

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
29 June 2006 (29.06.2006)

PCT

(10) International Publication Number  
**WO 2006/066355 A1**

(51) International Patent Classification:

**C07K 14/42** (2006.01) **A61P 31/00** (2006.01)  
**A01P 1/00** (2006.01) **A61P 31/04** (2006.01)  
**A01P 3/00** (2006.01) **C07H 21/04** (2006.01)  
**A61K 38/16** (2006.01) **C12N 15/29** (2006.01)

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(21) International Application Number:

PCT/AU2005/001967

(22) International Filing Date:

23 December 2005 (23.12.2005)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

2004907323 24 December 2004 (24.12.2004) AU

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: CHITIN-BINDING PEPTIDES

(57) Abstract: The invention provides plant chitin-binding peptides having activities not hitherto found in known chitin-binding peptides or proteins. The peptides according to the invention comprise the sequence  $X_1PX_2CSPAGX_3X_4YCNX_5GRCCSX_6X_7NWCX_8TAAYCX_9X_{10}X_{11}NCIAX_{12}CWZ$  wherein, each X is independently any amino acid residue other than C, and Z is a carboxy group or the dipeptide PG. The invention also provides DNA encoding the peptides, constructs comprising the DNA, host cells transformed with the foregoing constructs, transgenic plants comprising the DNA, and reproductive material of such plants. The invention further provides compositions of the chitin-binding peptides and use of the peptides in controlling microbial infestation of a subject mammal, or insect or microbial infestation of a plant.

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## CHITIN-BINDING PEPTIDES

### TECHNICAL FIELD

The invention relates to peptides capable of inhibiting the growth of fungi, Oomycetes and bacteria, inhibiting infection by plant viruses, and inhibiting the activity of certain  
5 metabolic enzymes. In particular, the invention relates to newly identified representatives of the plant chitin-binding class of antimicrobial peptides, which representatives can be isolated from seeds of Australian endemic gymnosperms such as *Araucaria bidwillii*, *Agathis robusta* and *Podocarpus elatus*. The invention further relates to the purification and/or synthesis of the peptides, compositions comprising the peptides, and DNA encoding the peptides. The  
10 invention still further relates to the use of the peptides and/or DNA encoding the peptides in the prophylaxis or treatment of microbial or viral infestation of a plant. The invention also relates to the use of the peptides in the prophylaxis or treatment of microbial infection of an animal subject, particularly a human subject.

### BACKGROUND OF THE INVENTION

#### 15 Control of Pathogenic Microorganisms

Plant pathogens are those agents capable of causing diseases of plants. These may include fungi belonging to genera such as *Sclerotinia*, *Verticillium*, *Botrytis*, *Fusarium*, *Diaporthe*, *Macrophomina*, *Leptosphaeria*, *Mycosphaerella*, *Septoria* and others; Oomycetes belonging to genera such as *Phytophthora*, *Pythium*, *Albugo*, and the various downy mildew  
20 pathogens, bacteria belonging to genera such as *Pseudomonas*, *Xanthomonas*, *Erwinia*, *Ralstonia*, *Clavibacter*, and *Agrobacterium* and various viruses, phytoplasmas and nematodes. The symptoms produced on plants infected with such agents include damping-off, rotting of roots and shoots, malformation of plant organs, leaf and stem spots and others. Such infection of plants especially crop plants by plant pathogens such as these may cause lowering of yield  
25 both in quantity and quality. It is important therefore that plant pathogens be controlled.

Similarly microorganisms cause diseases in animals such as warm and cold blooded vertebrates. Fungal infection of animals are called mycoses and can be caused by fungi belonging to genera such as *Candida*, *Aspergillus*, *Fusarium*, *Xylohypha*, *Trichophyton*, *Scopulariopsis*, *Sporothrix*, *Histoplasma*, *Coccidioides*, *Cryptococcus* and others. These fungi  
30 can cause cutaneous, pulmonary, and systemic infections. As well fungal infection of food stuffs can cause toxicosis as toxins (mycotoxins) produced by the fungus are ingested by the feeding animal. Examples are the aflatoxins, trichothecenes, zearalenone, patulin and others

described in Matossian, M.K. [1989] '*Poisons of the past: molds, epidemics, and history*', Yale University Press, New Haven.

Control of many pathogens in plants can be obtained through manipulation of natural resistance genes, direct application of chemicals which inhibit germination of propagules and/or growth and reproduction, or use of other biological agents which either produce antibiotic molecules or compete for ecological niches used by plant pathogens. However, for many diseases effective or economic control measures are not available as natural genes conferring resistance have not been found or rapid changes in the virulence of the pathogen has rendered natural resistance ineffective or application of pesticides is too expensive or environmentally unacceptable. Although plants are constantly exposed to many microorganisms capable of causing diseases on many plant species they are resistant to many other pathogen species. Another option for the control of pathogens in any one plant species is to introduce genes from other species that confer resistance to those pathogens in those other species.

Plant chemicals may have a direct role in the defense of plants against plant pathogenic microorganisms. The earliest studied were the phytoalexins which low molecular weight secondary metabolites produced in response to pathogen challenge. More recently it has been shown that plants produce a large number of proteins and peptides which when isolated demonstrate antibiotic activity. These proteins have been categorised into several classes according to either their presumed mode of action and/or their amino acid sequence homologies. These classes include the following: chitinases (Roberts, W.K. *et al.* [1986] *Biochim. Biophys. Acta* 880: 161-170); chitin-binding proteins (De Bolle, M.F.C. *et al.* [1992] *Plant Mol. Biol.* 22: 1187-1190 and Van Parijs, J. *et al.* [1991] *Planta* 183: 258-264);  $\beta$ -1,3-glucanases (Manners, J.D. *et al.* [1973] *Phytochemistry* 12: 547-553); ribosome-inactivating proteins (Leah, R. *et al.* [1991] *J. Biol. Chem.* 266: 1564-1573);  $\alpha$  and  $\beta$  thionins (Fernandez de Caley, R. *et al.* [1972] *Appl. Microbiol.* 23: 998-1000 and Bohlmann, H. *et al.* [1988] *EMBO J.* 7:1559-1565); permatins, thaumatin-like and osmotin-like proteins (Woloshuk, C.P. *et al.* [1991] *Plant Cell* 3: 619-628 and Hejgaard, J. [1991] *FEBS Letts.* 291: 127-131 and Vigers, A.J. *et al.* [1991] *Mol. Plant-Microbe Interact.* 4:315-323); non-specific lipid transfer proteins (Molina, A. *et al.* [1993] *FEBS Letts.* 316: 119-122); 2s Albumins (Terras, F.R.G. *et al.* [1992] *J. Biol. Chem.* 267: 15301-15309) and plant defensins (Terras, F.R.G. *et al.* [1995] *Plant Cell* 7:5723-588). Other antimicrobial proteins from plants that have yet to be classified into broad groups include the peptide Mi-AMP1 (*Macadamia integrifolia*- Antimicrobial

Protein 1) ( Marcus, J.P. *et al.* [1997] *Eur. J. Biochem* 244:743-749), the neurotoxin-like antimicrobial knottin-type peptides from *Mirabilis jalapa* (Cammue, B.P.A. *et al.* [1992] *J. Biol. Chem.* 67:2228-2233), the peptides of *Impatiens balsamina* (Tailor, R.H *et al.* [1997] *J. Biol. Chem.* 272:24480-24487) and snak-in-1 from potato tubers (Segura *et al.* [1999] *Mol. Plant Micr. Int.* 12:16-23)

### **Chitin-Binding Peptides**

Plants produce a number of proteins that have binding affinity for certain carbohydrates. These so-called lectins include some with affinity to poly (*N*-acetyl-D-glucosamine) commonly known as chitin. The chitin binding proteins in plants have been reviewed by Raikhel and Broekaert (1993) [pp 407-423 in *Control of Plant Gene Expression*, DPS Verma (ed.) CRC Press] and Raikhel *et al.* (1993) [*Ann. Rev. Plant Phys. Plant Mol. Biol.* 44:591-615]. All include a cysteine/glycine-rich region thought to represent the chitin binding domain. Some of the proteins have multiple domains and are capable of agglutinating cells while others such as chitinases combine the chitin binding domain with a catalytic domain capable of hydrolysing chitin. Small non-enzymatic lectins have also been isolated from many plant species including cereals such as wheat, barley or rice (Rice and Etzler [1974] *Biochem Biophys Res Comm.* 59:414-419; Peumans *et al* [1982] *Biochem J.* 203: 139-143; Tsuda [1979] *J Biochem.* 86:1451-1461) and stinging nettle (Peumans *et al.* [1983] *FEBS Lett.* 177:99-103). Many of these small chitin-binding peptides have been shown by *in vitro* bioassay to be anti-fungal. These include a peptide, called hevein, from the latex of the rubber tree (Van Parijs *et al.* [1991] *Planta* 183: 258-264) and peptides from the leaves of *Ginkgo* (Huang *et al.* [2000] *FEBS Lett.* 478:123-126), bark of the spindle tree (Van den Bergh *et al.* [2002] *FEBS Lett.* 530:181-185), leaf intercellular washing fluids (Nielsen *et al.* [1997] *Plant Physiol.* 113: 83-91), seeds of *Amaranthus caudatus* (De Bolle *et al.* [1993] *Plant Mol. Biol.* 22: 1187-1190), *Atriplex nummularia* (Last and Llewellyn [1997] *New Zealand Journal of Botany* 35: 385-394), *Pharitis nil* (Koo *et al.* [1998] *Biochim Biophys Acta* 1382: 80-90) and *Capsicum* and *Briza* (International Patent Application Publication Number WO94/11511).

The chitin-binding class of anti-microbial proteins are herein defined as monomeric proteins about 30-45 amino acids lacking enzymatic activity with cysteine backbone of 6-10 cysteine residues forming disulphide bridges, basic pI, small size around 3-5000Da. Structural studies performed on hevein (Andersen *et al.* [1993] *Biochemistry* 32:1407-1422) and Ac-AMP2 from *Amaranthus caudatus* (Martins *et al.* (1996) *J Mol. Biol.* 258: 322-333) show that these peptides, although different in length, consist of 3 antiparallel  $\beta$  sheets with varying loops

and helical turns all stabilised by disulphide bridges. The conserved presence of one serine and three aromatic amino acid residues are necessary for chitin binding. Mutagenesis of these residues reduces or removes chitin affinity (Muraki *et al.* [2000] *Protein Engineering* 13: 385-389).

5           The recognised biology activity of chitin-binding peptides includes being anti-fungal, anti-Gram-positive bacteria (Van den Bergh *et al.* [2002] *FEBS Lett.* 530:181-185) and possibly anti-insect. Many of these activities are thought to be due to interaction with of chitin within the cell walls of many fungi and in the exoskeletons of insects and *N*-acetylglucosamine as a component of peptidoglycan of bacterial cell walls. This, however, does not explain  
10 activity against Oomycetes which lack chitin. It may be the basic iso-electric points of these peptides or that the peptides also have affinity for other carbohydrates that are present that contribute to the inhibitory activity against these organisms (Van den Bergh *et al.* [2004] *Planta* 219: 221-232). The peptides have been found non-toxic to *in vitro* cultures of human cells (Broekart *et al.* [1992] *Biochemistry* 31: 4308-4314).

#### 15   **Use of Antimicrobial Peptides to Control Pathogenic Microorganisms**

Once purified, the amino acid sequence of an antimicrobial peptide can be determined using a method such as Edman degradation N-terminal sequencing. The amino acid sequence provides information that can be used in the subsequent production of the peptide. The genes encoding the peptides in their natural substrates can be identified by methods such as RT-PCR,  
20 3' and 5' RACE. The gene can be used to produce the peptide by manipulating the genetics of a cell system such that with expression of the gene the cell system produces the desired peptide. The cell system could be a whole plant such that expression of the antimicrobial peptide confers some protection from infection and subsequent ingress by pathological agents. Agricultural and horticultural plants might be used to express the peptides including sunflower,  
25 maize, sorghum, canola, wheat, cotton, grape, rice and all others.

Published information describing the effect of incorporating antimicrobial proteins and peptides into plants include reports of the expression of a plant chitinase (Broglie, K. *et al.* [1991] *Science* 254:1194-1197), a plant chitinase in combination with a plant glucanase (Zhu, Q. *et al.* [1994] *Bio/technology* 12: 807-812), a fungal chitinase (Terakawa, T. *et al.* [1997] *Plant Cell Reports* 16: 439-443), a ribosome-inactivating protein (Logemann, J. *et al.* [1992] *Bio/Technology* 10:305-308), a human lysozyme (Nakajima, H. *et al.* [1997] *Plant Cell Reports* 16: 674-679), a peptide derived from a moth (Huang, Y. *et al.* [1997] *Phytopathology* 87: 494-499), a plant lipid transfer protein (Molina, A. and Garcia-Olmedo, F. [1997] *Plant Journal* 12:

669-675), a peptide derived from horseshoe crabs (Allefs, S.H.J.M. *et al.* [1996] *Molecular Breeding* 2: 97-105), a plant  $\alpha$ -thionin (Carmona, M.J. *et al.* [1993] *Plant Journal* 3: 457-462) and a chitin-binding peptide (Lee, O.S. *et al.* [2003] *Phytochemistry* 62: 1073-1079).

Alternatively, the cell system could consist of cells maintained in culture such that expression of the gene would require purification of the peptide either from the cell or the medium in which the cells are growing. Such purified peptide could be used ectopically to protect plants, for example as a spray, or animals including humans from infection by infectious agents particularly where those agents are fungi. The peptide could be incorporated in some diluent and applied or encapsulated to provide timed release.

Peptides can also be manufactured by chemically synthesising the amino acid sequence. The above techniques allow the amino sequence of the peptides to be manipulated either through changing the nucleotide sequence of the encoding gene, that is, through site-directed or random mutagenesis or by controlling the incorporation of different amino acids during chemical synthesis. Such altered peptides may have enhanced efficacy against microorganisms (Cuervo, *et al* [1988] *Peptide Research* 1: 81-86).

#### **The Desirability of Having Available Further Chitin-Binding Peptides**

Although a large number of individual chitin-binding peptides are known, it would be desirable to have available further chitin-binding peptides so that an antimicrobial peptide can be chosen having an activity that is appropriate for the particular circumstance. Specifically, it would be desirable to have available new chitin-binding peptides which, *inter alia*:

- have selective activity against a particular pathogen of a particular plant or animal;
- have enhanced activity against a particular pathogen compared with other members of the class; and
- have activities not hitherto found in chitin-binding peptides such as antiviral activity and inhibitory activity against metabolic enzymes.

A number of plant chitin-binding peptides have been described in the patent literature. For example, in International Patent Application No. PCT/GB93/02179 (Publication No. WO 94/11511) by Zeneca Limited, the applicant describes two "biocidal chitin binding proteins". None of the disclosures referred to in the previous paragraph relate to chitin-binding peptides having the desirable properties set out above.

The invention described herein relates to previously unidentified peptides with anti-microbial activity. These peptides can be isolated from *Araucaria bidwillii* (Ab), *Agathis robusta* (Ar) or *Podocarpus elatus* (Pe) plants especially from the seed of these plants.

*Araucaria bidwillii* and *Agathis robusta* are members of the Araucariaceae family. The former produces an edible kernel known commonly as the bunya nut. The latter is known as the South Queensland Kauri Pine. *Podocarpus elatus* is a member of the Podocarpaceae family and is known as the Brown pine.

