

A family of antimicrobial peptides is produced by processing of a 7S globulin protein in *Macadamia integrifolia* kernels

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Summary

A new family of antimicrobial peptides has been discovered in *Macadamia integrifolia*. The first member of this new family to be purified from nut kernels was a peptide of 45 aa residues, termed MiAMP2c. This peptide inhibited various plant pathogenic fungi *in vitro*. cDNA clones corresponding to MiAMP2c encoded a 666 aa precursor protein homologous to vicilin 7S globulin proteins. The deduced precursor protein sequence contained a putative hydrophobic N-terminal signal sequence (28 aa), an extremely hydrophilic N-proximal region (212 aa), and a C-terminal region of 426 aa which is represented in all vicilins. The hydrophilic portion of the deduced protein contained the sequence for MiAMP2c as well as three additional segments having the same cysteine spacing pattern as MiAMP2c. Each member of the MiAMP2 family (i.e. MiAMP2a, b, c and d) consisted of approximately 50 amino acids and contained a C-X-X-X-C-(10–12)X-C-X-X-X-C motif. Subsequent isolations from seed exudates led to the purification of the predicted family members MiAMP2b and 2d, both of which also exhibited antimicrobial activity *in vitro*. These results suggest that some vicilins play a role in defence during seed germination.

Introduction

Plants are exposed to pathogens at every stage in their lifecycle and appear to use a variety of defences to resist attack. Antimicrobial peptides have been implicated as a defence mechanism in plants and have been detected in a wide array of species and plant tissues (Broekaert *et al.*, 1997). The widespread localization of antimicrobial peptides in a variety of plant tissues and their potent antimicrobial activity *in vitro* suggests that

they may serve a general protective role against plant pathogens. Another indication that these peptides act to protect plants against pathogens comes from the observation that the expression of certain antimicrobial peptides is induced both locally and systemically by pathogen attack (Bohlmann *et al.*, 1998; Penninckx *et al.*, 1996).

The germination stage is an especially vulnerable one for the developing plant. The seed must survive in a moist and pathogen rich environment during the critical stages of imbibition and germination. As seeds take up water, a significant amount of solutes are released into the surrounding medium, including sugars, organic acids, ions, amino acids and proteins (Bewley and Black, 1994). The increased concentration of these nutrients could lead to an increased growth of fungi and bacteria and give rise to a pathogenic interaction. To counter this, the plant seed has defence mechanisms to protect against pathogens.

Antimicrobial proteins have been divided into protein (> 100 aa) and peptide classes (< 100 aa) (Broekaert *et al.*, 1997). Representatives of both classes have been isolated from seeds. Thionins and plant defensins are well-known subclasses of antimicrobial peptides found in many different seeds (Osborn *et al.*, 1995; Ponz *et al.*, 1986; Terras *et al.*, 1992). Other antimicrobial peptides that have been isolated from seeds include proteinase inhibitors (Joshi *et al.*, 1998), lipid-transfer proteins (Cammue *et al.*, 1995), chitin-binding proteins (Broekaert *et al.*, 1992), knottin-type peptides (Chagolla-Lopez *et al.*, 1994), and several 4-cysteine-type peptides (reviewed in Broekaert *et al.*, 1997). The large number of antimicrobial peptides isolated from seeds suggests that they have important roles in protecting seeds before and during germination; evidence that plant defensins function to protect seeds during this critical period in germinating radish seeds has been reported previously (Terras *et al.*, 1995).

Multiple protein processing events occur during seed germination and storage proteins are utilized as a source of nutrients for germination and growth. Vicilins, also called 7S globulins, comprise one well-known class of storage proteins and can constitute as much as 70–80% of total seed protein (Bewley and Black, 1994). These proteins are cleaved into smaller fragments before and during the germination process

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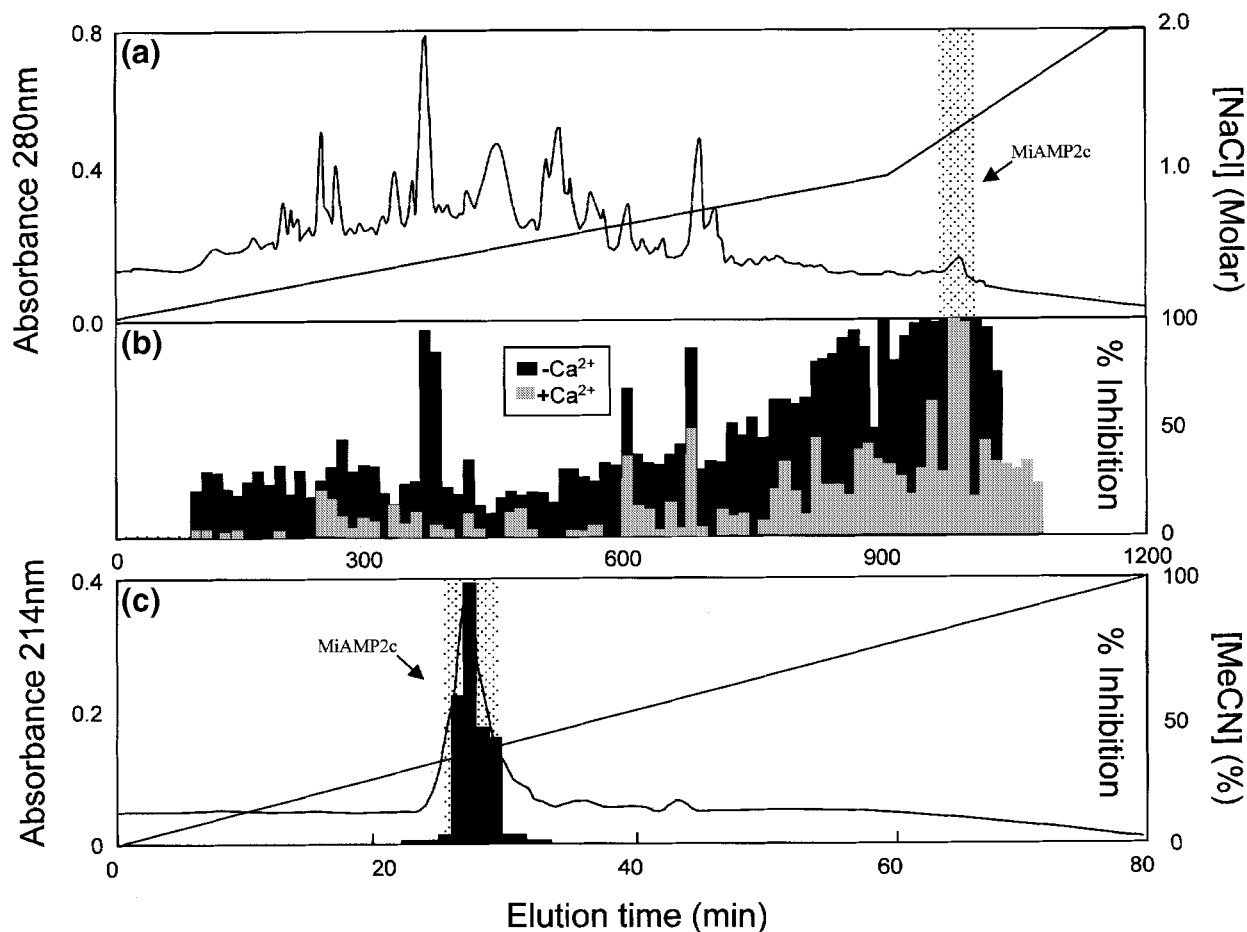


Figure 1. Purification of MiAMP2c from Macadamia crude extract.

