leu Gly
1 5 10 15

Leu Ile Arg Ile Gln Gly Thr Leu Phe Cys Thr Ala Asp Gly Asn Ile
20 25 30

Gly Ala Asn Gly Thr Ala Thr Pro Val Phe Pro Asn Ala Leu Val Gln
35 40 45

Leu Gln Cys Gly Gly Asn Val Val Ser Thr Ser Thr Thr Asn Gly Ser
50 55 60

Gly Met Phe Ser Ile Leu Leu Asp Pro Leu Ser Tyr Ile Leu Ser Ser
65 70 75 80

Ile Leu Ser Asp Cys Asn Leu Lys Val Asp Thr Pro Leu Ile Ser Cys
85 90 95

Asn Ser Ser Leu Pro Ala Val Gly Gly Leu Leu Ser Pro Leu Arg Phe
100 105 110

Ile Gly Asn Thr Ala Leu Gly Ala Val Leu Ser Val Ala Asn Ile Ile
115 120 125

Pro Ala Gly Phe Arg Phe Val Pro Ser Asn
130 135

<210> SEQ ID NO 47
<211> LENGTH: 132
<212> TYPE: PRT
<213> ORGANISM: Soybean clone

<400> SEQUENCE: 47

Gln Leu Gly Ile Leu Ser Gly Leu Leu Gly Ser Val Ser Asn Ile Gln
1 5 10 15

Gly Thr Val Phe Cys Thr Ser Lys Asp Asn Met Gly Val Lys Gly Ala
20 25 30

Ser Val Pro Val Phe Pro Asn Ala Gln Val Gln Leu Val Cys Gly Gly
35 40 45

Lys Glu Leu Ser Asn Ala Lys Thr Asn Asp Asp Gly Thr Phe Ser Met
50 55 60

Met Met Asp Pro Leu Leu Leu Asp Leu Ala Ser Leu Leu Ser Gly Cys
65 70 75 80

Asn Leu Val Val Ala Thr Pro Leu Ser Asn Cys Asn Ala Lys Leu Pro
85 90 95

Ser Thr Gly Gly Leu Ile Ser Thr Leu Asn Phe Ala Gly Ile Thr Ser
100 105 110

Val Gly Thr Gln Thr Met Ala Asn Ile Ile Pro Ser Gly Phe His Phe
115 120 125

-continued

Leu Pro Ser Ile
130

<210> SEQ ID NO 48
<211> LENGTH: 118
<212> TYPE: PRT
<213> ORGANISM: H. vulgare

<400> SEQUENCE: 48

Lys Leu Gly Arg Leu Val Val Ser Gly Val Ala Pro Cys Asn Thr Gly
1 5 10 15
Ser Leu Ile Asp Ile Ala Thr Ser Pro Ala Phe Pro Asn Ala Glu Val
20 25 30
Glu Leu Arg Cys Ala Gly Gln Val Val Ala Gly Ala Thr Thr Asn Thr
35 40 45
Asn Gly Ser Phe Thr Met Glu Ala Asp Leu Thr Ser Ala Leu Ala Ala
50 55 60
Phe Ile Gly Arg Cys Ser Leu Val Val Asp Thr Pro Leu Ile Lys Cys
65 70 75 80
Asp Ala Gln Leu Pro Pro Ala Gly Arg Leu Val Ser Tyr Leu Gln Gly
85 90 95
Pro Leu Thr Arg Leu Leu Gly Gly Ile Phe His Leu Phe Pro Ala Gly
100 105 110
Phe Ser Phe His Ser Arg
115

<210> SEQ ID NO 49
<211> LENGTH: 1249
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: promoter sequence

<400> SEQUENCE: 49

cccattccac tatgaacttc ccggaattca attctgacta tgcgtacaag tcataatgaa 60
gctgcacata gccttcatat cgctaaacga cgtgctaggt ctcaaaacga cctgtcgggg 120
tcgttacatt agaggtgatt aacttcgtgt atacttgtgc aagtgttcta taacaatttc 180
aggccaacct agtaagagta gaaatagtag atggcacata acaaacgata accacgaaat 240
gtacatgata taactcacac aaggtaggca cgctactaga caattaccaa taacaacaat 300
gcctaggaca tcacaagata tgaaaaatca atccttacta tcacggttga gttgtaacgt 360
gtaagaatat ttcacacttt ttagggcact aagatcactc caccaacatt tcaagagaat 420
cactggcact gccaaaaagc cctctacact gtagtgaatt tttgttagtt atctaaagtt 480
aattattcac ttagtattct ttacattaggt tccccctt ctaggtcctg cacgtaacta 540
gattgaatgg attggtccac tctattatta cagagtaatt attaaatatt tatttgacta 600
ggcaacacta attgcactat caacaagta ttagttctag ccttctgggt acttcatacc 660
tatgcaaatg ataattttat ttaaaacaat agatgtacat ggatataaat acctatgaaa 720
attaaataaa atataactaa gaaaaaaaaa ttaaagttca ctccctaagat atcgggttat 780
tacatgacca aacacaattt gtttatcaaa tactttcaaa agaatttgtc aaacgtaaat 840
tatttttctc caaagtgact tatgaattac tatgttgata aaatactttt caaagtaact 900
aatgtttaga agtcaaggat gggcttcttt tgattattga agttttagtc aattgtatgt 960
agttatagtc agggtagca ccagcatctc atatagcaat acacaagtgg gtttagcgtat 1020

-continued

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ttgaaatttc aattagttca ttcaaatata cacgtaatag cattataagc cactttcaca 1080
acagatagat taggggtttt aaaatttcaa ccaatgatat ttactataaa ttgatcatgc 1140
acaaacctta attgagcaac acaatttctt acagcaataa ctatcacata taacaataac 1200
tgccatggct tcagcaaaaa ttttcttgat tttccttttg gctgcatta 1249

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<210> SEQ ID NO 50
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: promoter sequence

```

```

<400> SEQUENCE: 50

```

```

Met Ala Ser Ala Lys Ile Phe Leu Ile Phe Leu Leu Ala Ala Leu
1           5           10          15

```

What is claimed is:

1. An isolated polypeptide comprising a sequence that is at least 95% identical to SEQ ID NO:18, wherein the polypeptide is a disease- or pest-resistance conferring protein.

2. The isolated polypeptide of claim 1, wherein the polypeptide comprises a sequence that is at least 98% identical to SEQ ID NO: 18, wherein the polypeptide is a disease- or pest-resistance conferring protein.

3. The isolated polypeptide of claim 1, wherein the polypeptide comprises SEQ ID NO:18.

4. The isolated polypeptide of claim 1, wherein the polypeptide comprises the amino acid sequence selected

from the group consisting of: amino acid sequence from residue 22 to 150 of SEQ ID NO: 18, amino acid sequence from residue 23 to 150 of SEQ ID NO: 18, and amino acid sequence from residue 24 to 150 of SEQ ID NO: 18.

5. A composition comprising a polypeptide of claim 1 and a pharmaceutically acceptable carrier.

6. A polypeptide produced by culturing a recombinant host cell genetically engineered to contain an isolated polynucleotide encoding the polypeptide of claim 1, wherein the culturing is performed under conditions to promote expression of the polypeptide.

* * * * *