5

## SUMMARY OF THE INVENTION

An object of the invention is to provide new plant chitin-binding peptides having activities not hitherto found in known chitin-binding peptides.

It is also an object of the present invention to provide peptides that can inhibit the activity of some or all of the following:  $\alpha$ -amylase enzymes of different origins; proteinase  
10 enzymes especially of the serine proteinase class; and, protein synthesis in cell free systems.

It is a still further object of the invention to provide DNA capable of expressing the aforementioned peptides in cell systems especially plant cells.

According to a first embodiment of the invention, there is provided an isolated or synthetic peptide comprising the sequence:

15  $X_1PX_2CSPAGX_3X_4YCNX_5GRCCSX_6X_7NWCGX_8TAAYCX_9X_{10}X_{11}NCIAX_{12}CWZ$   
(SEQ ID NO: 1)

wherein, each X is independently any amino acid residue other than C, and Z is a carboxy group or the dipeptide PG.

According to a second embodiment of the invention, there is provided an isolated or  
20 synthetic DNA which encodes a peptide according to the first embodiment.

According to a third embodiment of the invention, there is provided a DNA construct which includes at least one DNA according to the second embodiment operatively linked to elements for the expression of peptide encoded by said DNA.

According to a fourth embodiment of the invention, there is provided a host cell  
25 transformed with a DNA construct according to the third embodiment.

According to a fifth embodiment of the invention, there is provided a transgenic plant transformed with a DNA construct according to the third embodiment.

According to a sixth embodiment of the invention, there is provided reproductive material of a transgenic plant according to the fifth embodiment.

30 According to a seventh embodiment of the invention, there is provided a composition comprising at least one peptide according to the first embodiment together with an agriculturally acceptable carrier, diluent or excipient.

According to an eighth embodiment of the invention, there is provided a composition comprising at least one peptide according to the first embodiment together with a pharmaceutically acceptable carrier, diluent or excipient.

According to a ninth embodiment of the invention, there is provided a method of  
5 controlling insect, microbial or viral infestation of a plant, the method comprising:  
i) introducing a DNA construct according to the third embodiment into said plant; or  
ii) treating said plant with a peptide according to the first embodiment or a composition according to the seventh embodiment.

According to a tenth embodiment of the invention, there is provided a method of  
10 controlling microbial infestation of a mammalian subject, the method comprising treating the subject with a peptide according to the first embodiment or a composition according to the eighth embodiment.

With regard to the first embodiment defined above, it is preferred that:

15 X<sub>1</sub> is a polar hydrophilic negatively charged amino acid residue (D or E); and/or  
X<sub>2</sub> is a polar, hydrophilic, neutral amino acid residue, preferably T or S; and/or  
X<sub>3</sub> is a polar, hydrophilic, positively charged or neutral amino acid residue, preferably R or Q; and/or  
X<sub>4</sub> is selected from F, I or Q; and/or  
X<sub>5</sub> is a polar hydrophilic amino acid residue, preferably D, N or K; and/or  
20 X<sub>6</sub> is a polar hydrophilic, positively charged amino acid residue, preferably R or K; and/or  
X<sub>7</sub> is a neutral amino acid residue, preferably S or F; and/or  
X<sub>8</sub> is a polar, hydrophilic, neutral amino acid residue, preferably N or S, and/or  
X<sub>9</sub> is selected from K, A or Q; and/or  
25 X<sub>10</sub> is a polar hydrophilic, positively charged amino acid residue, preferably R or K; and/or  
X<sub>11</sub> is a neutral amino acid residue, preferably P or G; and/or  
X<sub>12</sub> is a polar, hydrophilic, neutral amino acid residue, preferably N or Q.

Particularly preferred peptides comprise the following sequences:

30 Asp-Pro-Thr-Cys-Ser-Pro-Ala-Gly-Arg-Phe-Tyr-Cys-Asn-Asp-Gly-Arg-Cys-  
Cys-Ser-Arg-Ser-Asn-Trp-Cys-Gly-Asn-Thr-Ala-Ala-Tyr-Cys-Lys-Arg-Pro-  
Asn-Cys-Ile-Ala-Gln-Cys-Trp-Pro-Gly (SEQ ID NO: 2); or

Asp-Pro-Ser-Cys-Ser-Pro-Ala-Gly-Gln-Gln-Tyr-Cys-Asn-Asn-Gly-Arg-Cys-  
 Cys-Ser- Lys-Phe-Asn-Trp-Cys-Gly-Ser-Thr-Ala-Ala-Tyr-Cys-Gln-Lys-Pro-  
 Asn-Cys-Ile-Ala-Gln-Cys-Trp-Pro-Gly (SEQ ID NO: 3); or  
 Glu-Pro-Thr-Cys-Ser-Pro-Ala-Gly-Arg-Ile-Tyr-Cys-Asn-Lys-Gly-Arg-Cys-  
 Cys-Ser-Lys-Phe-Asn-Trp-Cys-Gly-Asn-Thr-Ala-Ala-Tyr-Cys-Ala-Lys-Gly-  
 Asn-Cys-Ile-Ala-Asn-Cys-Trp (SEQ ID NO: 4).

Other embodiments of the invention include methods for obtaining or producing the subject peptide, and use of the peptide in the preparation of a medicament for controlling microbial infestation of a mammalian animal. Still further embodiments of the invention relate to the purification and/or synthesis of nascent (pre-translationally processed) protein comprising the subject peptide, compositions comprising the nascent protein, DNA encoding the nascent protein, and, inter alia, uses thereof as described in respect of the subject peptide. Still further embodiments of the invention will become apparent from a reading of the following detailed description and the examples of the invention. The examples include reference to the accompanying drawings briefly described in the following section of the specification.

### BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1 shows a cation exchange chromatogram for the purification of AbAFP1 and the associated graph of antifungal activity.

Figure 2A shows a cation exchange HPLC profile of purified AbAFP1

Figure 2B shows the results of mass spectrometric analysis of the native AbAFP1 SCX-HPLC peak.

Figure 3 shows the complete amino acid sequences of AbAFP1 and the nucleotide sequences of the cDNA encoding AbAFP1. In this figure, the putative translation initiation codon is underlined, the mature sequence as determined from N-terminal sequencing is boxed and the asterisk denotes a translation stop codon.

Figure 4 shows the cation exchange chromatogram for the purification of ArAFP1 and the associated graph of antifungal activity.

Figure 5A shows the SCX-HPLC profile of purified ArAFP1.

Figure 5B shows the results of mass spectrometric analysis of the native ArAFP1 SCX-HPLC peak.

Figure 6 shows the complete amino acid sequences of ArAFP1 and the nucleotide sequences of the putative partial cDNA encoding ArAFP1. In this figure, the mature sequence

as determined from N-terminal sequencing is boxed and the asterisk denotes a translation stop codon.

Figure 7 shows the cation exchange chromatogram for the purification of PeAFP1 and the associated graph of antifungal activity.

5 Figure 8A shows the SCX-HPLC profile of purified PeAFP1.

Figure 8B shows the results of mass spectrometric analysis of the native PeAFP1 SCX-HPLC peak.

10 Figure 9 shows the complete amino acid sequences of PeAFP1 and the nucleotide sequences of the putative partial cDNA encoding PeAFP1. In this figure, the mature sequence as determined from N-terminal sequencing is boxed and the asterisk denotes a translation stop codon.

15 Figure 10 shows the amino acid sequences of AbAFP1, ArAFP1 and PeAFP1 aligned with those of a number of other chitin-binding peptides. An asterisk denotes the presence of amino acid homology at that position in the three peptides while bold type indicates residues homologous for the whole group.

Figures 11 and 12 are bar graph summaries of control and test results of the effect of PeAFP1 on the growth of insect larvae. The Figure 11 results are for weight while the Figure 12 results relate to larval size.

Figure 13 comprises photographs of larvae in control and test experiments.

## 20 **BEST MODE AND OTHER MODES FOR CARRYING OUT THE INVENTION**

The following abbreviations are used hereafter:

|                |                                                                               |
|----------------|-------------------------------------------------------------------------------|
| Ab             | <i>Araucaria bidwillii</i>                                                    |
| Ar             | <i>Agathis robusta</i>                                                        |
| EDTA           | ethylenediaminetetra acetic acid                                              |
| 25 MALDI-TOF   | Matrix-assisted laser desorption ionisation- Time of flight mass spectrometer |
| MeCN           | methyl cyanide (acetonitrile)                                                 |
| MES            | 2-(N-morpholino) ethanesulphonic acid                                         |
| M <sub>r</sub> | molecular mass relative to 1/12 of the atomic mass <sup>12</sup> C            |
| 30 PCR         | polymerase chain reaction                                                     |
| Pe             | <i>Podocarpus elatus</i>                                                      |
| ppm            | Parts per million equivalent to µg/mL concentration                           |
| RACE           | rapid amplification of cDNA ends                                              |

|    |          |                                                                                             |
|----|----------|---------------------------------------------------------------------------------------------|
|    | RP HPLC  | reversed phase high pressure liquid chromatography                                          |
|    | RT PCR   | reverse transcriptase PCR                                                                   |
|    | SCX      | strong cation exchange                                                                      |
|    | SDS-PAGE | sodium dodecyl sulphate polyacrylamide gel electrophoresis                                  |
| 5  | SE       | standard error as estimate of standard distribution of sample mean based on population mean |
|    | SPE      | solid phase extraction                                                                      |
|    | T-DNA    | transfer DNA                                                                                |
|    | TFA      | trifluoroacetic acid                                                                        |
| 10 | Tris     | Tris[hydroxymethyl]-aminomethane hydrochloride                                              |

The present inventors have identified three new peptides (also referred to herein as proteins) obtainable from the seed of Australian native conifers which have antimicrobial and anti-insect activity. The invention provides peptides *per se* as well as DNA sequences encoding each of the peptides. The invention further provides the amino acid sequences of the peptides (Example 3). From these sequences, the sequence of DNA encoding each peptide can be derived by reverse translating the amino-acid sequence. DNA sequences coding for these peptides can be deduced using standard codon tables. DNA having a nucleotide sequence encoding the peptides can be synthesised biochemically or isolated from plant tissue of the conifers using standard cloning methods as described in laboratory manuals such as *Current*

15 *Protocols in Molecular Biology* (copyright 1987-1995 edited by Ausabel, F.M. *et al.* and published by John Wiley & Sons, Inc. printed in the U.S.A.). The deduced DNA sequence can be used to design oligonucleotide probes or primers that can be used to isolate the encoding gene/s and control sequences.

The gene/s, under control of a tissue specific constitutive or inducible promoter, can be

25 cloned into a biological system which allows expression of the peptides. Transformation methods allowing for the peptides to be expressed in a variety of systems are known. The peptides can then be expressed in any suitable system for the purpose of producing peptide for further use. Suitable hosts for the expression of peptides include *E. coli*, fungal cells, insect cells, mammalian cells, plant cells and plants. Such methods for expressing peptides in such

30 hosts are described in a variety of texts including *Current Protocols in Molecular Biology* (*supra*).

As indicated above, a new peptide isolated from the seed of *Araucaria bidwillii* has been identified. This peptide has potent antimicrobial and anti-insect activity and has the following sequence:

5                   Asp-Pro-Thr-Cys-Ser-Pro-Ala-Gly-Arg-Phe-Tyr-Cys-Asn-Asp-Gly-Arg-Cys-  
Cys-Ser-Arg-Ser-Asn-Trp-Cys-Gly-Asn-Thr-Ala-Ala-Tyr-Cys-Lys-Arg-Pro-  
Asn-Cys-Ile-Ala-Gln-Cys-Trp-Pro-Gly (*AbAFP1*; SEQ ID NO: 2)

An additional antimicrobial and anti-insect peptide has been isolated from the seed of *Agathis robusta*, the sequence of this peptide being as follows:

10                   Asp-Pro-Ser-Cys-Ser-Pro-Ala-Gly-Gln-Gln-Tyr-Cys-Asn-Asn-Gly-Arg-Cys-  
Cys-Ser-Lys-Phe-Asn-Trp-Cys-Gly-Ser-Thr-Ala-Ala-Tyr-Cys-Gln-Lys-Pro-  
Asn-Cys-Ile-Ala-Gln-Cys-Trp-Pro-Gly (*ArAFP1*; SEQ ID NO: 3)

A still further antimicrobial and anti-insect peptide has been isolated from the seed of *Podocarpus elatus*, the sequence this peptide being as follows:

15                   Glu-Pro-Thr-Cys-Ser-Pro-Ala-Gly-Arg-Iso-Tyr-Cys-Asn-Lys-Gly-Arg-Cys-  
Cys-Ser- Lys-Phe-Asn-Trp-Cys-Gly-Asn-Thr-Ala-Ala-Tyr-Cys-Ala-Lys-Gly-  
Asn-Cys-Ile-Ala-Asn-Cys-Trp (*PeAFP1*; SEQ ID NO: 4)

Isolation of the foregoing peptides is detailed below in Example 1.

20                   The peptides are highly basic with predicted pI values for *AbAFP1* of 8.5, for *ArAFP1* of 8.2 and for *PeAFP1* of 10.1. Each peptide has 8 cysteine residues which are presumed to be involved in four disulphide linkages for stabilisation of the three-dimensional structures of the peptides (Example 2). The relative molecular mass of the peptides has been determined by mass spectrometry to be *AbAFP1* 4,726±2 Da, *ArAFP1* 4,640±2 Da and for *PeAFP1* 4,458±2 Da.

25                   The amino acid sequences share varying degrees of similarity with previously described peptides/proteins in sequence databases (Swiss Prot and non-redundant databases) searched using the Blast algorithm (Altschul, S.F. *et al.* [1990] *J. Mol. Biol.* 215: 403). Identity was found with many peptides for which only cDNA or genomic DNA sequences are known. The proteins with which the subject peptides have identity include the antimicrobial chitin-binding peptides, chitinases and certain plant agglutinins.

30                   The peptides described herein show a wide range of anti-fungal activity (Examples 5 and 6) including many fungi that cause serious and economically damaging plant diseases. These peptides may therefore have application in the control of such diseases either by being expressed in the host or through topological application to plant parts. Similarly, the peptides

can be used for the control of animal pathogens through topological application or intravenous injection.

The peptides also have potent activity against insects, particularly insect pests of plants. While the efficacy of the peptides in this regard has been demonstrated against lepidopteran species, the peptides are likely to have activity against all insects by virtue of their ability to bind to chitin and hence interfere with chitin-synthesising enzymes. European corn borer (*Ostrinia nubilalis*) and corn root worm (*Diabrotica virgifera*) are examples of insects against which the present peptides have activity.

With specific reference to the embodiments of the invention defined above, the peptides according to the invention can be isolated by any of the methods known to those of skill in the art including the method exemplified herein. The peptides can be alternatively synthesised either chemically or enzymatically. These methods will again be known to those of skill in the art.

DNA according to the second embodiment of the invention can be isolated using any of the techniques known to those of skill in the art. An advantageous method is to amplify the relevant gene sequence from genomic DNA after identifying that gene using probes designed from the amino acid sequence of the peptide. DNA sequences encoding the peptides can also be chemically synthesized using methods that will be known to the skilled person.

With reference to the third embodiment of the invention, the term "construct" includes vectors such as plasmids, cosmids, viruses, and the like as well as naked DNA *per se*. Control elements which can be included in constructs will be known to those of skill in the art. Examples of such elements are promoters, enhancers, polyadenylation signals and transcription terminators.