(a) Cation-exchange separation of the basic fraction of macadamia crude extract. The shaded region indicates the material that was further processed by reverse-phase chromatography as seen in (c).

(b) *In vitro* antimicrobial activity of fractions from the cation exchange separation shown in (a). The inhibition (%) of growth of *Phytophthora cryptogea* in the presence (+Ca²⁺) and absence (-Ca²⁺) of an additional 1 mM calcium chloride added to the growth media (which normally contains 50 µM calcium chloride) is shown.

(c) Reverse-phase separation of the cation-exchange fractions indicated by shading in (a). The inhibition of growth of *Phytophthora cryptogea* is shown by the histogram.

(Müntz, 1996; Shewry *et al.*, 1995). However, little is known regarding what roles other than nutrient storage these seed proteins assume. Some recent reports have raised the possibility that vicilins may, in fact, also play defensive roles in germinating seeds (Chung *et al.*, 1997; Yamada *et al.*, 1999).

We have previously reported the discovery of a completely novel antimicrobial peptide (MiAMP1) from seeds of *Macadamia integrifolia* (Marcus *et al.*, 1997; McManus *et al.*, in press). The presence of antimicrobial activity in other fractions encouraged us to look for new antimicrobial peptides from this source. Here, we describe the isolation of a new family of 4-cysteine-type antimicrobial peptides that are produced from a large precursor protein related to 7S globulins in other plants.

These observations demonstrate a potential role for some vicilin proteins in plant defence.

Results

Purification and characterization of antimicrobial peptides from Macadamia

Antimicrobial activity was previously detected in cation-exchange fractions of macadamia extracts and an antimicrobial peptide labeled MiAMP1 was isolated (Marcus *et al.*, 1997). Further fractionations and bioassays were carried out in an effort to identify other novel antimicrobial peptides. Figure 1(a) shows the elution profile from the cation-exchange separation of the crude basic protein

Table 1. Concentrations of MiAMP2c at which 50% inhibition of growth was observed

Organism	IC ₅₀ (µg ml ⁻¹)	
	- Ca ²⁺	+ Ca ²⁺
<i>Alternaria helianthi</i>	5–10	ND
<i>Ceratocystis paradoxa</i>	20–50	> 50
<i>Cercospora nicotianae</i>	5–10	5–10
<i>Chalara elegans</i>	2–5	10–20
<i>Fusarium oxysporum</i>	10	20–50
<i>Leptosphaeria maculans</i>	25	> 50
<i>Sclerotinia sclerotiorum</i>	20–50	> 50
<i>Verticillium dahliae</i>	5–10	> 50
<i>Saccharomyces cerevisiae</i>	20–50	> 50
<i>Phytophthora cryptogea</i>	5–10	10–25
<i>Phytophthora parasitica nicotianae</i>	10–20	> 50
<i>Clavibacter michiganensis</i>	50	> 50
<i>Ralstonia solanacearum</i> ^a	> 50	> 50
<i>Escherichia coli</i> ^a	> 50	> 50

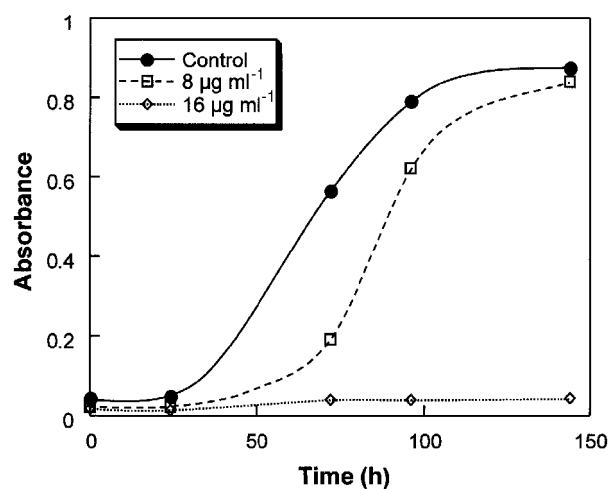
Growth inhibition measurements were made in the presence of low (-Ca²⁺) and high (+Ca²⁺) concentrations of calcium chloride (i.e. 50 µM and 1 mM, respectively).

^aGrowth inhibition was not detected in these organisms at 50 µg ml⁻¹. The specification >50 generally indicates that little or no inhibition was evident when 50 µg ml⁻¹ concentrations were used.

extracted from Macadamia nut kernels. Inhibition of the test pathogen was detected in a number of regions throughout the profile. Among the fractions exhibiting antimicrobial activity, a cation-exchange peak eluting at approximately 1.3M NaCl showed a high level of activity in both the presence and absence of calcium ions (Figure 1b). This peak was further purified using reverse-phase HPLC (Figure 1c). The main eluting component from preparative RP-HPLC coincided with significant inhibitory activity and constitutes MIAMP2 (*Macadamia integrifolia antimicrobial protein 2*).

Purified MIAMP2 was tested *in vitro* against a range of fungal and bacterial plant pathogens (Table 1). These *in vitro* tests yielded IC₅₀ values as low as 2–5 µg ml⁻¹ against some pathogens (e.g. *Chalara elegans*). The antimicrobial peptide was active against a range of fungal pathogens at concentrations of 5–10 µg ml⁻¹. Of the various bacterial pathogens that were tested, activity was only detected against *Clavibacter michiganensis* with an IC₅₀ value of 50 µg ml⁻¹.

Since Ca²⁺ ions are known to reduce the inhibitory activity of many antimicrobial peptides against a number of pathogens (Broekaert *et al.*, 1997), we also tested the strength of inhibitory activity in the presence of 1 mM Ca²⁺ (Table 1). In most cases, the activity of MIAMP2 was significantly reduced; for example, in the presence of calcium, the IC₅₀ against *Verticillium dahliae* was increased

**Figure 2.** Inhibition dynamics of various concentrations of MiAMP2c against *Fusarium oxysporum* grown in culture.

from 5 to 10 µg ml⁻¹ to over 50 µg ml⁻¹. However, in some cases, the effect of calcium on IC₅₀ was less evident, as in the case of *Cercospora nicotianae*.

One aspect of the inhibition that is not apparent from the IC₅₀ values is the dynamic nature of the growth inhibition process. At lower peptide concentrations, inhibition of fungal growth tended to be transient in nature, lasting approximately 48 h and then gradually diminishing in effect. However, when the peptide concentrations were sufficiently high, the pathogen did not overcome the inhibitory effect of the peptide. An example of this effect can be seen in Figure 2 where growth of the fungus *Fusarium oxysporum* was monitored. At 8 µg ml⁻¹, the fungus is strongly inhibited during the first 48 h of the bioassay; subsequently, the level of inhibition diminished dramatically and net growth was not affected over 96 h. In contrast, when the peptide was tested at 16 µg ml⁻¹, the fungus did not overcome inhibition (Figure 2).