Constructs according to the invention include chimeric genes which are defined herein as genes that do not exist in nature. A chimeric gene typically comprises the following elements in 5' to 3' orientation: a promoter functional in a host cell, as defined above operably linked to a DNA encoding a peptide according to the invention in a sense or antisense orientation and a termination and/or polyadenylation signal functional in said cell. Other elements, for example an enhancer or an intron, or other regulatory sequences, may also be present. These chimeric genes may be incorporated into recombinant replicable constructs.

Operably linked refers to the association of DNA sequences on a single nucleic acid fragment so that the function of one sequence is affected by the other.

According to the invention and as indicated above, it is also possible to use, in combination with the promoter regulatory sequence of constructs, other regulatory sequences which are situated between the promoter and the coding sequence. These regulatory sequences include transcription activators (enhancers) such as, for example, the translation activator of the tobacco mosaic virus (TMV) which is described in International Application No. WO 87/07644, the tobacco etch virus (TEV) described by Carrington and Freed (see *J. Virol.* 64(4),1590-1597 [1990]), or the figwort mosaic virus described in US Patent No. 5,994,521.

Particularly preferred introns for inclusion in chimeric genes are those which promote gene expression in monocotyledonous plants such as intron 1 of the actin gene described in International Application No. PCT/FR98/02820 (Publication No. WO 99/34005).

The chimeric gene may also comprise a sequence encoding a signal peptide or a transit peptide. Such sequences allow the encoded polypeptide to be directed to a specific subcellular compartment or aid its secretion. The role of such sequences has been in *Plant Molecular Biology*, Vol., 38 (1998)—see the articles by the following authors: Neuhaus, J.-M. and Rogers, J.C., pp127-144; Heese-Peck, A. and Raikhel, N.V., pp 145-162; Soll, J. and Tien, R., pp 191-207; Robinson, C. *et al.*, pp 209-221; and, Glaser, E. *et al.*, pp 311-338. These transit peptides can be single or double as described in Patent Application No. EP 0 508 909.

With regard to polyadenylation or terminator regulatory sequences, these may be any suitable sequence of bacterial origin, such as for example the *Agrobacterium nos* terminator, or alternatively of plant origin, such as for example a histone terminator as described in Patent Application No. EP 0 633 317.

The host cells of the fourth embodiment of the invention include plant and animal cells. Plant cells can be transformed with DNA constructs of the invention according to a variety of known methods such as *Agrobacterium*-mediated, electroporation, micro-injections, sonication, micro-projectile, and the like. For expression in plants, the DNA sequences encoding a protein would be used in conjunction with a DNA sequence encoding the native or a heterologous signal peptide sequence which would target the protein to a cellular compartment such as the vacuole or extracellularly to the apoplast. These coding sequences can be ligated to a plant promoter sequence that would ensure strong expression in plant cells. The promoter sequence might ensure strong constitutive expression of the protein in most or all plant cells, it may be a promoter which ensures expression in specific tissues or cells or it may also be a promoter which ensures strong induction of expression during the infection process. The expression cassette will also include a transcription termination codon and polyadenylation

signal sequence to allow efficient production and stabilisation of the transcribed mRNA. Efficient expression of a peptide can also be facilitated by inclusion of its DNA sequence into a sequence encoding a much larger protein which is processed *in planta* to release the antimicrobial or anti-insect peptide.

5           Gene cassettes can be ligated into binary vectors carrying: i) left and right border sequences that flank the T-DNA of the *Agrobacterium tumefaciens* Ti plasmid; ii) a suitable selectable marker gene for the selection of transformed cells or plants; iii) origins of replication that function in *A. tumefaciens* or *Escherichia coli*; and, iv) antibiotic resistance genes that allow selection of plasmid transformed cells of *E. coli* and *A. tumefaciens*. Such binary vectors  
10       can be introduced either by electroporation or tri-parental mating into *A. tumefaciens* strains carrying disarmed Ti plasmids such as strains LBA4404, GV3101 and AGL1 or into *A. rhizogenes* strains such as R4 and NCCP1885. These *Agrobacterium* strains can be co-cultivated with suitable plant explants or intact plant tissue and the transformed plant cells and/or regenerant shoots selected using an agent that allows the presence of the selectable  
15       marker gene to be determined. Suitable selectable marker genes can be used to confer resistance to antibiotics or herbicides or to produce a molecule that can be assayed fluorometrically or chemically. The expression of the subject peptides in transgenic plants can be detected using antibodies raised against the peptide or by using antimicrobial assays.

          Other methods of gene transfer in plants use direct insertion of the gene into plant cells.  
20       The encoding gene cassette can be micro-injected into isolated plant cells which are then selected for introgression of the gene into the genome. Alternatively, the gene cassette can be co-precipitated onto gold or tungsten particles along with a plasmid encoding a chimeric selectable marker gene. The coated particles or projectiles are accelerated into plant cells or tissues.

25           With regard to methods of transforming plant cells and of regenerating plants, there may be mentioned in particular the following patents and patent applications: US 4,459,355; US 4,536,475; US 5,464,763; US 5,177,010; US 5,187,073; EP 267,159; EP 0 604 662; EP 0 672 752; US 4,945,050; US 5,036,006; US 5,100,792; US 5,371,014; US 5,478,744; US 5,179,022; US 5,565,346; US 5,484,956; US 5,508,468; US 5,538,877; US 5,554,798; US  
30       5,489,520; US 5,510,318; US 5,204,253; US 5,405,765; EP 0 442 174; EP 0 486 233; EP 0 486 234; EP 0 539 563; EP 0 674 725; EP 1 171 621; and the international applications having the publication numbers WO 91/02701, WO 95/06128 and WO 00/22148.

Regenerant plants can be selected for presence of the marker gene and expression of the peptide can be detected using antibodies raised against the peptide or by using anti-microbial assays.

The transformed cells and plants according to the invention can comprise, in addition to the sequence encoding a subject peptide, other heterologous sequences encoding proteins of interest such as additional peptides which are capable of conferring on the plant resistance to diseases of bacterial or fungal origin. The heterologous sequences can furthermore encode proteins for tolerance to herbicides and/or resistance to insects, such as the *Bt* proteins in particular (WO 98/40490). Sequences encoding disease resistance polypeptides such as the polynucleotide encoding oxalate oxidase (described in US 5,866,778, US 6,229,065, US 6,235,530 or EP 0 531 498) can be included in plant cells, as well as polynucleotides encoding fungicidal or bactericidal peptides. Such peptides are described in the international applications having the publication numbers WO 97/30082, WO 99/24594, WO 99/02717, WO 99/53053 and WO 99/09189.

Other polynucleotides encoding agronomic traits can also be inserted, such as a polynucleotide encoding a delta-6 desaturase (US 5,552,306; US 5,614,313, WO 98/46763 and WO 98/46764), a polynucleotide encoding a serine acetyltransferase (SAT) (see WO 00/01833), or a polynucleotide encoding acyltransferase (see WO 94/13814).

Genes for tolerance to herbicides are well known to persons skilled in the art and are in particular are described in the following patent applications: EP 0 115 673; WO 87/04181; EP 0 337 899; WO 96/38567; and, WO 97/04103.

The other sequences referred to above may be integrated into a plant or cells thereof by means of any suitable vector. For example, the vector can comprise a chimeric gene which comprises a first sequence encoding a peptide according to the invention and at least one other sequence encoding another peptide or protein of interest.

Further information on the expression of heterologous genes in plants is given in *Plant Molecular Biology* (copyright 1994, 2<sup>nd</sup> ed., edited by Gelvin, S.B. and Schilperoort, R.A., published by Kluwer Academic Publishers, Dordrecht, The Netherlands).

Transgenic plants according to the invention may also be obtained by crossing parental strains. For example, one parental strain carrying a gene encoding a peptide according to the invention can be crossed with another strain carrying a gene encoding at least one other peptide or protein of interest.

Mammalian cells that can be transformed with constructs according to the invention will be known to those of skill in the art. Like plant cell transformation, mammalian cells can be transformed using any of the techniques known to those of skill in the art.

5 With regard to the fifth embodiment of the invention, both monocotyledonous and dicotyledonous plants can be transformed and regenerated. Plants which can be genetically modified include grains, forage crops, fruits, vegetables, oil seed crops, palms, forestry, and vines. Specific examples of plants which can be modified follow: maize, banana, peanut, field  
10 peas, sunflower, tomato, canola, tobacco, wheat, barley, oats, potato, soybeans, cotton, carnations, sorghum, lupin, rice, and oilseed rape. These, as well as other agricultural plants, can be transformed with genes encoding the peptides such that they exhibit a greater degree of resistance to pathogen attack. Alternatively, the peptides can be used for the control of microbial or insect infestation of a plant by topological application to the subject plant or to tissue thereof.

15 With reference to the sixth embodiment of the invention, reproductive material of a transgenic plant includes seeds, pollen, ovules, progeny plants and clonal material.

With reference to the seventh and eighth embodiments of the invention, those with skill in the art will know the nature of carriers, diluents or excipients which can be included in compositions for administration to plants and animals. Similarly, those with skill in the art will know the best way of applying the composition or peptide *per se* to achieve disease control. In  
20 the present context, the term "control" is used to denote the prophylaxis or treatment of a microbial disease or infestation of a plant or mammalian subject, or insect infestation of a plant.

Compositions according to the seventh and eighth embodiments can comprise any one of the subject peptides or any combination thereof. Compositions for administration to  
25 mammals can furthermore comprise other antimicrobial agents known to those of skill in the art, while compositions for application to plants can include other insect control agents.

Non-limiting examples of the invention follow.

## GENERAL METHODS

### Antifungal activity assays

#### 30 *Method A*

Antifungal bioassays were conducted using microspectrophotometry essentially as described by Cammue *et al.*[1992] *J. Biol. Chem.* 276: 2228-2233 in a defined fungal growth medium (FGM) consisting of K<sub>2</sub>HPO<sub>4</sub> (2.5 mM), MgSO<sub>4</sub> (50 mM), CaCl<sub>2</sub> (50 mM), FeSO<sub>4</sub>

(5mM), CoCl<sub>2</sub> (0.1 mM), CuSO<sub>4</sub> (0.1 mM), Na<sub>2</sub>MoO<sub>4</sub> (2 mM), H<sub>3</sub>BO<sub>4</sub> (0.5 mM), KI (0.1 mM), ZnSO<sub>4</sub> (0.5 mM), MnSO<sub>4</sub> (0.1 mM), sucrose (10 g/L), asparagine (1 g/L), methionine (20 mg/L), myo-inositol (2 mg/L), biotin (0.2 mg/L), thiamine-HCl (1 mg/L) and pyridoxine-HCl (0.2 mg/L). Some fungi which did not grow satisfactorily in the defined growth medium were  
 5 bioassayed in half strength potato dextrose broth (1/2 PDB). Yeasts were assayed in quarter strength yeast peptone broth (1/4 YPD). Bioassays were performed in 96 well microtitre plates where 50 µL of filter-sterilised protein test samples dissolved in water were added to 50 µL of fungal inoculum. In control wells 50 µL of sterile water was added to 50 µL of fungal inoculum. Fungal inoculum consisted of spores (50,000 spores/mL), yeast cells (50,000  
 10 cells/mL) or mycelial fragments (produced by blending a mycelial mass grown in broth and passing the macerate through a fine screen to remove larger hyphal fragments).

Microtitre plates were incubated at 25°C. Filamentous fungi were incubated stationary while yeasts were gently shaken on a microtitre plate shaker. Fungal growth in the microtitre plate wells was assessed by measuring the change in absorbance at 600 nm (A<sub>600</sub>) over time.  
 15 The A<sub>600</sub> at each assessment time was corrected by deducting the absorbance at time zero. Inhibition of growth was calculated as corrected absorbance of the test wells as a percentage of the control wells. Results are expressed as either IC<sub>50</sub> or MIC values which are the concentrations of protein required to give corrected absorbances 50% and less than 10% of control absorbances, respectively, at the first assessment when the corrected A<sub>600</sub> of the control  
 20 wells exceeded 0.4. This point was reached anywhere from 48 to 144 hours incubation depending on the growth rate of the different fungi.

#### Method B

Microtiter biotests were performed *in vitro* to evaluate the antifungal spectrum and efficacy of the peptides studied. One microtiter plate per fungus was prepared. The peptide  
 25 solution (5, 10, 20 ppm) was added to 10 µl of fungi in potato dextrose broth (PDB). The microtiter plates were then incubated in the dark at room temperature (21-22°C) except for *Michrodochium nivale* for which the microtiter plates were incubated in the dark at 4-10°C.

The absorbance at 620 nm was measured 5 days after inoculation. The fungal growth was expressed as the difference between the optical density at 620 nm at a given time (OD<sub>(620)t</sub>)  
 30 and at the beginning of the experiment (OD<sub>(620)i</sub>). The inhibition efficacy of a peptide was calculated as follows:

$$\text{Efficacy}\% = 100(\text{OD}_{(620)t} - \text{OD}_{(620)i})(\text{UTC}) - (\text{OD}_{(620)t} - \text{OD}_{(620)i})(\text{peptide}) / (\text{OD}_{(620)t} - \text{OD}_{(620)i})(\text{UTC})$$

where  $(OD_{(620)t} - OD_{(620)i})(\text{peptide})$  is the growth of the fungus in presence of peptide and  $(OD_{(620)t} - OD_{(620)i})(\text{UTC})$  is the growth of the same fungus without peptide (untreated control).

#### Anti-bacterial activity assays

Bacterial stocks were streaked onto tryptic soy agar (TSB, Difco) and incubated at 28°C except for *E. coli* and *Bacillus subtilis* which were incubated at 37°C. Single colonies were transferred to 25mL of TSB and shaken for 18 hr. Aliquots were diluted in TSB to  $A_{600}=0.1$  and shaken for another 3–4hr until  $A_{600}=0.2-0.4$ . Cells were pelleted by centrifugation for 10 min at 3000g and 4°C. Pellets were resuspended in double strength (2X) TSB to  $A_{600}=0.1$ . Aliquots of 50 µL were added to protein dilutions in microtitre plate wells. Plates were incubated with shaking at 28°C except for *E. coli* which was incubated at 37°C.  $A_{600}$  measurements were recorded at the start of the assay and at 6 hr intervals until the  $A_{600}$  of the control wells (no protein addition) reached  $A_{600}=0.5$ . Calculations of inhibition were performed as described for fungal assays in Example 1.

### Example 1

#### Isolation of Subject Peptides

In this example, we describe the extraction of the subject peptides (proteins) from seeds of *A. bidwillii*, *A. robusta* and *P. elatus* and the purification of the peptides from those extracts.

#### Extraction of basic protein fraction from seeds of native Australian conifers

One kilogram each of *Araucaria bidwillii* and *Agathis robusta* seeds were purchased from the Queensland DPI Forestry Tree Seed Centre, Queensland, Australia. Seed of *Podocarpus elatus* was collected from a tree growing on the campus of The University of Queensland, St. Lucia, Queensland, Australia. Seeds of *A. bidwillii* and *P. elatus* were dehulled before further processing while those of *A. robusta* was used as obtained. Seeds were flaked in a domestic food processor (Big Oscar, Sunbeam Appliances). Since the seed of *A. robusta* contains appreciable amounts of lipids the resulting meal was extracted for 1 hr with 2.5 L petroleum ether (30-40°C BP) at room temperature. Petroleum ether was added periodically to replace that lost through evaporation. The dissolved lipids were removed in the solvent by drawing through a sintered glass funnel under vacuum. The seed meal of *A. bidwillii* and *P. elatus* was not defatted.

The seed meals were extracted twice with excess 0.05 M  $H_2SO_4$  for 1hr at room temperature with occasional stirring. After each extraction the liquid phase was decanted off and centrifuged for 10 min at 10,000g. The two supernatants for each seed meal were pooled,

adjusted to 20 mM MES at pH 6 with NaOH. After standing at 4°C overnight the supernatants were centrifuged for 60 min at 4°C at 10,000g. This fraction was further purified as described in the following section.

#### **Cation-exchange chromatography of acid extracts of seeds of native Australian conifers**

5 Protein samples were passed through 0.45 µm filters to remove any particulate matter before cation exchange chromatography.

Cation exchange chromatography of the extracts was performed on Source 15S media in a HR16/10 column (Pharmacia) equilibrated with 20 mM MES pH6. Aliquots of 250 mL of clarified extract were loaded. Following loading of the sample the column was washed with 10 mM MES pH 6 until  $A_{280}$  measurements reached >0.05 AUFS. Bound proteins were eluted by passing a linear gradient of 0 to 2 M NaCl in 20 mM MES pH 6 over 90 min at 5 mL/min. The eluate was monitored by online measurement of the absorbance at 280 nm and eluted proteins were collected in either 10 or 20 mL fractions.

Five millilitre aliquots of the fractions were desalted and concentrated by passing over 15 Bond Elut™ (Varian) or Strata (C18e)™ (Phenomenex) SPE columns that were equilibrated with 20 mM MES pH 6, the salt was removed by washing with 3 column volumes of 5% MeCN in 0.1% TFA and the bound proteins were eluted with 50% MeCN in 0.1% TFA. The eluants were dried in a rotary vacuum concentrator before being resuspended in MilliQ H<sub>2</sub>O and redried to remove residual TFA. Resuspension and drying was repeated 3 times. The 20 fractions were finally resuspended in 1 mL MilliQ™ water before filter sterilisation through 0.22 µm filter into sterile tubes. Protein samples were stored at -20°C.

#### **Purification of proteins from cation-exchange fractions from seeds of native Australian conifers**

Fractions from the cation-exchange chromatography procedures outlined above were 25 bioassayed against the fungus *Sclerotinia sclerotiorum*. Protein concentrations were measured using the BCA assay (Pierce) against a standard curve generated using BSA and proteins solutions were diluted wherever possible to 50 µg/mL with sterile MilliQ™ water (Millipore Corporation).

Anti-fungal activity was found in several early eluting fractions in the cation-exchange 30 profile of *A. bidwillii* (Figure 1). Desalted fractions eluting between 50-100 mM NaCl completely inhibited growth of *Sclerotinia sclerotiorum* from ascospore inoculum, that is, MIC values were equal to or less than 25 µg/mL. Active fractions were subjected to high resolution cation-exchange HPLC to further purify anti-fungal activity. About 1 mg amounts of each of

the combined fractions were loaded on a Luna SCX™ (5 µm) column (150 x 4.6 mm) (Phenomenex) equilibrated with 10mM NH<sub>4</sub>Acetate in 20% MeCN (pH5) (=100%A). The column was eluted at 0.5 mL/min with a 35 mL linear gradient (70 min) from 100%A to 1M NH<sub>4</sub>Acetate in 20% MeCN (pH5) (=100%B). Eluate was monitored for protein by online measurement of absorbance at 280 nm. Fractions of 1 mL (2 min) of the eluate were collected, vacuum dried thrice after resuspension in MilliQ™ water to remove traces of NH<sub>4</sub>Acetate, resuspended in MilliQ™ water, filter sterilised and bioassayed against *S. sclerotiorum* and *B. cinerea* at concentrations of 50 µg/mL as described in General Methods.

The cation-exchange fractions of *A. bidwillii* that exhibited strong activity against *S. sclerotiorum* when subjected to cation-exchange HPLC provided simple chromatograms where in each case complete inhibition of fungal growth was provided by fractions eluting between 55-62 min (32-35 mS/cm) (Figure 2). The active factor in this peak was called AbAFP1 (*Araucaria bidwillii* antifungal protein 1).

Desalted fractions eluting between 20-200mM NaCl from the cation-exchange purification of the extract from *Agathis robusta* completely inhibited growth of *Sclerotinia sclerotiorum* (Figure 4). These were subjected to high resolution cation-exchange HPLC as described for the fractions from *A. bidwillii*. Among these fractions complete inhibition of growth of *S. sclerotiorum* and *Botrytis cinerea* was found in the major peak eluting between 33-37 min (16-19 mS/cm) (Figure 6). The active factor in this peak was called ArAFP1 (*Agathis robusta* antifungal protein 1).

Protein fractions from cation-exchange purification of the extract from *Podocarpus elatus* were subjected to high resolution cation-exchange HPLC as described for the fractions from *A. bidwillii*. A single major peak eluted from the column between 46-52 min (27-31mS/cm) (Figure 8A). The fractions corresponding to this peak completely inhibited growth of *S. sclerotiorum* and *Botrytis cinerea* at 25 µg/mL. The active factor in this peak was called PeAFP1 (*Podocarpus elatus* antifungal protein 1).

## Example 2

### Molecular Characterisation of the Purified Peptides

The peaks described as AbAFP1 (Example 1), ArAFP1 (Example 1) and PeAFP1 (Example 1) were subjected to mass spectroscopic analysis by MALDI-TOF to assess purity and mass. Approximately 50 µg of each peptide in 50% MeCN was used for testing. Analysis of AbAFP1 revealed the presence of a molecule with mass of 4,726Da ± 2 Da. Analysis of

ArAFP1 revealed a single peptide having a molecular mass of  $4,640\text{Da} \pm 2\text{ Da}$ . Analysis of PeAFP1 revealed a single peptide having a molecular mass of  $4,458\text{Da} \pm 2\text{ Da}$ . The actual mass spectrometric analyses described above are presented in Figures 2B, 5B and 8B.

5 The peptides were also subjected to reduction of possible disulphide bonds with dithiothreitol and alkylation of free thiol groups of cysteines with 4-vinylpyridine. Approximately  $50\text{ }\mu\text{g}$  of peptide were dissolved in  $800\text{ }\mu\text{L}$  reduction/alkylation buffer (6 M guanidinium-Cl in 100 mM Tris buffer pH 8, 0.01% EDTA) to which was added 4 mg DTT. The reduction reaction was conducted under argon at  $37^\circ\text{C}$  for 2 hr. Four microlitres of 4-vinylpyridine was added and the alkylation reaction was conducted overnight under argon, at  
10 room temperature and in darkness. The reduced and alkylated peptides were separated from reactants by reversed-phase HPLC on a Jupiter  $\text{C}_{18}^{\text{TM}}$  (Phenomenex) column ( $30 \times 4.6\text{ mm}$ ) and analysed by mass spectroscopy. The reduced and alkylated AbAFP1 sample gave a mass of  $5575 \pm 2\text{Da}$ . The mass of ArAFP1 increased to  $5492 \pm 2\text{Da}$  and that of PeAFP1 increased to  $5306 \pm 2\text{Da}$ . In all these peptides the mass increases over the native peptides were each 848-  
15 850 mass units. This gain in mass was interpreted as the reaction of eight 4-vinylpyridine groups (mass 106 Da) with 8 cysteine residues in each of the peptides. This conclusion has been confirmed by amino acid sequencing (Example 3) and nucleotide sequence of putative encoding genes (Example 7).

### Example 3

#### Amino Acid Sequencing of the Purified Peptides

Approximately 1 µg of each of reduced and alkylated peptides were subjected to Automated Edman degradation N-terminal sequencing. Sequencing continued until no discernible signal could be determined. Using the amino acid sequences obtained it is possible to compare molecular weights with those obtained with mass spectroscopy. The software program MacVector 6.0™ was used to predict the molecular weight of the peptides consisting of the amino acid sequences obtained. AbAFP1 was estimated to have a  $M_r$  of 4,734, ArAFP1 to have a  $M_r$  of 4,649 and PeAFP1 to have a  $M_r$  of 4,466. These predictions do not account for the presence of disulphide bonds. Given the presence of eight cysteines there is potential for the presence of 4 disulphide bonds with the loss of eight hydrogens or mass units providing masses of 4,726Da for AbAFP1, 4,641Da for ArAFP1 and 4,458Da for PeAFP1. These agree very closely with the masses obtained by mass spectroscopic analysis (Example 2) and it is proposed that these sequences are therefore correct and that each peptide is stabilised by 4 disulfides.

### Example 4

#### Similarity and Homology Searches

The amino acid and cDNA sequences (obtained in Examples 3 and 6) were subjected to comparison against published databases to evaluate whether the peptides described herein had been previously identified. Amino acid sequences were analysed using the BLASTP algorithm [Basic Logic Alignment Search Tool, Altscul *et al.* (1997) *Nucleic Acids Research* 25: 3389-3402] against the SwissProt database and non-redundant databases at NCBI. The BLAST searches identified a number of similarities of the peptides described herein and chitin-binding lectins of various sizes, including other chitin-binding peptides. An alignment of AbAFP1, ArAFP1 and PeAFP1 with previously known chitin-binding peptides is presented in Figure 10. The sequence IDs of the other chitin-binding peptides presented in this figure are as follows:

|               |               |
|---------------|---------------|
| <i>AcAMP1</i> | SEQ ID NO: 5  |
| <i>AcAMP2</i> | SEQ ID NO: 6  |
| <i>An1</i>    | SEQ ID NO: 7  |
| <i>BmAMP1</i> | SEQ ID NO: 8  |
| <i>BvIWF4</i> | SEQ ID NO: 9  |
| <i>CaAMP1</i> | SEQ ID NO: 10 |
| <i>EaAFP1</i> | SEQ ID NO: 11 |

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|               |               |
|---------------|---------------|
| <i>EaAFP2</i> | SEQ ID NO: 12 |
| <i>EeCBP1</i> | SEQ ID NO: 13 |
| Hevein        | SEQ ID NO: 14 |
| <i>PnAMP1</i> | SEQ ID NO: 15 |
| <i>PnAMP2</i> | SEQ ID NO: 16 |
| UDA           | SEQ ID NO: 17 |

**Example 5****Anti-fungal Potency of the Purified Peptides****Against Fungal Pathogens of Plants**

The activity of purified AbAFP1, ArAFP1 and PeAFP1 were tested against different plant pathogenic fungi in synthetic fungal medium (SFM) as described in General Methods using Method A. Table 1 presents the IC<sub>50</sub> of the peptides against a number of plant pathogenic fungi. In the table where a result is presented as '>200', this indicates that concentrations higher than 200 µg/mL (ie 200 ppm) were not tested.

Table 1

Anti-fungal activity of *AbAFP1*, *ArAFP1* and *PeAFP1* against plant pathogenic fungi

| Organism                                        | Inoculum   | Medium | <i>AbAFP1</i><br>(IC <sub>50</sub> ppm) | <i>ArAFP1</i><br>(IC <sub>50</sub> ppm) | <i>PeAFP1</i><br>(IC <sub>50</sub> ppm) |
|-------------------------------------------------|------------|--------|-----------------------------------------|-----------------------------------------|-----------------------------------------|
| Fungi:                                          |            |        |                                         |                                         |                                         |
| <i>Alternaria brassicicola</i>                  | conidia    | SFM    | 10                                      | 15                                      | 3                                       |
| <i>Bipolaris sorokiniana</i>                    | conidia    | SFM    | 10                                      | 30                                      | 3                                       |
| <i>Botrytis cinerea</i>                         | conidia    | SFM    | 10                                      | 5                                       | 4                                       |
| <i>Ceratocystis paradoxa</i>                    | conidia    | SFM    | 80                                      | 50                                      | 4                                       |
| <i>Colletotrichum falcatum</i>                  | conidia    | SFM    | 200                                     | >200                                    | 100                                     |
| <i>Eutypa lata</i>                              | conidia    | SFM    | >200                                    | >200                                    | 10                                      |
| <i>Fusarium oxysporum</i><br><i>vasinfectum</i> | conidia    | SFM    | 200                                     | 200                                     | 10                                      |
| <i>Phoma lingam</i>                             | conidia    | SFM    | 3                                       | 5                                       | 1.5                                     |
| <i>Sclerotinia minor</i>                        | ascospores | SFM    | 3                                       | 2                                       | 2                                       |
| <i>Vericillium dahliae</i>                      | conidia    | SFM    | 5                                       | 20                                      | 2                                       |

The results presented in the preceding table indicate that the prototype peptides have activity against a broad range of fungi. These fungi represent root, vascular and foliar

pathogens of pulse crops, grapes sunflower, sugar cane, banana, cotton, canola and most broad leaf crops. Against this panel of fungi PeAFP1 is the most potent generally causing a 50% reduction in hyphal growth at lower concentrations than the other two peptides.

### Example 6

#### Anti-fungal Potency of the Purified Peptide *AbAFP1*

##### Against Fungal Pathogens of Plants

The *in vitro* antifungal activity of *AbAFP1* was evaluated on seven fungal strains responsible for major field damage to crops using the second procedure described above in General Methods. The seven strains were: *Microdochium nivale*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium moniliforme subglutinans*, *Fusarium moniliforme proliferatum*, *Sclerotinia sclerotiorum*, *Septoria tritici*. The results of the evaluations are presented in the following table in which the data are for inhibition efficacy (%) at 5, 10 and 20 ppm.

Table 2

Inhibition efficacy (%) of *AbAFP1* against plant pathogenic fungi

| Fungus                             | Peptide (ppm) | Time (hours) |    |     |    |     |     |
|------------------------------------|---------------|--------------|----|-----|----|-----|-----|
|                                    |               | 24           | 48 | 72  | 96 | 120 | 144 |
| <i>Fusarium culmorum</i>           | 5             | 0            | 0  | 0   | 4  | 5   | 8   |
|                                    | 10            | 0            | 0  | 0   | 3  | 6   | 10  |
|                                    | 20            | 39           | 15 | 12  | 8  | 12  | 16  |
| <i>F. graminearum</i>              | 5             | 3            | 10 | 15  | 18 | 20  | 17  |
|                                    | 10            | 0            | 2  | 8   | 9  | 11  | 12  |
|                                    | 20            | 0            | 0  | 5   | 6  | 11  | 12  |
| <i>F. moniliforme proliferatum</i> | 5             | 0            | 0  | 0   | 0  | 0   | 0   |
|                                    | 10            | 0            | 0  | 0   | 0  | 0   | 0   |
|                                    | 20            | 0            | 0  | 0   | 0  | 4   | 0   |
| <i>F. moniliforme subglutinans</i> | 5             | 6            | 12 | 16  | 16 | 15  | 21  |
|                                    | 10            | 0            | 10 | 11  | 9  | 8   | 16  |
|                                    | 20            | 0            | 11 | 9   | 10 | 11  | 12  |
| <i>Microdochium nivale</i>         | 5             | 100          | 83 | 97  | 90 | 88  | 72  |
|                                    | 10            | 85           | 84 | 100 | 92 | 100 | 96  |
|                                    | 20            | 97           | 85 | 86  | 82 | 63  | 48  |

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|                                 |    |     |     |     |     |     |     |
|---------------------------------|----|-----|-----|-----|-----|-----|-----|
| <i>Sclerotinia sclerotiorum</i> | 5  | 100 | 100 | 100 | 100 | 100 | 100 |
|                                 | 10 | 100 | 100 | 100 | 100 | 100 | 100 |
|                                 | 20 | 100 | 100 | 100 | 100 | 100 | 100 |
| <i>Septoria tritici</i>         | 5  | 100 | 100 | 100 | 100 | 100 | 97  |
|                                 | 10 | 100 | 100 | 100 | 100 | 100 | 100 |
|                                 | 20 | 100 | 100 | 100 | 100 | 100 | 100 |

The results presented in the preceding table show that native AbAFP1 shows a degree of specificity of activity against the pathogens tested. Little activity was evident against the various *Fusarium* species at the concentrations tested. However, even at the lowest concentration tested (5ppm) the peptide had an efficacy of greater than 90% against *Steptoria tritici*, *Sclerotinia sclerotiorum* and *Microdochium nivale*. The efficacy was at its maximum between 3 and 5 days and decreased slightly thereafter, this decrease probably being due to degradation of the peptide.