Mass spectrometry of MIAMP2 revealed its mass to be 5931 Da. N-terminal sequencing of this peptide yielded 42 residues of amino acid sequence (out of 45 total); the remaining residues were deduced from cDNA clones and confirmed by mass spectrometry data (see below). This peptide was highly basic and had a predicted pI of 11.1. The sequence of this peptide exactly matched the deduced amino acid sequence obtained from a cDNA clone (see bracketed residues R118–Q162 in clone 3, Figure 3).

Further purification procedures using the crude extract led to the isolation of other abundant antimicrobial peptides which were length variants of the 5.9 kDa protein described above. Direct sequencing and mass spectrometric analysis of these variants revealed that the N-terminal sequences of the peptides were identical to the 5.9 kDa protein, but contained an additional 2 or 22 amino acid residues at the C terminus. The masses of these related

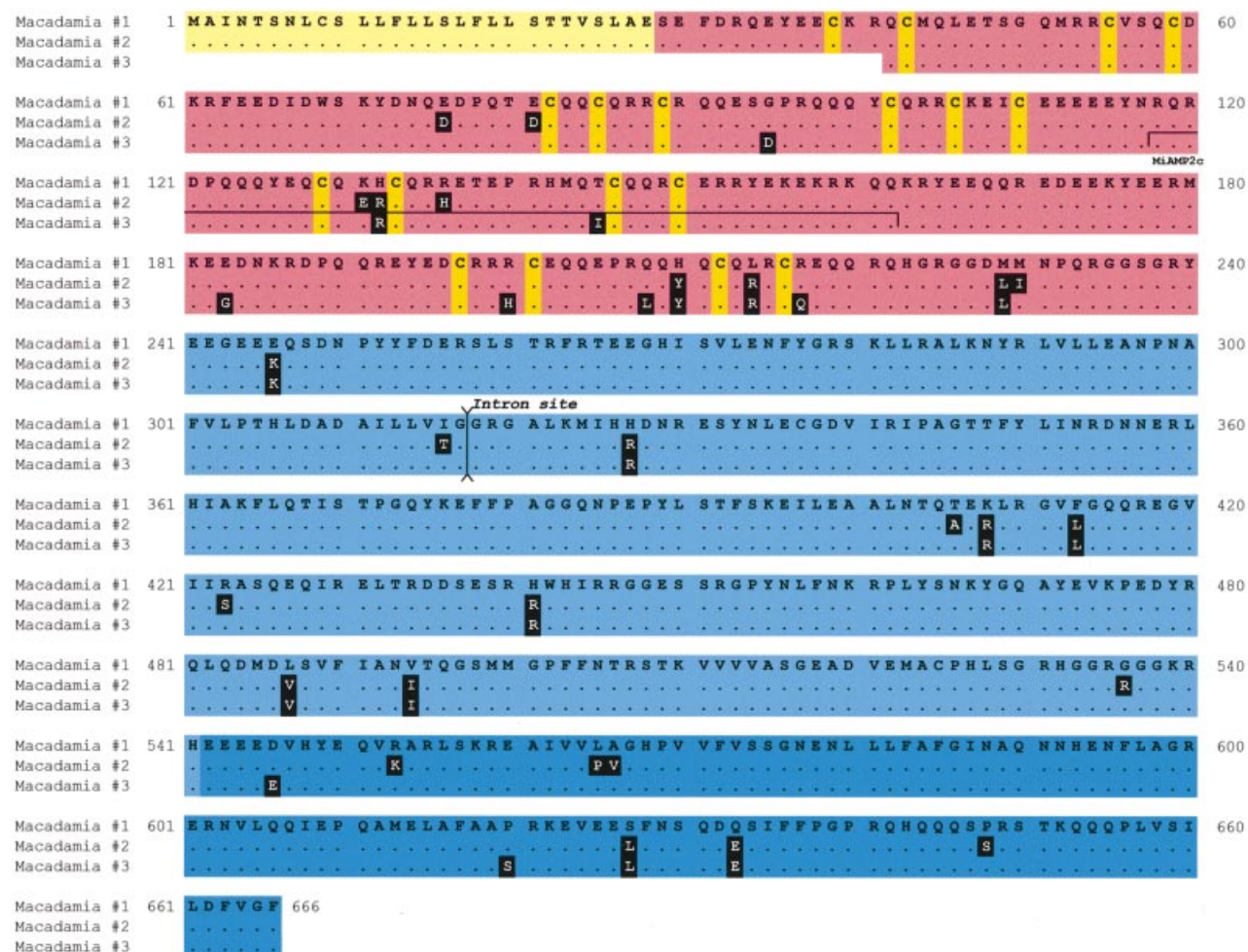


Figure 3. Deduced amino acid sequences of three macadamia clones.

Dots show where clones 2 and 3 are identical in sequence to clone 1; highlighted residues show differences. Colour coding: light yellow, putative signal peptide; pink, hydrophilic region containing antimicrobial peptide sequences; dark yellow, cysteine residues; light and dark blue, vicilin 7S globulin invariant regions. The boundary between the light blue and dark blue regions shows another putative processing site based on analogy with a processing site in pea vicilin. The exact sequence corresponding to MiAMP2c was found in clone 3 and is delineated by a vertical line over the sequence (R118–Q162). Longer versions of MiAMP2c were also isolated with 2 and 22 additional residues at the C-terminus (R118–R164/D184). Sequencing of a genomic clone revealed the presence of one intron located between codons for amino acids 317 and 318 (vertical line with inverted arrowheads). Sequences can be located as GenBank accession numbers AF161883, AF161884 and AF161885.

peptides were found to be 6216 Da and 8818 Da, respectively; these masses correspond to peptides represented by residues R118–R164 and R118–D184 in Figure 3. The longest of these variant peptides (i.e. R118–D184) was found to elute at approximately 0.8 M NaCl (700 min) in the cation-exchange separation of Figure 1a; whereas both of the shorter versions of MiAMP2c eluted later in the separation gradient.

MiAMP2 is derived from large precursor proteins homologous to 7S globulin proteins

Degenerate oligonucleotide primers were designed using the aa sequence of MiAMP2 and a cDNA fragment was amplified using RT-PCR. Northern analysis of RNA from

macadamia nut kernels using the cDNA as a probe revealed a transcript greater than 2 kb in size. A cDNA library was subsequently screened leading to the isolation and full nucleotide sequencing of three positive clones. The three cDNA clones had very similar sequences but varied in length. The two longest clones (i.e. clones 1 and 2) contained large open reading frames which encoded 666 amino acid proteins (see Figure 3). While deduced proteins on clones 1 and 2 contained amino acid sequences almost identical to MiAMP2 (42 and 43 out of 45 residues), the exact MiAMP2 sequence was found in the clone 3 protein (brackets, Figure 3).