### Example 7

#### Molecular Cloning and Sequencing of the Corresponding cDNAs

Mature seeds were used for RNA extraction. Megagametophyte tissue was obtained, sliced into 1mm thick slices and immersed in RNAlater™ (Ambion Inc) RNA stabilisation solution and stored at -80°C. Total RNA was extracted from 4-5g of frozen tissue by using the Hot Borate method of Wilkins and Smart (1996) [p 21-41 in *A Laboratory Guide to RNA: Isolation, Analysis and Synthesis*, PA Krieg (ed.), Wiley-Liss]. Poly (A)+ mRNA was purified by affinity separation using the Oligotex™ system (QIAGEN Pty Ltd). Double stranded cDNA was prepared from the mRNA using the SMART™ PCR cDNA synthesis kit (Clontech).

The software package MacVector 6.0™ was used to predict degenerate probes from the degenerate reverse translations from the amino acid sequences. The same software was then used to predict the suitability of the probes as PCR primers. Degenerate primers were synthesised for use in 3' RACE. The following degenerate oligonucleotide primer was designed from amino acids 9-15 present in the known sequence of AbAFP1.

AbAFP1 Degenerate: 5' GNT TYT AYT GYA AYG AYG G 3' (SEQ ID NO: 18)

This primer and the oligonucleotide primer specific to the cDNA prepared with the SMART™ system—ie, TS-PCR: 5' AAGCAGTGGTATCAACGCAGAGT 3' (SEQ ID NO: 19)—were used to amplify DNA fragments from the cDNA template. Control PCR reactions were also performed using only AbAFP1 Degenerate or TS-PCR primers with the cDNA template. The

thermocycling program included an initial step of 94°C for 5min, followed by 30 cycles of 92°C for 45sec, 50°C for 30sec and 72°C for 1 min before a final step of 72°C for 7min. Amplification products were separated by electrophoresis on 1.5% (w/v) agarose gel. Bands present in the amplifications using AbAFP1 Degenerate and TS-PCR primers together but  
5 absent when only or other primer was used in the PCR were excised from the gel, purified using a Concert™ DNA clean-up kit and cloned into the vector pGemT Easy™ (Promega) and transformed into E. coli (Top 10™, Invitrogen) using the procedures recommended by the various manufacturers. Inserts were sequenced using the fluorescent dideoxy terminator reaction procedure (PRISM™, ABI).

10 The following degenerate oligonucleotide primer designed from amino acids 8 – 15 present in the known sequence of ArAFP1 was used for performing 3' RACE of ArAFP1:

ArAFP1 Degenerate: 5' GNC ARC ART AYT GYA AYA AYG G 3' (SEQ ID NO: 20)  
PCR was performed as described for the AbAFP1 3' RACE.

Similarly, the following degenerate oligonucleotide primer designed from amino acids  
15 8 – 17 present in the known sequence of PeAFP1 was used for performing 3' RACE of PeAFP1:

PeAFP1 Degenerate: 5' GGN AGN ATH TAY TGY AAY AAR GGN CGN TG 3'  
(SEQ ID NO: 21)

PCR was performed as described for AbAFP1 3' RACE except that the annealing temperature  
20 used was 55°C.

Sequencing of amplification products provided sequences that when translated conformed to the known amino acid sequences of the C-terminal ends of the peptides. From these sequences the software package MacVector 6.0™ was used to predict suitable anti-sense primers for 5' RACE. The respective primers were:

25 AbAFP1 Anti-sense: 5' AGA CTT GCT CGA TCT ACA CG 3' (SEQ ID NO: 22)

ArAFP1 Anti-sense: 5' GTG AGG GCA GTA ACA CCG 3' (SEQ ID NO: 23)

PeAFP1 Anti-sense: 5' AAG CCA TTG GAT TGG AGG 3' (SEQ ID NO: 24)

The AbAFP1 and PeAFP1 anti-sense primers were situated in the 3' UTR while the ArAFP1 anti-sense primer was situated 5' of the stop codon.

30 These primers were used in conjunction with the TS-PCR primer to amplify the respective cDNA pools under the amplification conditions described for the 3' RACE reactions. Strong amplifications not present in the control reactions were cloned and sequenced. In each

case sequences complimentary to the respective sequences produced with 3' RACE were recovered. Contigs were created between the respective 3' and 5' RACE products.

The AbAFP1 Contig (SEQ ID NO: 25) was approximately 600 nucleotides in length and included an ORF (open reading frame) encoding a 94 amino acid sequence (SEQ ID NO: 26) within which are the 43 amino acids corresponding exactly to AbAFP1. The ORF also encodes 26 N-terminal amino acids with a predicted signal peptide structure obeying the rules of von Heijne (1985, *Mol. Biol.* 184: 99-105). There are also C-terminal 25 amino acids of unknown function. It is predicted therefore that AbAFP1 is part of a tripartite protein that undergoes post-translational processing before release of mature AbAFP1 (Figure 3).

The ArAFP1 Contig (SEQ ID NO: 27) was approximately 550 nucleotides in length and included a coding region (SEQ ID NO: 28) that when translated included a sequence of 43 amino acids corresponding exactly to ArAFP1. There are 29 amino acids N-terminal to this sequence and 30 amino acids C-terminal. It is predicted therefore ArAFP1 is part of a tripartite protein that undergoes post-translational processing before release of mature ArAFP1 (Figure 6).

The PeAFP1 Contig (SEQ ID NO: 29) was shorter at only 420 nucleotides. It is predicted that 5' RACE did not obtain the full 5' sequence. However, the sequence obtained when translated (SEQ ID NO: 30) included a sequence of 41 amino acids corresponding exactly to the sequence of PeAFP1 obtained though peptide sequencing (Example 3). There are at least 15 amino acids N-terminal and 29 amino acids C-terminal to the PeAFP1 coding sequence. It is predicted therefore PeAFP1 is part of a tripartite protein that undergoes post-translational processing before release of mature PeAFP1 (Figure 9).

### Example 8

#### Demonstration of Chitin-binding Ability

The similarity of AbAFP1, ArAFP1 and PeAFP1 to several classes of lectins (chitinases, agglutinins and the like) presented the possibility that these peptides might also possess such activity. A 1 ml column was packed with chitin (poly-N-acetylglucosamine from crab shells, Sigma C-3132) particles that passed through a 30 mesh screen. The column was extensively washed with alternate cycles of 50 mM NH<sub>4</sub>Acetate (pH 7) and 100 mM acetic acid (pH 2.8) and the eluant monitored until no material absorbing at 280nm passed from the column. The column was then equilibrated with 50 mM NH<sub>4</sub>Acetate and 200 µg of either AbAFP1, ArAFP1 or PeAFP1 dissolved in the same buffer was loaded. The column was washed with 50 mM NH<sub>4</sub>Acetate until the A<sub>280</sub> was less than 0.05 AUFS. Bound peptide was

eluted by applying 100 mM acetic acid. In each case a single  $A_{280}$  absorbance peak eluted from the column and was collected. The fraction was dried and subject to mass spectrometric analysis. In each case the  $M_r$  corresponded to the respective peptide thereby confirming that these peptides exhibit affinity to chitin.

5

### Example 9

#### Anti-insect Activity Determination

The peptide PeAFP1 exemplified above was tested for effects on lepidopteran larvae given that it has some similarity to a number of multivalent lectins that have been shown to possess anti-insect activity.

10

PeAFP1 was incorporated into the insect diet at 5 mg/mL and dispensed in 0.5 mL aliquots in small disposable plastic receptacles. Second instar larvae of *Helicoverpa armigera* were weighed and placed 1 per receptacle. Control insects were provided diet without PeAFP1 or with the addition of 5 mg/mL BSA as a control protein. Each day insects were checked for mortality, on days 2, 3, 4, 5, and 7 after enclosure larvae were assessed for developmental stage (instar) and on days 3 and 7 after enclosure larvae were weighed. Twenty-four replicate insects were used for each treatment.

15

The experimental results are presented in Tables 3 and 4 below and Figures 11 to 13. In Table 3, the numbers in the "Weight" and "Instar" columns represent observations made on the days shown in the "Day" row. Day 0 is considered to be the day during which the insect larvae were first enclosed and exposed to the respective diets.

20

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Table 3  
Descriptive Statistics

## 3A. Control Treatment

| Day   | Weight (mg) |              |               | Instar      |             |             |             |             |             |
|-------|-------------|--------------|---------------|-------------|-------------|-------------|-------------|-------------|-------------|
|       | 0           | 3            | 7             | 0           | 2           | 3           | 4           | 5           | 7           |
| Mean  | 1.3         | 8.3          | 58.7          | 2.0         | 2.3         | 2.7         | 2.8         | 3.2         | 3.7         |
| SE    | 0.1         | 1.0          | 9.0           | 0.0         | 0.1         | 0.1         | 0.1         | 0.1         | 0.1         |
| Range | 0.5-2.7     | 1.8-<br>21.5 | 4.9-<br>147.0 | 2.0-<br>2.0 | 2.0-<br>3.0 | 2.0-<br>3.0 | 2.0-<br>3.0 | 2.0-<br>4.0 | 2.0-<br>4.0 |

## 5 3B. BSA Treatment

| Day   | Weight (mg) |              |                | Instar      |             |             |             |             |             |
|-------|-------------|--------------|----------------|-------------|-------------|-------------|-------------|-------------|-------------|
|       | 0           | 3            | 7              | 0           | 2           | 3           | 4           | 5           | 7           |
| Mean  | 1.2         | 7.7          | 56.3           | 2.0         | 2.3         | 2.6         | 2.8         | 3.2         | 3.6         |
| SE    | 0.1         | 1.1          | 8.3            | 0.0         | 0.1         | 0.1         | 0.1         | 0.1         | 0.1         |
| Range | 0.8-2.4     | 2.2-<br>20.7 | 11.6-<br>122.0 | 2.0-<br>2.0 | 2.0-<br>3.0 | 2.0-<br>3.0 | 2.0-<br>4.0 | 3.0-<br>4.0 | 3.0-<br>4.0 |

## 3C. PeAFP1 Treatment

| Day   | Weight (mg) |         |              | Instar      |             |             |             |             |             |
|-------|-------------|---------|--------------|-------------|-------------|-------------|-------------|-------------|-------------|
|       | 0           | 3       | 7            | 0           | 2           | 3           | 4           | 5           | 7           |
| Mean  | 1.2         | 2.6     | 10.5         | 2.0         | 2.0         | 2.2         | 2.6         | 2.8         | 2.8         |
| SE    | 0.1         | 0.3     | 1.8          | 0.0         | 0.0         | 0.1         | 0.1         | 0.1         | 0.1         |
| Range | 0.6-2.1     | 0.8-7.2 | 0.9-<br>38.0 | 2.0-<br>2.0 | 2.0-<br>2.0 | 2.0-<br>3.0 | 2.0-<br>3.0 | 2.0-<br>3.0 | 2.0-<br>4.0 |

Table 4

## Summary

| Test    | Weight (mg) | SE  | Instar | SE  |
|---------|-------------|-----|--------|-----|
| Control | 58.7        | 9.0 | 3.7    | 0.1 |
| BSA     | 56.3        | 8.3 | 3.6    | 0.1 |
| PeAFP1  | 10.5        | 1.8 | 2.8    | 0.1 |

Increasing the amount of protein *per se* in the diets of the insects did not affect growth or development as can be seen from the use of BSA as the additive. However exposure of *H. armigera* larvae to PeAFP1 in the diet had a significant affect on growth as assessed by weight and development as assessed by instar. No mortality was observed in the 7 day period of the experiment.

The foregoing embodiments are illustrative only of the principles of the invention, and various modifications and changes will readily occur to those skilled in the art. The invention is capable of being practiced and carried out in various ways and in other embodiments. It is also to be understood that the terminology employed herein is for the purpose of description and should not be regarded as limiting.

The term “comprise” and variants of the term such as “comprises” or “comprising” are used herein to denote the inclusion of a stated integer or stated integers but not to exclude any other integer or any other integers, unless in the context or usage an exclusive interpretation of the term is required.

Any reference to publications cited in this specification is not an admission that the disclosures constitute common general knowledge in Australia.

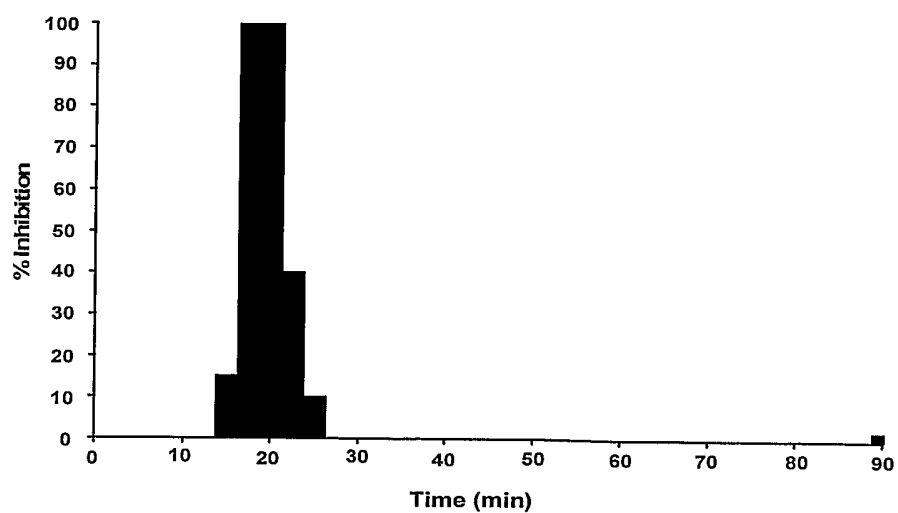
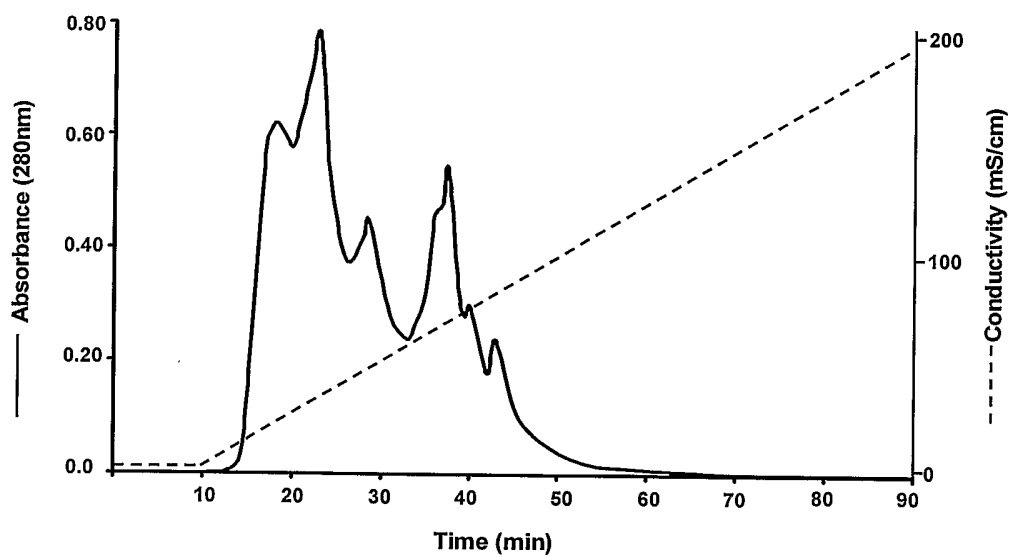
**CLAIMS**