The deduced amino acid sequence from the Macadamia cDNA clones were found to have extensive homology to a number of vicilin, 7S globulin proteins. In particular,

pumpkin, cotton and cocoa vicilin proteins (NCBI sequence accession numbers AB019195, 137578 and 384341, respectively) showed the highest degree of homology to the macadamia protein, with 216 (51%), 193 (45%) and 183 (43%) amino acid identities to the vicilin portion of macadamia cDNA clone 1 (residues 241–666 in Figure 3). Other 7S globulin proteins from species such as zamia, spruce, pea, soybean, peanut, barley and maize (NCBI accession numbers 949978, 99473, 297170, 99889, 1168390, 167004, and 121205, respectively) exhibited lower but significant degrees of homology to the deduced macadamia protein.

MiAMP2 is one of four repeated segments comprising an extremely hydrophilic N-terminus

The deduced amino acid sequences for the MiAMP2 clones exhibit a predicted signal peptide of about 28 amino acids (Figure 3). The signal peptide is followed by an extremely hydrophilic region comprising amino acids 29–240 (hydrophilic region is colour-coded pink in Figures 3 and 4). This region contains 47% charged amino acid side chains (i.e. asp, glu, his, lys and arg) and only 11% hydrophobic residues (i.e. ala, val, leu, ile, pro, met, phe and trp). A similar hydrophilic region proximal to the N-terminus is present in some, but not all, 7S globulins (Dure, 1990). Figure 4 shows hydrophilicity plots of various 7S globulin proteins containing the hydrophilic region as well as an example of a 7S globulin without it. The remainder of the protein (i.e. residues 241–666) is much less marked in its hydrophilicity, with 27% charged residues and 38% hydrophobic residues (the region is colour-coded blue in Figures 3 and 4). While many 7S globulins do not contain a hydrophilic region (Dure, 1990), they all contain segments with high homology to the C-terminal end of the MiAMP2 precursor protein (i.e. aa 241–666).

The amino acid sequence for MiAMP2 is found in the hydrophilic portion of the protein. Interestingly, this region of the mature macadamia protein contains four repeated segments with the four-cysteine motif C-X-X-X-C-(10–12)X-C-X-X-X-C that is present in the MiAMP2 protein (see MiAMP2a, b, c and d; Figure 5). Each of these repeated segments is separated by a stretch of amino acids containing four to five acidic residues and a nearby asparagine residue. MiAMP2 constitutes the third of the four segments. Therefore, from here on, MiAMP2 will be referred to as MiAMP2c. Together, the four repeated segments comprise the hydrophilic region of the translated MiAMP2c precursor protein. Pumpkin, cotton, cocoa and other 7S globulins contain similar segments possessing the four-cysteine motifs in the N-terminal portions of the mature proteins (Figure 5). Cotton has three repeated

segments while pumpkin and cocoa have two. Other vicilin proteins reported to date contain either one or no segments at all.

Other MiAMP2 family members are released during imbibition of macadamia kernels

Since the MiAMP2c fragment was present in extracts from macadamia kernels and the deduced amino acid sequence data from the cDNA clone showed multiple repeats with the same cysteine spacing as MiAMP2c, it seemed reasonable that other fragments having the same cysteine spacing might also be present in the seed (i.e. MiAMP2a, b and d). In addition, since other 7S globulin proteins are known to be processed during the germination process (Gatehouse *et al.*, 1983), it is likely that the macadamia precursor protein also undergoes processing into smaller fragments before or during germination. If such processing occurred then it may be expected that the predicted fragments could be found in seed exudates after seed imbibition.

In order to establish whether the 666 aa macadamia protein is processed into smaller fragments upon seed imbibition, we examined the exudates of macadamia seeds after they were allowed to imbibe water. SDS-PAGE analysis of these samples showed an abundance of low molecular weight peptides in the seed exudate (Figure 6). Antibodies raised against MiAMP2c were found to react with a portion of the low molecular weight material in Western analysis suggesting that MiAMP2c is released from the seed during imbibition (Figure 6). In addition to finding immunoreactive material in seed exudates, low molecular weight peptides reacting with the antiserum were also detected in nut extracts. However, the relative abundance of low molecular weight peptides was lower in seeds than in the exudate (Figure 6).

The seed exudate was also assayed for antimicrobial activity. When total exudate was added to test fungus, significant inhibition was obtained with protein concentrations of $25\mu\text{g ml}^{-1}$ (Table 2). Since the exudate exhibited antimicrobial activity and MiAMP2c-immunoreactive low molecular weight peptides were present in the exudate, it was decided to attempt the purification of other related fragments predicted to arise from the 666 aa precursor protein (i.e. the predicted fragments MiAMP2a, b, c and d). When the exudate was separated by cation exchange, greater than 90% inhibition of the test fungus was observed in two pools of fractions eluting at approximately 125–140 min and 350–700 min (shaded regions in Figure 7a). Material from each of these pools was further fractionated by reverse-phase chromatography.

The reverse-phase profile of the first cation-exchange pool with antimicrobial activity is shown in Figure 7(b). A