1. An isolated or synthetic peptide comprising the sequence:  
X<sub>1</sub>PX<sub>2</sub>CSPAGX<sub>3</sub>X<sub>4</sub>YCNX<sub>5</sub>GRCCSX<sub>6</sub>X<sub>7</sub>NWCGX<sub>8</sub>TAA<sub>Y</sub>CX<sub>9</sub>X<sub>10</sub>X<sub>11</sub>NCIAX<sub>12</sub>CWZ  
(SEQ ID NO: 1)
- 5 wherein, each X is independently any amino acid residue other than C, and Z is a carboxy group or the dipeptide PG.
2. The peptide of claim 1, wherein X<sub>1</sub> is D or E.
3. The peptide of claim 1, wherein X<sub>2</sub> is a polar, hydrophilic, neutral amino acid residue.
4. The peptide of claim 3, wherein X<sub>2</sub> is T or S.
- 10 5. The peptide of claim 1, wherein X<sub>3</sub> is a polar, hydrophilic, positively charged or neutral amino acid residue.
6. The peptide of claim 5, wherein X<sub>3</sub> is R or Q.
7. The peptide of claim 1, wherein X<sub>4</sub> is F, I or Q.
8. The peptide of claim 1, wherein X<sub>5</sub> is a polar hydrophilic amino acid residue.
- 15 9. The peptide of claim 8, wherein X<sub>5</sub> is D, N or K.
10. The peptide of claim 1, wherein X<sub>6</sub> is a polar hydrophilic, positively charged amino acid residue.
11. The peptide of claim 10, wherein X<sub>6</sub> is R or K.
12. The peptide of claim 1, wherein X<sub>7</sub> is a neutral amino acid residue.
- 20 13. The peptide of claim 12, wherein X<sub>7</sub> is S or F.
14. The peptide of claim 1, wherein X<sub>8</sub> is a polar, hydrophilic, neutral amino acid residue.
15. The peptide of claim 14, wherein X<sub>8</sub> is N or S.
16. The peptide of claim 1, wherein X<sub>9</sub> is K, A or Q.

17. The peptide of claim 1, wherein X<sub>10</sub> is a polar hydrophilic, positively charged amino acid residue.
18. The peptide of claim 17, wherein X<sub>10</sub> R or K.
19. The peptide of claim 1, wherein X<sub>11</sub> is a neutral amino acid residue.
- 5 20. The peptide of claim 19, wherein X<sub>11</sub> is P or G
21. The peptide of claim 1, wherein X<sub>12</sub> is a polar, hydrophilic, neutral amino acid residue.
22. The peptide of claim 21, wherein X<sub>12</sub> is N or Q.
23. The peptide of claim 1 comprising the sequence  
Asp-Pro-Thr-Cys-Ser-Pro-Ala-Gly-Arg-Phe-Tyr-Cys-Asn-Asp-Gly-Arg-Cys-  
10 Cys-Ser-Arg-Ser-Asn-Trp-Cys-Gly-Asn-Thr-Ala-Ala-Tyr-Cys-Lys-Arg-Pro-  
Asn-Cys-Ile-Ala-Gln-Cys-Trp-Pro-Gly (SEQ ID NO: 2).
24. The peptide of claim 1 comprising the sequence  
Asp-Pro-Ser-Cys-Ser-Pro-Ala-Gly-Gln-Gln-Tyr-Cys-Asn-Asn-Gly-Arg-Cys-  
Cys-Ser- Lys-Phe-Asn-Trp-Cys-Gly-Ser-Thr-Ala-Ala-Tyr-Cys-Gln-Lys-Pro-  
15 Asn-Cys-Ile-Ala-Gln-Cys-Trp-Pro-Gly (SEQ ID NO: 3).
25. The peptide of claim 1 comprising the sequence  
Glu-Pro-Thr-Cys-Ser-Pro-Ala-Gly-Arg-Ile-Tyr-Cys-Asn-Lys-Gly-Arg-Cys-  
Cys-Ser-Lys-Phe-Asn-Trp-Cys-Gly-Asn-Thr-Ala-Ala-Tyr-Cys-Ala-Lys-Gly-  
Asn-Cys-Ile-Ala-Asn-Cys-Trp (SEQ ID NO: 4).
- 20 26. An isolated or synthetic DNA which encodes a peptide according to claim 1.
27. A DNA construct which includes at least one DNA according to claim 26 operatively linked to elements for the expression of peptide encoded by said DNA.
28. A host cell transformed with a DNA construct according to claim 27.
29. A transgenic plant transformed with a DNA construct according to claim 27.
- 25 30. Reproductive material of a transgenic plant according to claim 29.

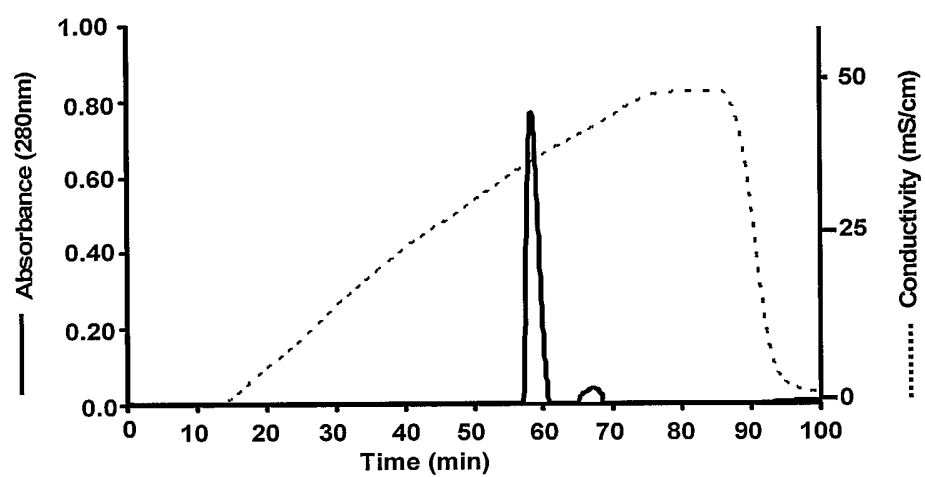
31. A composition comprising at least one peptide according to claim 1 together with an agriculturally acceptable carrier, diluent or excipient.
32. A composition comprising at least one peptide according to claim 1 together with a pharmaceutically acceptable carrier, diluent or excipient.
- 5 33. A method of controlling insect, microbial or viral infestation of a plant, the method comprising:
- i) introducing a DNA construct according to claim 27 into said plant; or
  - ii) treating said plant with a peptide according to claim 1 or a composition according to claim 31.
- 10 34. A method of controlling microbial infestation of a mammalian subject, the method comprising treating said subject with a peptide according to claim 1 or a composition according to claim 32.
35. Use of a peptide according to claim 1 in the preparation of a medicament for controlling microbial infestation of a mammalian subject.
- 15 36. The method of claim 34 or the use of claim 35, wherein said subject is a human subject.

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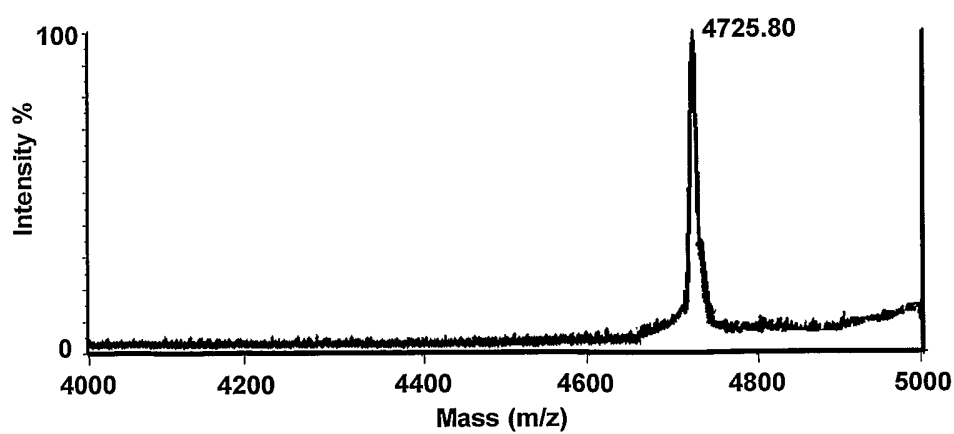
*Fig. 1*

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A.



B.

*Fig. 2*

1 GCCTGTCCGAAATCGAGTAACAGCATTGAAATTGAAAGTTCTGCAACGGAGTG ATG GGA TAC AAG AAT TTC  
M G Y K N F

73 GTG TGG GTA GTA CTG GTT TTG GCT GTG GTA ATT GGG GAA AAC AGT GTA ATC GTA AAA GGG  
V W V V L V L A V V I G E N S V I V K G

103 GAT CCG ACA TGC AGT CCA GCG GGA CGA TTC TAT TGC AAT GAT GGG AGA TGC TGC AGC AGG  
D P T C S P A G R F Y C N D G R C C S R

163 TCC AAC TGG TGC GGA AAC ACT GCT GCT TAC TGT AAA AGG CCT AAT TGT ATT GCT CAG TGT  
S N W C G N T A A Y C K R P N C I A Q C

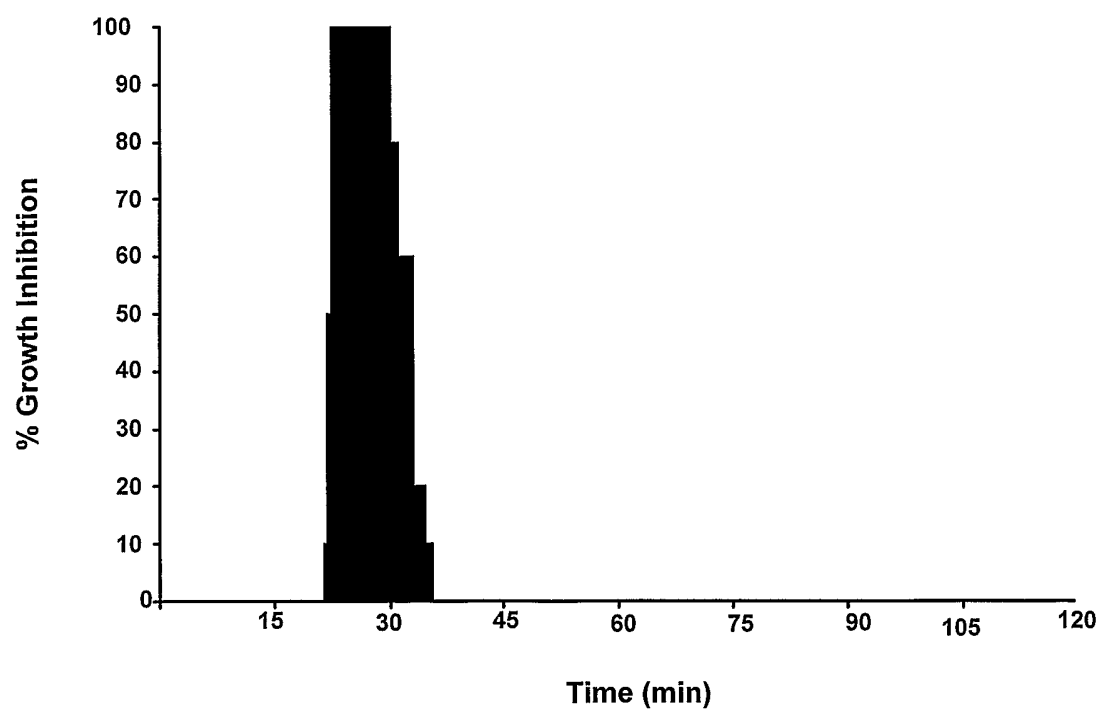
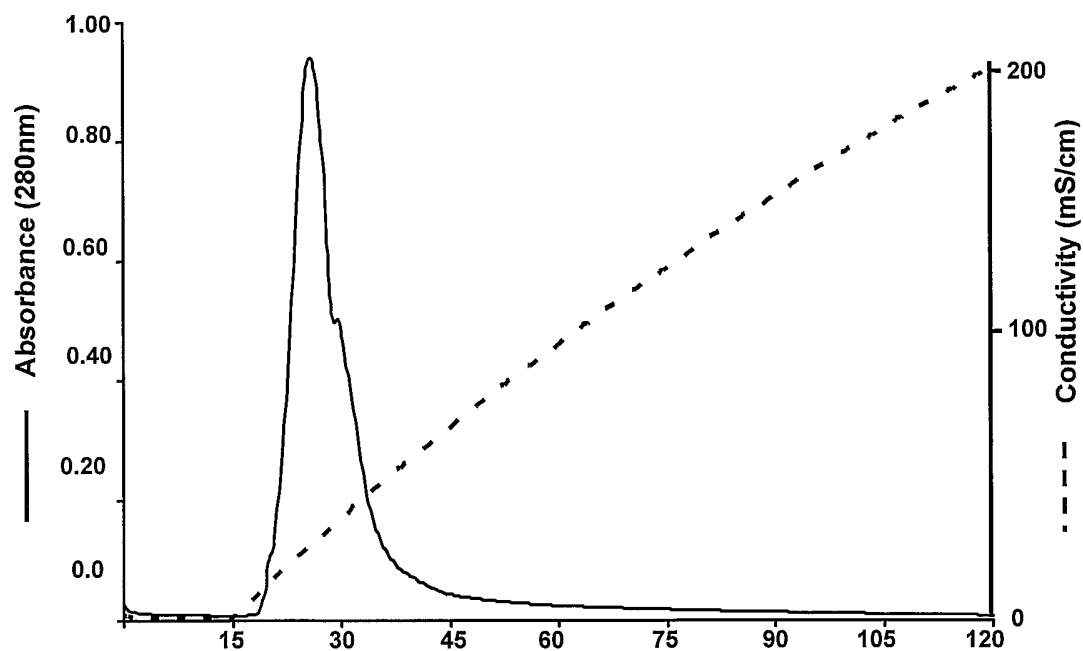
223 TGG CCC GGT GTT ACT CCC CTC ACT CTC TCT GAA TAC CGA CTC TCT TCT TCC AAC TAT ACT  
W P G V T P L T L S E Y R L S S N Y T

283 TCA CAA GCC ACC GCC AAT AAT CCG TAG TCGTTACTCGCTGCCACCGCAGTACGTACATAACCCGTGTAG  
S Q A T A N N P \*

380 ATCGAGCAAGTCTAAATATAATAAATAATCTTGCTCGGTATAATGGATATATGTGTTTCTATACCAGTTAGAATAGT  
459 ACGAATAGTAATTGTCAGGTTTGTATCCGGTTTCTTATGTATGTAATCAATAAATTTGGGGAATGGACAATGAATA  
538 GTTAAATAGTATTATTATTAATAAAAAAAAAAAAAA

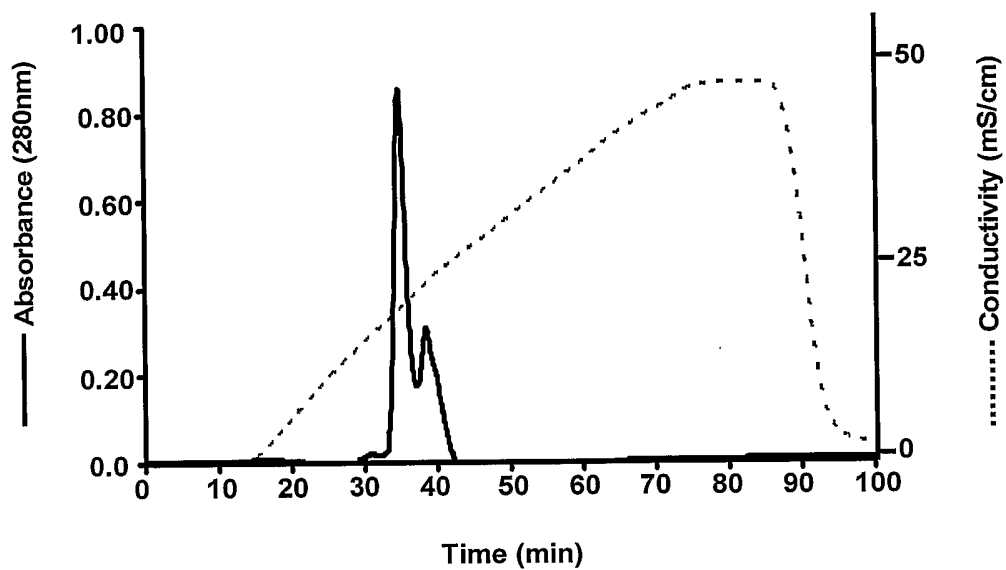
Fig. 3

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*Fig. 4*

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A.