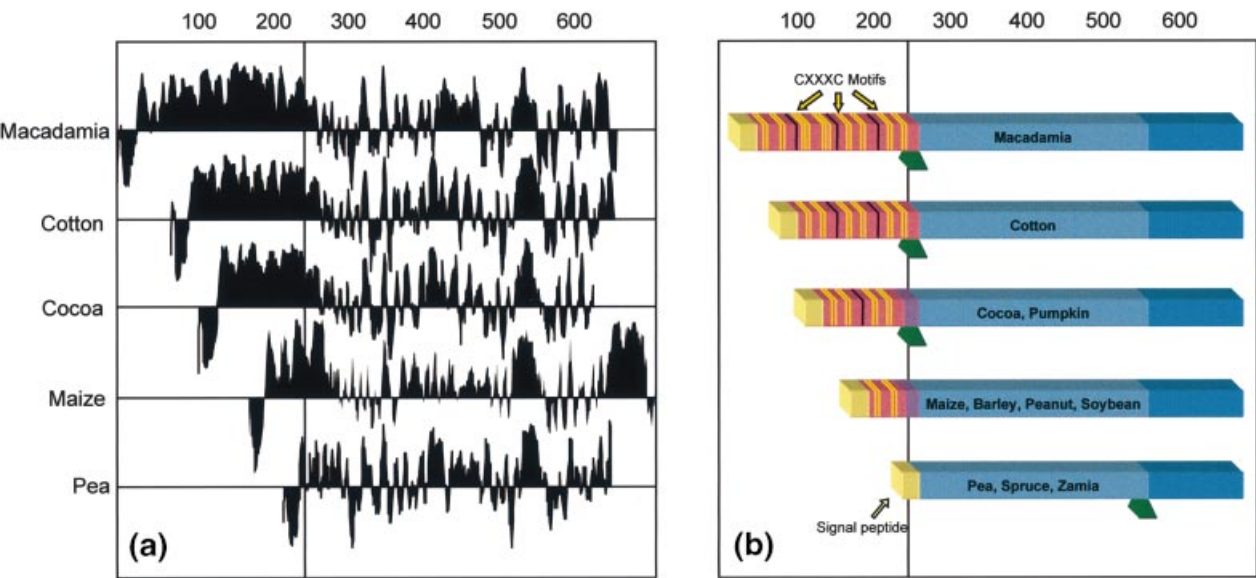


Figure 4. Overview of hydrophilicity and sequence relationships of vicilin proteins from macadamia, cotton (Chlan *et al.*, 1987), cocoa (Spencer and Hodge, 1992), maize (Kriz, 1989), barley (Heck *et al.*, 1993), peanut (Burks *et al.*, 1995), pumpkin (Yamada *et al.*, 1999), soybean (Leievre *et al.*, 1992), pea (Spencer and Hodge, 1992), zamia (Braun *et al.*, 1996) and spruce (Newton *et al.*, 1992).
(a) Hydrophilicities of various vicilin 7S proteins.
(b) Diagrammatic representation of homologies exhibited between vicilin molecules and the domains of the molecule. Light yellow, signal peptides; pink, hydrophilic segments containing paired CXXXC motifs (bright yellow lines); light and dark blue, vicilin 7S globulin invariantly represented regions. Green arrowheads show known processing regions of various vicilin proteins. The purple region indicates a domain of maize, barley, peanut, soybean and pumpkin vicilins that did not align with the macadamia sequences.

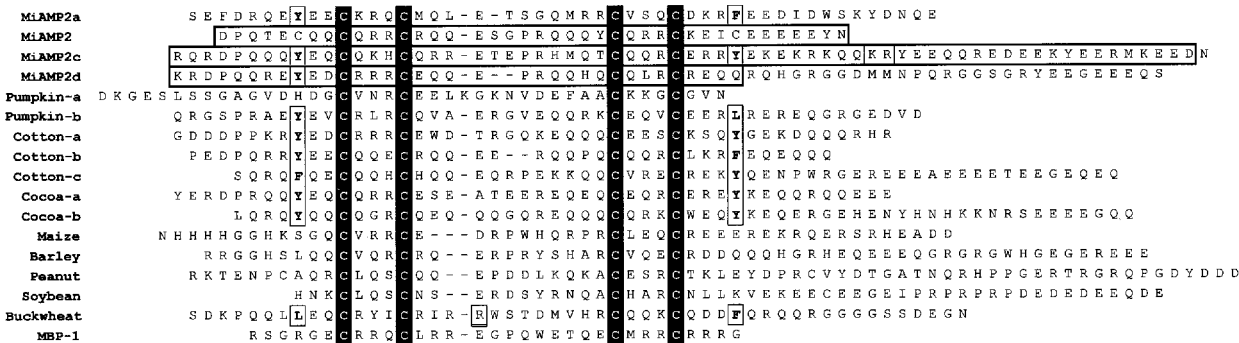


Figure 5. Sequence alignment of predicted fragments from the hydrophilic region of macadamia and other vicilin-like molecules. Sequences were as for Figure 4, with the addition of maize basic protein-1 (Duvick *et al.*, 1992) and buckwheat trypsin inhibitor (Park *et al.*, 1997). Cysteine residues are highlighted with inverted text to show the spacing pattern. Aromatic amino acids with highly conserved spacing relative to the cysteine residues are bold and boxed. Actual peptides that have been purified from seed extracts and exudates are shown with heavy boxes around the sequence. The two vertical dotted lines in the MiAMP2c boxed sequence delineate alternate C-termini of peptides that have been isolated.

peak exhibiting significant antifungal activity ($>50\%$ inhibition at $25\mu\text{g ml}^{-1}$) towards the test fungi was characterized (shaded region in Figure 7b). The N-terminal sequence of the peptide was found to be DEPTEC which corresponds exactly to the N-terminus of MiAMP2b (i.e. the second segment of the four present in the macadamia clone; see Table 2). The peptide exhibited a mass of 5139 Da which was 19 Da more than expected based on

comparison with the clone sequences (Table 2). However, this discrepancy in mass (i.e. 5139 Da versus 5120 Da) could be explained by supposing a single variation in amino acid sequence (for example, a one nucleotide change could give an isoleucine to methionine substitution at position 1109; see Figure 3). Overall, the evidence strongly suggested that the peptide was a closely related variant of MiAMP2b (Figure 3, D77–N117).

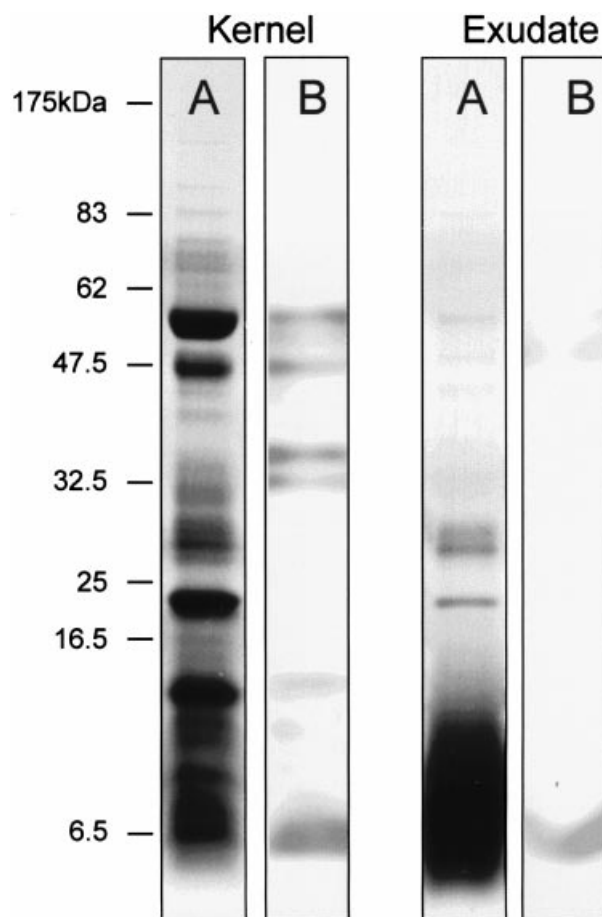


Figure 6. SDS-PAGE and Western analysis of imbibed whole macadamia kernels and exudates after 2 days of imbibition. Lane A, Coomassie-blue stained SDS-PAGE gel. Lane B, Western analysis of SDS-PAGE gel with antibodies raised to MiAMP2c.

Fractionation of the second cation-exchange pool exhibiting antimicrobial activity is shown in Figure 7(c). Although all of the peaks showed some antifungal activity, the peak eluting at 35 min was characterized because it was the main eluting component. The peptide was found to have a mass of 4576 Da and an N-terminal sequence of KRDPQ. Both the mass and the N-terminal sequence match exactly with the N-terminal sequence of MiAMP2d (Figure 3, K186–Q220 in clone 3) predicted to arise from processing of the hydrophilic portion of the protein (Table 2). The data thus identify this peptide as MiAMP2d. Both MiAMP2b and MiAMP2d showed antimicrobial activity *in vitro* (Table 2).

Organization of the *Macadamia vicilin* gene

Sequencing of a genomic clone obtained using PCR techniques revealed the presence of one intron containing 203 bases located between nucleotides 963 and 964 relative to cDNA clone 1 (i.e. exactly between the codons for amino acids G317 and G318) (Figure 3). Although the *Macadamia* cloned sequences show significant homology with a number of vicilin-like proteins, the presence of only one intron in the *Macadamia* gene contrasts with the number of introns reported for other vicilin genes. For example, the genes for cotton and cocoa vicilins, which show the high degrees of similarity to the macadamia protein, contain four and five introns, respectively (Chlan *et al.*, 1986; McHenry and Fritz, 1992). Indeed, the *Macadamia* gene is the only vicilin-like gene reported to date which contains only one intron. Other vicilin genes, including those from legumes, as well as from wheat and maize contain either four or five introns (McHenry and Fritz, 1992). The location

Table 2. Inhibition, mass and sequence data for MiAMP2 fragments purified from seed exudate material

Sample	Source	Inhibition versus <i>Verticillium</i>	Inhibition versus <i>Leptosphaeria</i>	Mass Actual (Expected)	N-terminal sequence (Expected)
Crude exudate	Seed exudate (SE)	NA	>50% at 25 µg ml ⁻¹	NA (6–8 kDa)	NA
MiAMP2a	Not found	NA	NA	NA (5988]	NA (SEFRQEY)
MiAMP2b	Purified from SE	>90% at 25 µg ml ⁻¹	>90% at 25 µg ml ⁻¹	5139 (5120]	DEPTEC (DEPT(E/D)C)
MiAMP2c	Purified from whole seed extract	~50% at 5–10 µg ml ⁻¹	~50% at 25 µg ml ⁻¹	5931 6216 8818 (same as above)	RQRDPQ (RQRDPQ)
MiAMP2d	Purified from SE	>50% at 25 µg ml ⁻¹	>50% at 25 µg ml ⁻¹	4576 (4576)	KRDPQ (KRDPQ)

IC₅₀ values are from measurements made in the presence of low Ca²⁺ (i.e. 50 µM).
NA, not available or not applicable.

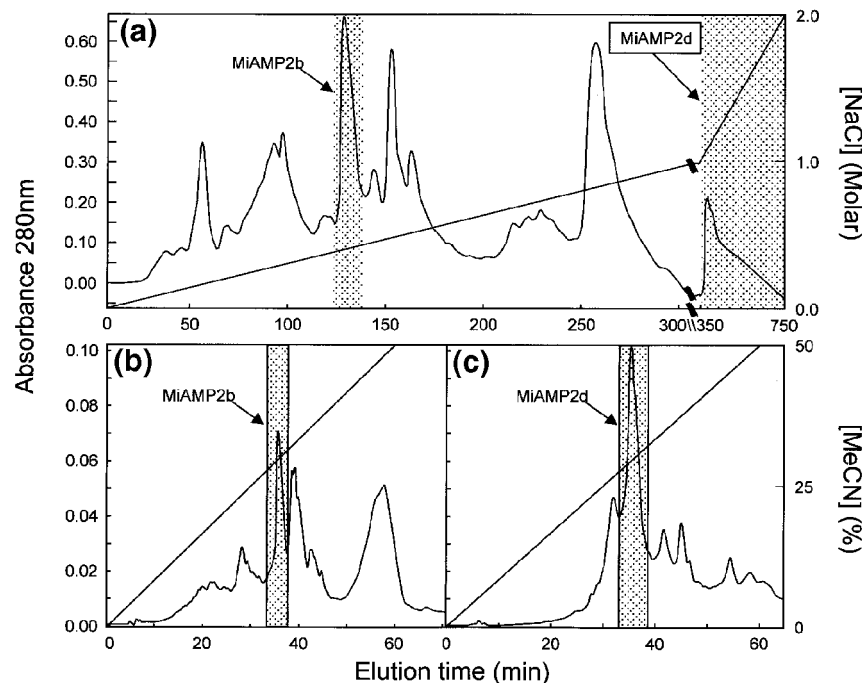


Figure 7. Purification of subunits MiAMP2b and MiAMP2d from imbibed seed exudates. (a) Cation-exchange separation of seed exudate. Shaded regions show *Verticillium*-active fractions that were further processed by reverse-phase chromatography (RP-HPLC shown in b and c). The elution profile from 350 to 750 min is condensed for better clarity of presentation. (b) Reverse phase purification of MiAMP2b. Shaded peak shows the location of MiAMP2b. (c) Reverse phase purification of MiAMP2d. Shaded peak shows the location of MiAMP2d.

of the *Macadamia* intron matches the first intron position in both the cotton and cocoa vicilin genes.

Southern analysis of macadamia DNA (from leaves of an individual tree) using a portion of the cDNA clone 3 revealed one major hybridizing band as well as two to three bands showing a low level of hybridization to the probe (Figure 8). This result is consistent with a small family of genes encoding the vicilin-like *Macadamia* protein(s). In contrast, the pea genome (*Pisum sativum*) has been reported to contain 11 genes for vicilin (Domoney and Casey, 1985).

Discussion

Vicilin proteins are abundant seed storage proteins present in the seeds of many plant species. Several domains have been recognized in vicilin proteins, and the presence of a cysteine-rich hydrophilic region proximal to the N-terminal signal peptide is a feature of vicilin proteins from several plant species, including peanut, barley, maize, cotton, cocoa and pumpkin. In this paper we have shown that the vicilin protein from macadamia contains an unusually large hydrophilic N-proximal region that is processed to release a family of antimicrobial peptides, at least some of which are released into seed exudates. The proximal N-terminal hydrophilic regions of vicilins from macadamia and some other plant species contain segments with a characteristic four-cysteine motif of C-X-X-X-C-(10–12X)-C-X-X-X-C (Figure 5). The macadamia protein contains four segments each with the four-cysteine motif in the N-proximal hydrophilic region. Three

of these four segments were purified and each purified segment exhibited antimicrobial activity *in vitro*; this suggests that they may have a potential role in defence.

The production of potentially defensive peptides from large precursor proteins in seeds may, in fact, be common to many plants. Processing of a precursor protein from the seeds of *Impatiens balsamina* has been found to be the source of a novel family of antimicrobial peptides (Tailor *et al.*, 1997). A precursor protein from the stigmas of *Nicotiana glauca* is processed into a number of trypsin and chymotrypsin inhibitors that presumably operate to protect reproductive organs from pests and possibly pathogens (Atkinson *et al.*, 1993). Similarly, various partially characterized antimicrobial peptide fragments of vicilin proteins have been reported in cotton seeds (Chung *et al.*, 1997). More recently, a pumpkin vicilin has been shown to contain a hydrophilic N-proximal region which is processed into multiple fragments, some of which contain the C-X-X-X-C motifs (Yamada *et al.*, 1999). One of these fragments was reported to exhibit trypsin inhibition. Another similar peptide from buckwheat has also been shown to inhibit trypsin activity (Park *et al.*, 1997). In contrast, the macadamia peptides did not inhibit trypsin activity (J. Marcus, unpublished results). On the whole, these studies suggest that the production of multiple defensive peptides from larger precursor proteins may be widespread in plant seeds.