B.

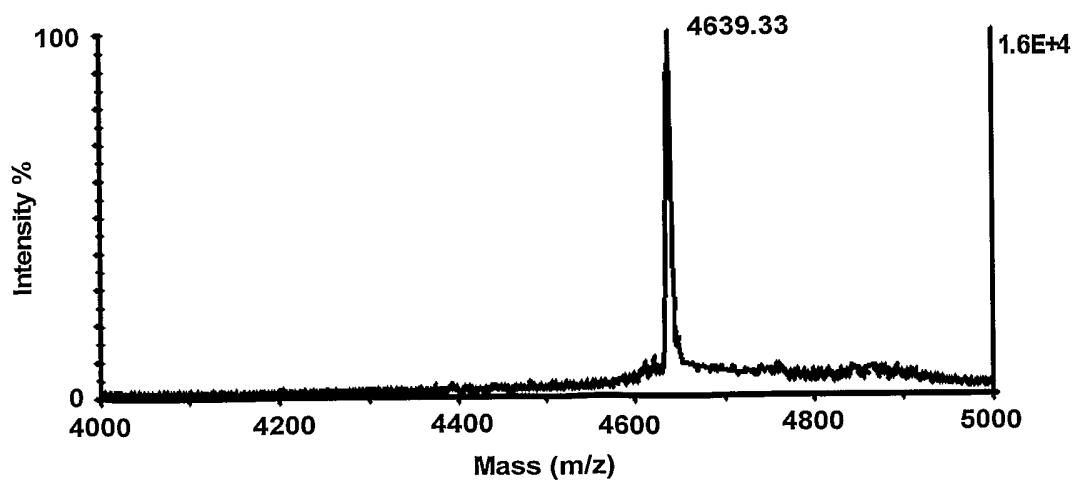


Fig. 5

... AAT TGG GCC CGA CGT CGC ATG CTC CCG GCC ATG GCG GCC GGA ATT CGA TTA  
N W A R R R M L P A A M A A A G I R L

61 AGC AGT GGT ATC AAC GCA GAG TAC GCG GGG GAT CCG TCG TGC AGT CCG GCC GGA CAA CAG  
S S G I N A E Y A G D P S C S P A G Q Q

121 TAT TGT AAC AAC GGG AGA TGT TGC AGC AAG TTC AAC TGG TGC GGA AGC ACT GCT GCT TAC  
Y C N N G R C C C S K F N W C G S T A A Y

181 TGT CAG AAA CCT AAT TGC ATT GCT CAG TGT TGG CCC GGT GTT ACT GCC CTC ACT CTC TCT  
C Q K P N C I A Q C W P G V T A L T L S

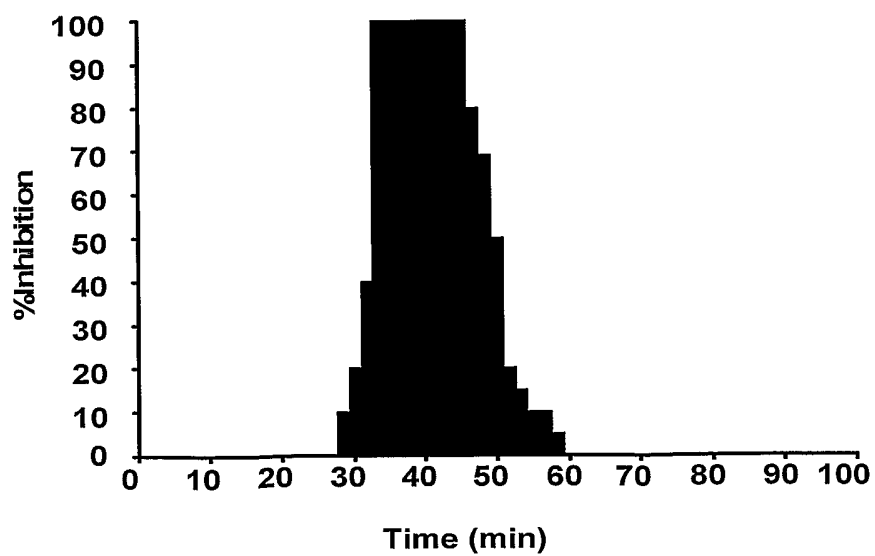
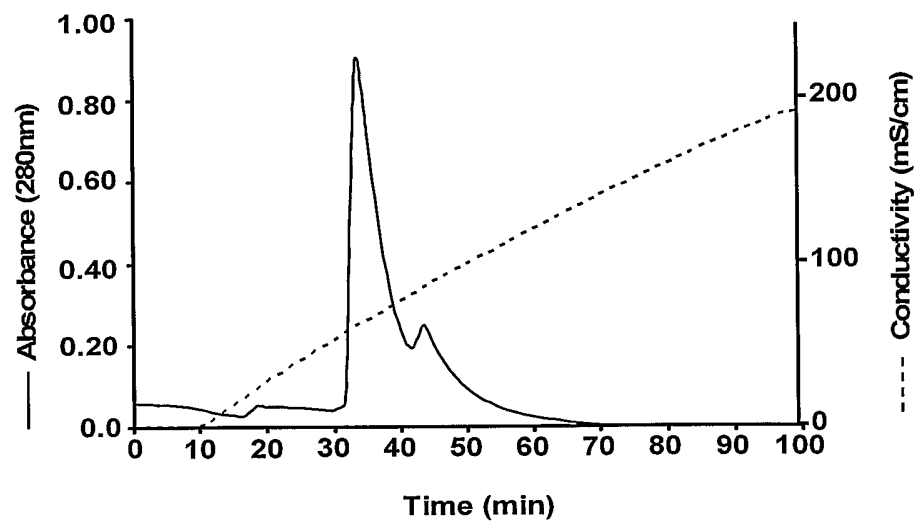
241 GAA TCC CTA CTC TCT CTT CAA AAC TAC ACT TCA GAA GCC ACC ATG GCC GCT GAA AAT  
E S L L S L Q N Y T S E A A T M A A E N

301 AAT AAT CCA TAG TCATCACTCCTCCGCACTACACGTACGTCCCCTAAGCCATGCAGATCGAGCAGTCTAGAAGAGA  
N N P \*

376 ATAACATGCTCGGGTTTAAAGCAAGTCTAATGAGTGCAATTTTAGTGCTTTGTTCATTATTACAGTTGTT  
455 ATATCATGTTATGATTATTTCAGTGTTGTATATCATATAAGCATAACAATATCATGTTATATCATATAAGCATAACAATG  
534 ATCATAGAAATTTTTCCTCTAAAAAIAAAAAAAAAA

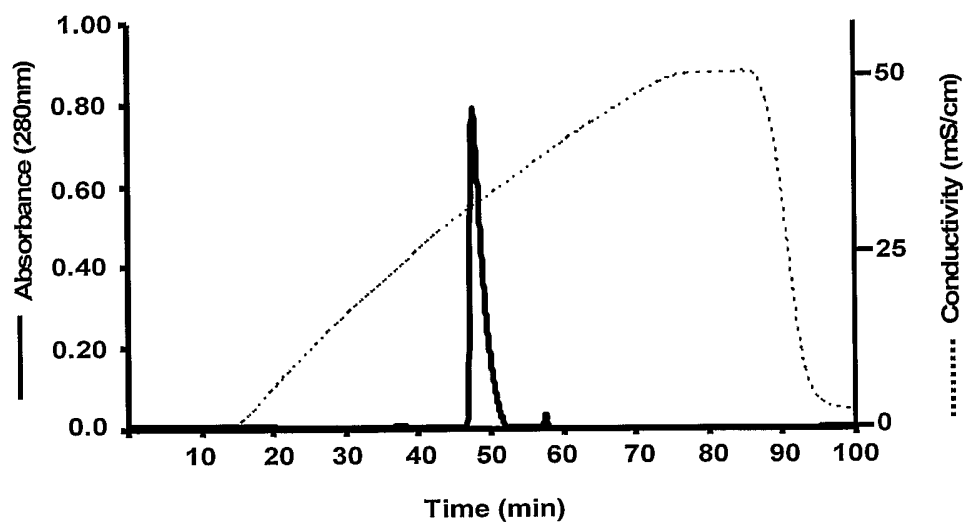
**Fig. 6**

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*Fig. 7*

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A.



B.

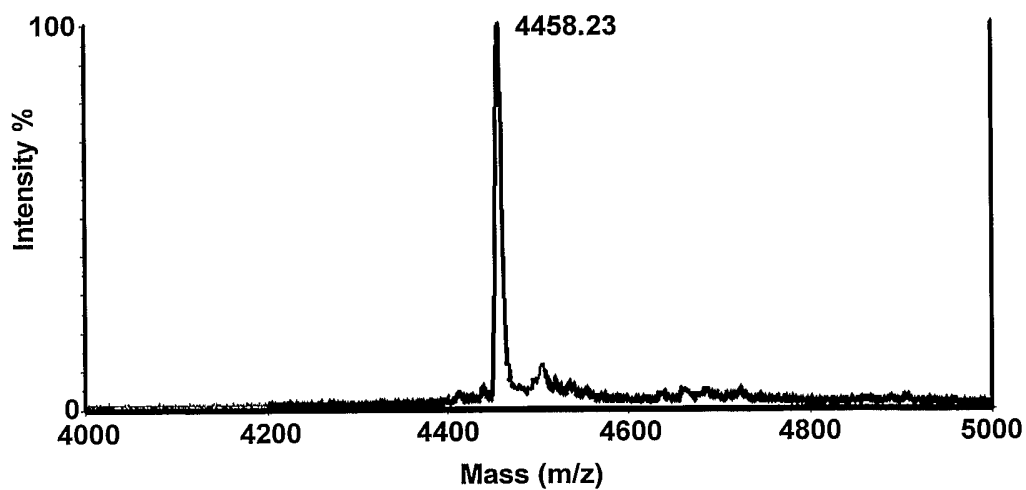


Fig. 8

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|     |                                                                                 |
|-----|---------------------------------------------------------------------------------|
| 1   | ... ATG GTG TTT GCA ATG GCC ATG GTT ATG ATA ATG GTA GAA GGA GAA CCG ACT TGC     |
|     | M V F A M A M V M M I M V E G                                                   |
|     | E P T C                                                                         |
| 61  | AGT CCA GCC GGG AGG ATT TAC TGT AAC AAA GGT AGA TGC TGC AGC AAG TTT AAC TGG TGT |
|     | S P A G R I Y C N K G R C C S K F N W C                                         |
| 121 | GGC AAT ACT GCT GCC TAC TGT GCA AAA GGT AAC TGC ATT GCT AAC TGT TGG AAA GCC ACC |
|     | G N T A A Y C A K G N C I A N C W                                               |
| 181 | TCT CCT CTC GCC CAA TCC CTT CTG TCC ATT ACT AAT TAC ACG GCT GAT GTC GAG         |
|     | S P L P L A Q S L L S I T N Y T A D V E                                         |
| 241 | GCT CAT AAT CCG AAT CCG TAG AGTAGAAAAATAAACTTGTA CTGCTGTATAAAGGGGTGCCATACCC     |
|     | A H N P N P *                                                                   |
| 313 | CACTACATATAAATAAGGCATGCCCTCCAATCCAATGGCTTCCCATTATGGGATATGTAGTTAATTATTTCTAGTG    |
| 392 | ATAATAAACGGTTGTAGCTCGGTGGTCAAAAAAAAAAAAAA                                       |

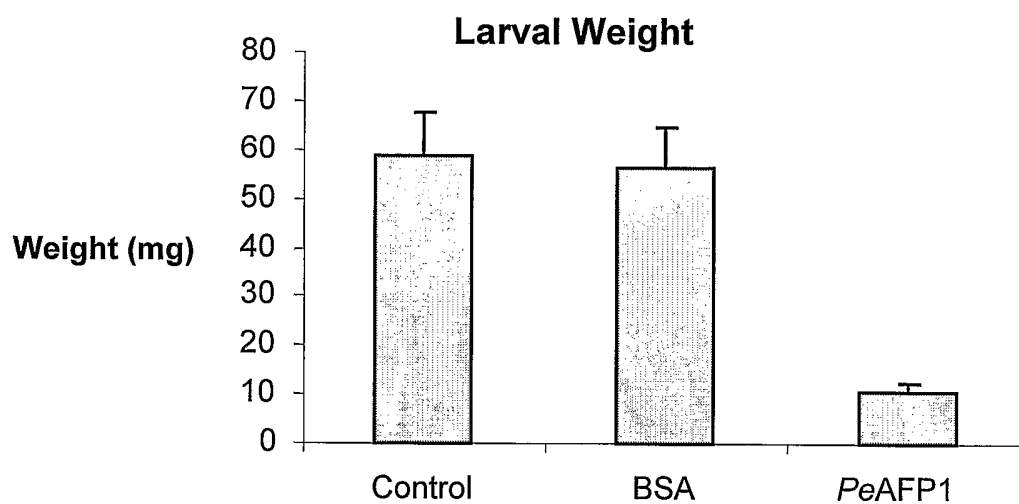
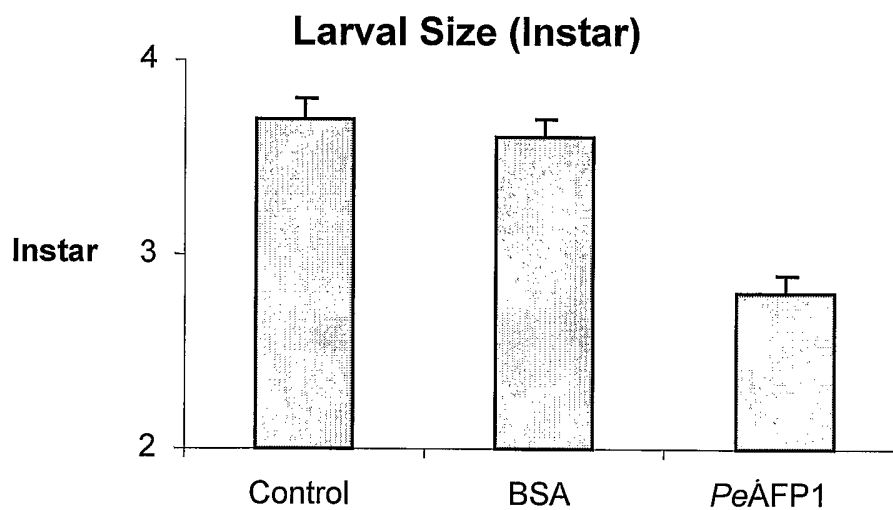
**Fig. 9**