The protein-rich macadamia seed exudate had significant antimicrobial activity that may have a role in inhibiting the growth of potential pathogens around the germinating seed. Direct evidence that the two macadamia

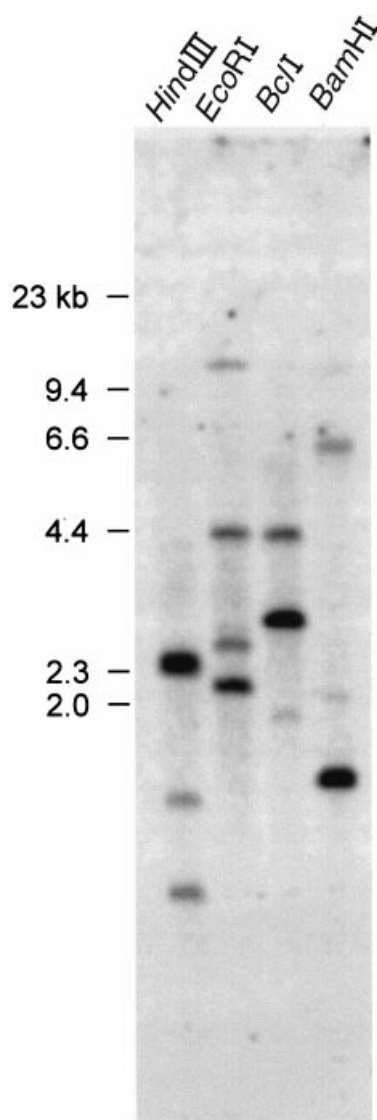


Figure 8. Southern analysis of *Macadamia integrifolia* genomic DNA using a fragment of MiAMP2 cDNA as a hybridisation probe indicates a small gene family encoding MiAMP2.

vicilin segments MiAMP2b and MiAMP2d were released from the germinating seed was obtained herein by their purification from seed exudates. Evidence for the presence of the MiAMP2c segment in seed exudates was also obtained indirectly by Western blotting. Previously, we have demonstrated that an antimicrobial β -barrelin peptide, termed MiAMP1, was present in the kernels of macadamia (Marcus *et al.*, 1997; McManus *et al.*, in press). This 76 amino acid polypeptide is unrelated in sequence to the MiAMP2 peptides, but Western blotting has shown that MiAMP1 is also present in macadamia seed exudates (J. Marcus and J. Anderson, unpublished results). This suggests that the seed may release a diverse array of

antimicrobial peptides into the surrounding medium during seed imbibition and germination. A different type of antimicrobial peptide, the plant defensins, were shown to be released into the surrounding media by germinating radish seeds, suggesting that this may be a general defence mechanism for seeds (Terras *et al.*, 1995). The release of antimicrobial peptides into seed exudates may be of particular importance for macadamia seeds that would naturally germinate in a habitat of moist, microbially rich soils in subtropical and tropical forests.

That various fragments are released during the processing of precursor vicilin proteins is well known. (Chlan *et al.*, 1986; Chung *et al.*, 1997; Gatehouse *et al.*, 1983; Kermode *et al.*, 1995; Scholz *et al.*, 1983; Yavuz *et al.*, 1996). Examination of vicilin fragment sizes and fragment-associated amino acid sequences has allowed the delineation of major processing regions in several vicilin precursor proteins. While the exact sequence requirements for cleavage are not known for certain, it is clear that in each case where the cleavage process has been examined, a stretch of hydrophilic charged residues plays a key role. In particular, glutamic acid residues are abundant in the region of processing sites (Chlan *et al.*, 1986; Gatehouse *et al.*, 1983). These stretches of acidic residues are more marked in some vicilin molecules than in others. Significantly, the examination of alignments (data not shown) shows that many vicilins from a variety of species contain acidic stretches in regions that have been shown to be processing sites for at least one vicilin. In the macadamia protein, these potential cleavage sites match positions 241–246 and 542–546 (Figure 3) which are rich in glutamic acid residues. In addition, the cleavage sites for the purified antimicrobial peptides MiAMP1b–d are also rich in acidic residues. However, cleavage of the antimicrobial peptides appears to be imprecise and longer versions of MiAMP2c were also purified and appeared to represent cleavage events at other sites rich in acidic residues. This imprecision in cleavage produces great diversity in the antimicrobial peptides produced but it is not certain whether this has any functional significance. For pumpkin vicilin, cleavage occurs at N-Q motifs in the hydrophilic portion of the sequence (Yamada *et al.*, 1999). Although the macadamia peptides contain asparagine residues, their connection with glutamine residues is only seen at the C-terminus of MiAMP2a.

In light of the above considerations concerning probable cleavage sites, the mature macadamia vicilin protein can be divided into four major segments (Figures 3 and 4): (i) a 28 aa hydrophobic N-terminal signal sequence; (ii) a hydrophilic domain proximal to the N terminus comprising residues 29 to approximately 240 (which can be subdivided into four cysteine-rich segments); (iii) a middle region comprising residues at approximately 241–546; and (iv) a C-terminal region delineated at residues 547–666. Each of the

latter three segments is separated by a stretch of acidic amino acids. These delineations appear to apply to many other vicilins, but not all, because in some (e.g. pea, Dure (1990), Jack bean, Bassuner *et al.* (1987), spruce, Newton *et al.* (1992) and Zamia, Braun *et al.* (1996)), the N-proximal hydrophilic domain of the vicilin protein appears to be absent. However, because of similarities in protein structure and putative processing sites, it seems likely that many vicilin molecules may resemble the macadamia protein and be multifunctional with possible roles in plant defence in addition to the established roles in nutrient storage.

Experimental procedures

Purification of MiAMP2c from *Macadamia integrifolia*

A crude basic ($pI > 9$) protein preparation was made from 25 kg of macadamia nut kernels as described previously (Marcus *et al.*, 1997). This protein fraction was further purified by cation-exchange chromatography as shown in Figure 1. About 4 g of the basic protein fraction dissolved in 20 mM sodium succinate (pH 4) was applied to an S-Sepharose High Performance column (5×60 cm) (Pharmacia) previously equilibrated with the succinate buffer. The column was eluted at 17 ml min^{-1} with a linear gradient of 20 l from 0 to 2 M NaCl in 20 mM sodium succinate (pH 4). Two hundred ml fractions were collected and portions of each fraction were desalted using C8 solid-phase extraction cartridges. These fractions were subsequently tested for antifungal activity against *Phytophthora cryptogea* at a concentration of $100 \mu\text{g ml}^{-1}$ in the presence (Figure 1a) and absence (Figure 1b) of 1 mM Ca^{2+} . Fractionation yielded a number of unresolved peaks eluting between 0.05 and 2 M NaCl. A peak eluting at about 16 h (fractions 90–92) showed significant antimicrobial activity. Fractions showing significant antimicrobial activity were further purified by reversed-phase chromatography. Aliquots of fractions 90–92 were loaded onto a Pep-S (C2/C18), column (25×0.93 cm, Pharmacia) equilibrated with 95% $\text{H}_2\text{O}/5\%$ MeCN/0.1% TFA (= 100%A). The column was eluted at 3 ml min^{-1} with a 240 ml linear gradient (80 min) from 100%A to 100%B (= 5% $\text{H}_2\text{O}/95\%$ MeCN/0.1% TFA). Individual peaks were collected, vacuum dried and subsequently resuspended in water for use in bioassays. Figure 1(c) shows the HPLC separation of fraction 92 (cation-exchange fraction, Figure 1). Protein elution was monitored at 214 nm. Individual peaks were bioassayed for antimicrobial activity at $15 \mu\text{g ml}^{-1}$ of protein from each of the fractions. The active protein (MiAMP2c) eluted at approximately 27 min (approximately 30% MeCN/0.1% TFA). Peptide purity was confirmed by analytical HPLC and mass spectrometry.

Antimicrobial activity assays

Bioassays to assess antifungal and antibacterial activity were carried in 96-well microtiter plates (Terras *et al.*, 1995). Unless otherwise stated, incubations were carried out at 25°C for 48 h. All fungi including yeast were grown at 25°C . *Escherichia coli* were cultured at 37°C and other bacteria were cultured at 28°C . Typically, measurements were taken at 24 h intervals and the period from 24 to 48 h was used for %Inhibition measurements. Bacterial cultures were monitored every 1–4 h for growth inhibition.

DNA manipulations

Using the reverse-translated nucleotide sequences derived from the MiAMP2c amino acid sequence, degenerate primers were made for use in PCR reactions with genomic DNA from Macadamia. Degeneracy of the primers was reduced taking into account the fact that G can pair with T in addition to C. Primers JPM17 and JPM20 were used in PCR amplifications (the primer JPM17 sequence was 5' CAG CAG CAG TAT GAG CAG TG 3' and the primer JPM20 degenerate sequence was 5' TTT TTC GTA (T/T)C(T/G) (G/T)C(T/G) TTC GCA 3'). Using this approach, it was possible to amplify a 102 bp fragment of DNA. Direct sequencing of this nucleotide fragment yielded the nucleotide sequence corresponding to a portion of the amino acid sequence of the antimicrobial protein MiAMP2c. Total RNA was purified from macadamia nut kernels using a guanidine thiocyanate/cesium chloride protocol (Kingston, 1998). Messenger RNA was then purified from total RNA using a spun column mRNA purification kit (Pharmacia). A cDNA library was constructed using a lambda ZAP cloning kit and screened according to the manufacturer's instructions (Stratagene) using the MiAMP2c PCR fragment as a hybridisation probe. Of 20 positive clones, 15 showed inserts of about 2 kb in size. Of these, three clones were fully sequenced and 12 were partially sequenced. To isolate genomic clones that spanned the coding region, PCR primers JPM42 and JPM43 (respective sequences: 5'-TC-TAG-ATC-TAA-AGT-TGG-AAA-GCT-GTT-CC-3' and 5'-TCT-AGA-TCT-ACA-ACA-ATG-GCT-ATC-AAT-ACA-TC-3') containing *Bgl*III and *Xba*I restriction sites were used to specifically amplify genomic DNA obtained from leaves of *Macadamia integrifolia*. Amplifications yielded a PCR product of 2.4 kb that was cloned and then fully sequenced. Translated amino acid sequences were searched against non-redundant databases using a gapped-BLAST algorithm (Altschul and Gish, 1996). Sequences showing significant similarities were aligned using the ClustalW program (Thompson *et al.*, 1994). Signal peptide cleavage sites were predicted according to the method of Von Heijne (Von Heijne, 1986). Hydrophobicity analysis (Figure 5) was carried out using a window size of 7 amino acids with the criteria of Kyte and Doolittle (1982) with MacVector 6.5 sequence analysis software.

For Southern blot analysis, $20 \mu\text{g}$ quantities of Macadamia DNA were digested with the restriction enzymes *Bam*HI, *Bcl*I, *Eco*RI and *Hind*III, separated on a 0.8% agarose gel and transferred to a nylon membrane. A 399 bp probe corresponding to bases 384–782 was amplified from cDNA clone 1 using primers JPM22 and JPM29 (sequences: 5'-CAG-TAT-GAG-CAG-TGT-CAG-AAG-C-3', 5'-TCG-TTC-GTC-GAA-GTA-GTA-GG-3'). The PCR product was used as a hybridization probe and results detected by a phosphorimager.

Immunodetection of MiAMP2c-like peptides in seeds and seed exudates

Seed exudate was prepared as follows: 50 intact nut kernels from freshly cracked macadamia nuts were surface sterilized with 70% ethanol for 2 min, rinsed in sterile water and individually immersed in 5 ml sterile water. The imbibition fluid was collected after incubating for 24 h at room temperature and replaced with fresh sterile water for 48 h. The pooled imbibition fluid (i.e. the exudate) was filtered, sterilised and stored at -20°C . The exudates and extracts from nut kernels were made to 1.5 M Tris-HCl (pH 8.8), 12% glycerol, 4% SDS and 0.015% Coomassie Blue G, centrifuged at 4000 g for 15 min and the supernatant analysed on Tris-Tricine 10–20% gradient polyacrylamide gels and then

transferred to nitrocellulose filters for Western blot analysis by standard procedures. MiAMP2c antibodies were obtained from rabbits injected with MiAMP2c conjugated with Diphtheria toxoid (Chiron Mimotopes) and suspended in Freund's incomplete adjuvant (Sigma). Typically, a 1/1000 dilution of the antisera was used in primary detection reactions. Reactive proteins were detected using alkaline phosphatase-linked goat anti-rabbit IgG.

Isolation of predicted MiAMP2 fragments from seed exudates

Seed exudate was prepared for cation-exchange chromatography by adding glycine to a final concentration of 50 mM (pH 3.0) and clarifying the solution by centrifugation at 10 000 *g* for 30 min. The clarified exudate was then loaded onto an S-Sepharose Fast Flow column (5 × 60 cm, Pharmacia) previously equilibrated with 50 mM glycine (pH 3). The column was subsequently eluted with an 8 l gradient at 10 ml min⁻¹ from 0 to 2 M NaCl (Figure 7a). Cation-exchange fractions were desalted using 1 ml C8 solid-phase extraction cartridges (Varian) using 50% acetonitrile plus 0.1% TFA to elute bound proteins. Desalted fractions were lyophilized, resuspended in water and then diluted for protein estimation, immunoblotting and bioassay. Both *Leptosphaeria maculans* and *Verticillium dahliae* were used in the bioassay of these cation-exchange fractions with protein concentrations of 25 µg ml⁻¹ in the bioassay. Cation-exchange fractions exhibiting significant antimicrobial activity were further purified by reverse-phase chromatography (C2/C18 PepS column, Pharmacia) to obtain individual peaks which were subsequently tested for antimicrobial activity and sequenced.

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GenBank accession numbers for MiAMP2 cDNAs are AF161883, AF161884 and AF161885.