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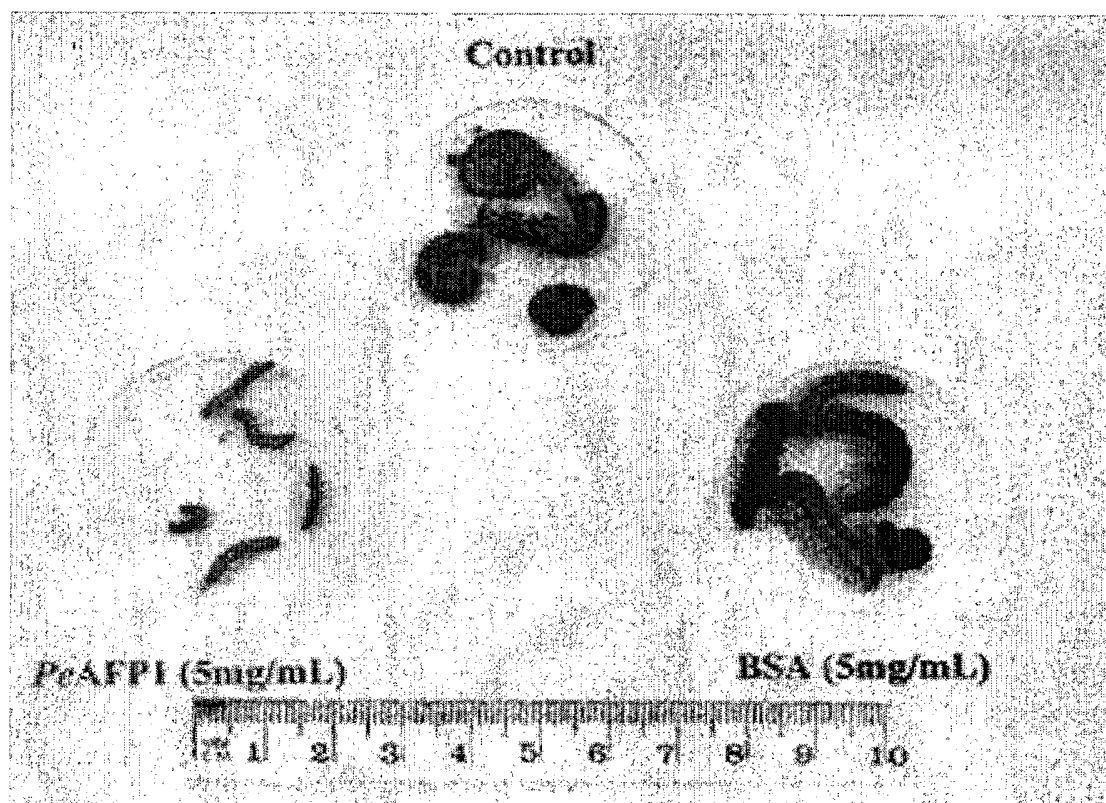
|        |                                                                                                                                    |
|--------|------------------------------------------------------------------------------------------------------------------------------------|
| AbAFP1 | DPT <b>C</b> SPAGRFY- <b>C</b> NDGR <b>CCS</b> RSNW <b>C</b> GN <b>TAA</b> <b>Y</b> CKR-PNCIA- <b>Q</b> CWPG                       |
| ArAFP1 | DP <b>S</b> CSPAGQ̄QY- <b>C</b> NNGR <b>CCS</b> KFNW <b>C</b> G <b>STAA</b> <b>Y</b> CQK-PNCIA- <b>Q</b> CWPG                      |
| PeAFP1 | EPT <b>C</b> SPAGRIY- <b>C</b> NKGR <b>CCS</b> KFNW <b>C</b> GN <b>TAA</b> <b>Y</b> CAK-GNCIA- <b>N</b> CW                         |
|        | * * * * *                                                                                                                          |
|        | * * * * *                                                                                                                          |
|        | * * * * *                                                                                                                          |
| AcAMP1 | VGE <b>C</b> VR- <b>---</b> GRCP <b>S</b> GM <b>CCS</b> Q̄FGY <b>C</b> GKGP <b>K</b> <b>Y</b> CG                                   |
| AcAMP2 | VGE <b>C</b> VR- <b>---</b> GRCP <b>S</b> GM <b>CCS</b> Q̄FGY <b>C</b> GKGP <b>K</b> <b>Y</b> CGR                                  |
| An1    | -GE <b>C</b> GR- <b>---</b> GRSCPPGL <b>CCS</b> RYGY <b>C</b> GTGPD <b>Y</b> CGR                                                   |
| BmAMP1 | <b>---</b> <b>C</b> SS- <b>---</b> HNP <b>C</b> PRHQ̄ <b>CCS</b> KYGY <b>C</b> GLGSD <b>Y</b> CGL- <b>---</b> GCRGGP <b>C</b> DR   |
| BvIWF4 | SGE <b>C</b> NM- <b>---</b> YGRCP <b>P</b> GY <b>CCS</b> KFGY <b>C</b> GVGR <b>Y</b> CG                                            |
| CaAMP1 | QE <b>Q</b> CGNQAGGRACANRL <b>CCS</b> Q̄YGY <b>C</b> G <b>S</b> TR <b>Y</b> CGV- <b>---</b> GCQ <b>S</b> - <b>N</b> CGR            |
| EaAFP1 | -Q̄ <b>T</b> CASRCPRP- <b>C</b> NAGL <b>CCS</b> IYGY <b>C</b> GSGNA <b>Y</b> CGA-GNCR- <b>C</b> Q̄CRG                              |
| EaAFP2 | -Q̄ <b>T</b> CASRCPRP- <b>C</b> NAGL <b>CCS</b> IYGY <b>C</b> GSGAA <b>Y</b> CGA-GNCR- <b>C</b> Q̄CRG                              |
| FeCBP1 | -Q̄ <b>Q</b> CGRQAGNRR <b>C</b> PNNL <b>CCS</b> Q̄FGY <b>C</b> GRTNE <b>Y</b> CTGF <b>G</b> CQ <b>S</b> - <b>N</b> CRRCG           |
| Hevein | -E <b>Q</b> CGRQAGGKL <b>C</b> PNNL <b>CCS</b> Q̄WGW <b>C</b> G <b>S</b> TDE <b>Y</b> CSPDH <b>N</b> CQ <b>S</b> - <b>N</b> CKDS   |
| PnAMP1 | -Q̄ <b>Q</b> CGRQASGRL <b>C</b> GNRL <b>CCS</b> Q̄WGW <b>C</b> G <b>S</b> TAS <b>Y</b> CGA- <b>---</b> GCQ <b>S</b> - <b>Q</b> CRS |
| PnAMP2 | -Q̄ <b>Q</b> CGRQASGRL <b>C</b> GNRL <b>CCS</b> Q̄WGW <b>C</b> G <b>S</b> TAS <b>Y</b> CGA- <b>---</b> GCQ <b>S</b> - <b>Q</b> CR  |
| UDA    | -Q̄ <b>R</b> CSQGGGGT <b>C</b> PALW <b>CCS</b> IWGW <b>C</b> GDSE <b>P</b> <b>Y</b> CG- <b>---</b> RTCEN- <b>K</b> CWSG            |

Fig. 10

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*Fig. 11**Fig. 12*

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*Fig. 13*

## SEQUENCE LISTING

<110> The University of Queensland  
The State of Queensland acting through its Department of  
Primary Industries and Fisheries  
Commonwealth Scientific and Industrial Research  
Organisation  
BSES Limited  
Charles Darwin University  
Australian National University  
The Commonwealth of Australia acting through the Department  
of Agriculture Fisheries and Forestry  
The Northern Territory of Australia acting through its  
Department of Primary Industry, Fisheries and Mines  
Biogemma SAS

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<170> PatentIn version 3.3

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&lt;220&gt;

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&lt;223&gt; Xaa can be any naturally occurring amino acid

&lt;400&gt; 1

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|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Xaa | Pro | Xaa | Cys | Ser | Pro | Ala | Gly | Xaa | Xaa | Tyr | Cys | Asn | Xaa | Gly | Arg |
| 1   |     |     |     | 5   |     |     |     | 10  |     |     |     |     |     | 15  |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Cys | Cys | Ser | Xaa | Xaa | Asn | Trp | Cys | Gly | Xaa | Thr | Ala | Ala | Tyr | Cys | Xaa |
|     |     |     | 20  |     |     |     |     | 25  |     |     |     |     | 30  |     |     |

|     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Xaa | Xaa | Asn | Cys | Ile | Ala | Xaa | Cys | Trp | Glx |
|     |     | 35  |     |     |     |     | 40  |     |     |

&lt;210&gt; 2

&lt;211&gt; 43

&lt;212&gt; PRT

&lt;213&gt; Araucaria bidwillii

&lt;400&gt; 2

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Asp | Pro | Thr | Cys | Ser | Pro | Ala | Gly | Arg | Phe | Tyr | Cys | Asn | Asp | Gly | Arg |
| 1   |     |     |     | 5   |     |     |     |     | 10  |     |     |     |     | 15  |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Cys | Cys | Ser | Arg | Ser | Asn | Trp | Cys | Gly | Asn | Thr | Ala | Ala | Tyr | Cys | Lys |
|     |     |     | 20  |     |     |     |     | 25  |     |     |     |     | 30  |     |     |

|     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Arg | Pro | Asn | Cys | Ile | Ala | Gln | Cys | Trp | Pro | Gly |
|     |     | 35  |     |     |     |     | 40  |     |     |     |

&lt;210&gt; 3

&lt;211&gt; 43

&lt;212&gt; PRT

&lt;213&gt; Agathis robusta

&lt;400&gt; 3

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|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Asp | Pro | Ser | Cys | Ser | Pro | Ala | Gly | Gln | Gln | Tyr | Cys | Asn | Asn | Gly | Arg |
| 1   |     |     |     | 5   |     |     |     | 10  |     |     |     |     |     | 15  |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Cys | Cys | Ser | Lys | Phe | Asn | Trp | Cys | Gly | Ser | Thr | Ala | Ala | Tyr | Cys | Gln |
|     |     |     | 20  |     |     |     |     | 25  |     |     |     |     | 30  |     |     |

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20 25 30

Lys Gly Asn Cys Ile Ala Asn Cys Trp  
35 40

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1 5 10 15

Gln Phe Gly Tyr Cys Gly Lys Gly Pro Lys Tyr Cys Gly Arg  
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Arg Tyr Gly Tyr Cys Gly Thr Gly Pro Asp Tyr Cys Gly Arg  
20 25 30

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Gly Pro Cys Asp Arg  
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1 5 10 15

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Leu Cys Cys Ser Gln Tyr Gly Tyr Cys Gly Ser Thr Arg Ala Tyr Cys  
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Gly Val Gly Cys Gln Ser Asn Cys Gly Arg  
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Gln Thr Cys Ala Ser Arg Cys Pro Arg Pro Cys Asn Ala Gly Leu Cys  
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Cys Ser Ile Tyr Gly Tyr Cys Gly Ser Gly Asn Ala Tyr Cys Gly Ala  
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Gly Asn Cys Arg Cys Gln Cys Arg Gly  
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Gly Asn Cys Arg Cys Gln Cys Arg Gly  
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20 25 30

Thr Gly Phe Gly Cys Gln Ser Asn Cys Arg Arg Cys Gly  
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Cys Cys Ser Gln Trp Gly Trp Cys Gly Ser Thr Asp Glu Tyr Cys Ser  
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&lt;210&gt; 20

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 tgctgcagca ggtccaactg gtgcggaaac actgctgctt actgtaaaag gcctaattgt 240  
 attgctcagt gttggcccgg tgttactccc ctactctct ctgaataccg actctcttct 300  
 tccaactata cttcacaagc caccgccaat aatccgtagt cgttactcgc tgccaccgca 360  
 cgtacgtaca taaccctgt agatcgagca agtctaaata tataaataaa tcttgctcgt 420  
 ggtataatgg atatatgtgt ttctatacca gttagaatag tacgaatagt aattgtcagg 480  
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20 25 30

Ala Gly Arg Phe Tyr Cys Asn Asp Gly Arg Cys Cys Ser Arg Ser Asn  
35 40 45

Trp Cys Gly Asn Thr Ala Ala Tyr Cys Lys Arg Pro Asn Cys Ile Ala  
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Gln Cys Trp Pro Gly Val Thr Pro Leu Thr Leu Ser Glu Tyr Arg Leu  
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 35 40 45

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Cys Ile Ala Gln Cys Trp Pro Gly Val Thr Ala Leu Thr Leu Ser Glu  
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 cctcttctc tcgccaatc ccttctgtcc attactaatt acacggctga tgtcgaggct 240  
 cataatccga atccgtagag tagaaaataa acttgactg ctgttgtgta taaagggtgt 300

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aaaaaaaaaa aa 432

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20 25 30

Cys Ser Lys Phe Asn Trp Cys Gly Asn Thr Ala Ala Tyr Cys Ala Lys  
35 40 45

Gly Asn Cys Ile Ala Asn Cys Trp Lys Ala Thr Ser Pro Leu Pro Leu  
50 55 60

Ala Gln Ser Leu Leu Ser Ile Thr Asn Tyr Thr Ala Asp Val Glu Ala  
65 70 75 80

His Asn Pro Asn Pro  
85

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2005/001967

## A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

*C07K 14/42* (2006.01)      *A61K 38/16* (2006.01)      *C07H 21/04* (2006.01)  
*A01P 1/00* (2006.01)      *A61P 31/00* (2006.01)      *C12N 15/29* (2006.01)  
*A01P 3/00* (2006.01)      *A61P 31/04* (2006.01)

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

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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

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

File Registry subsequence search based on sequence defined in Claim 1

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                 | Relevant to claim No. |
|-----------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| A         | K. K. NIELSON et al., "Characterization of a New Antifungal Chitin-Binding Peptide from Sugar Beet Leaves", Plant Physiol., 1997, 113, pp. 83-91.<br>See whole document.                                                           | 1-36                  |
| A         | M. MURAKI et al., "Chemically prepared hevein domains: effect of C-terminal truncation and the mutagenesis of aromatic residues on the affinity for chitin", Protein Engineering, 13(6), 2000, pp. 385-389.<br>See whole document. | 1-36                  |

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Further documents are listed in the continuation of Box C

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See patent family annex

|                                                                                                                                                                         |                                                                                                                                                                                                                                                  |
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Date of the actual completion of the international search  
23 January 2006

Date of mailing of the international search report  
10 FEB 2006

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