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A maritime pine antimicrobial peptide involved in ammonium nutrition

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ABSTRACT

A large family of small cysteine-rich antimicrobial peptides (AMPs) is involved in the innate defence of plants against pathogens. Recently, it has been shown that AMPs may also play important roles in plant growth and development. In previous work, we have identified a gene of the AMP β-barrelin family that was differentially regulated in the roots of maritime pine (*Pinus pinaster* Ait.) in response to changes in ammonium nutrition. Here, we present the molecular characterization of two AMP genes, *PpAMP1* and *PpAMP2*, showing different molecular structure and physicochemical properties. *PpAMP1* and *PpAMP2* displayed different expression patterns in maritime pine seedlings and adult trees. Furthermore, our expression analyses indicate that PpAMP1 is the major form of AMP in the tree, and its relative abundance is regulated by ammonium availability. In contrast, *PpAMP2* is expressed at much lower levels and it is not regulated by ammonium. To gain new insights into the function of *PpAMP1*, we overexpressed the recombinant protein in *Escherichia coli* and demonstrated that PpAMP1 strongly inhibited yeast growth, indicating that it exhibits antimicrobial activity. We have also found that PpAMP1 alters ammonium uptake, suggesting that it is involved in the regulation of ammonium ion flux into pine roots.

Key-words: *Pinus*; β-barrelins; ammonium uptake; nitrogen metabolism; plant antimicrobial protein.

INTRODUCTION

Recent advance in whole-genome sequencing indicates the presence in plants of a large number of small secreted cysteine-rich peptides (CRPs) that ressemble antimicrobial peptides (AMPs) (Silverstein *et al*. 2007). Specifically, 825 genes were identified in the *Arabidopsis* genome and 598 in the rice genome. These proteins are characterized by their small size (<100 Aa), a secretion signal peptide in the amino terminal region, charged or polar mature peptides and conservation of the cysteine residues in their sequences. The number and position of cysteine residues are used to classify AMPs (Silverstein *et al*. 2007). For example, peptides with eight cysteines form a family called defensins, the most ancient group of cysteine-rich AMPs. However, plants possess many other types of AMPs that have been classified into different families based on their sequence and tridimensional structure (Hammami *et al*. 2009).

Marcus *et al*. (1997) discovered a novel type of AMP in *Macadamia integrifolia* (MiAMP1) that has a strong antimicrobial activity and was structurally different from any other plant AMP. In particular, MiAMP1 has eight β-strands arranged in two Greek key motif, which are associated to form a Greek key β-barrel (McManus *et al*. 1999). In the last few years, several conifer genes related to defence against pathogenic fungi and structurally similar to MiAMP1 have been discovered (Asiegbu *et al*. 2003; Ekramoddoullah, Liu & Zamani 2006). The expression of these genes is activated during infection by pathogens and their expression products secreted into the apoplast (Manners 2009). A new family within AMP called β-barrelins includes all these novel plant AMPs (McManus *et al*. 1999; Hammami *et al*. 2009).

In our laboratory, we are interested in studying the nitrogen metabolism and its regulation in the conifer maritime pine (*Pinus pinaster*). Because pine trees live in forest ecosystems where ammonium ions are the main source of available inorganic nitrogen, we are particularly interested in the assimilation of this nutrient (Cánovas *et al*. 2007). In a recent paper, we investigated the effects of the ammonium availability on the transcriptome of maritime pine roots using a combination of microarray analysis and suppressive subtraction hybridization (Canales *et al*. 2010). Thereby, a group of defence-related genes was identified, whose expression was up-regulated in response to high levels of ammonium and in coordination with some genes involved in nitrogen metabolism. *PpAMP1*, a member of the plant AMP β-barrelin family, was one of these defence-related genes, and we hypothesized that its expression product, PpAMP1, could have a role in the regulation of ammonium transport into maritime pine roots. Little is known about the signalling and sensing mechanisms that control ammonium acquisition, but it has recently been suggested that there may be an unknown sensor that is able to detect the external ammonium status and down-regulate the activity of ammonium transporters (AMTs) to prevent toxic accumulation of this molecule (Ho & Tsay 2010). Given this

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background, we decided to characterize the AMP family in maritime pine and further study whether these proteins are related to the regulation of ammonium uptake in root cells.

MATERIALS AND METHODS

Plant material

Seeds from maritime pine (*P. pinaster* Ait.) were provided by the 'Centro de Recursos Genéticos Forestales El Serranillo', Ministerio de Medio Ambiente, Medio Rural y Marino, Spain. Seed germination was carried out as described elsewhere (Cañas *et al*. 2006). Thirty days after germination, seedlings were individually transferred to a 0.2 L pot with vermiculite as a substrate. A group of 35 seedlings was then watered once a week with a nutrient solution described by supplemented with 3 mM ammonium provided as 1.5 mM $(NH4)_2SO_4$, and another group was supplemented with 10 mM ammonium provided as 5 mM $(NH_4)_2SO_4$ (Canales *et al*. 2010). The samples from roots, hypocotyls and cotyledons were collected 2 months after germination and were stored at –80 °C until processing.

Samples from 25-year-old maritime pine trees were collected during May 2009 in Sierra Bermeja (Málaga, Spain). Telescoping pruning shears were used to collect following samples from the apex of the tree: bud (AB), female strobili (FE), needles (AN) and stems (AS); and stems (LS) and needles (LN) from the branches. Samples from 12 different individuals were frozen in liquid nitrogen, transported on dry ice and then stored in a freezer at –80 °C.

RNA isolation, rapid amplification of the cDNA ends (RACE) and cDNA cloning

All samples were ground in liquid nitrogen, and then 100 mg from each sample was extracted with a 650 μL of a CTAB extraction buffer in a 1.5 mL tube [3% (w/v) CTAB, 100 mM Tris pH 8.0, 2 M NaCl, 2% (w/v) PVP40, 30 mM ethylenediaminetetraacetic acid (EDTA)] according to the protocol by Liao *et al*. (2004). Total RNA was quantified using a NanoDrop spectrophotometer, and RNA quality was checked by agarose gel electrophoresis. Residual genomic DNA was removed from RNA samples by a treatment with RQ1 RNase-Free DNase (Promega Biotech Ibérica, Madrid, Spain) following the manufacturer's instructions.

To clone the full-length cDNA of *PpAMP1* and *PpAMP2*, a 5′ RACE PCR was performed using the FirstChoice RLM-RACE Kit (Ambion, Madrid, Spain) with the oligonucleotides: AMP15′Outer 5′-AGTTGGCGGTG TTGTAAGCA-3′ and AMP15′Inner 5′-CGCAGTGAA CATAACTGCCCT-3′ for *PpAMP1* and AMP25′Outer 5′-AGCTCACAACAATAGGCAG-3′ and AMP25′Inner 5′-GATGCACATATCATCACACGG-3′ for *PpAMP2*. The AMP15′ oligonucleotides were designed from a maritime pine EST (accession number GW575412), and AMP25′ oligonucleotides were designed from a *Picea sitchensis* full-length cDNA (accession number EF676834). The PCRs were carried out with the high-fidelity enzyme AccuSure DNA polymerase (Bioline, London, UK) in an MJ Mini Personal Thermal Cycler (Bio-Rad, Madrid, Spain) with the following program: one cycle of 95 °C for 5 min and 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min. The reaction volume was 25 μL, and each oligonucleotide was present at 0.5 μM. The blunt-end PCR products were cloned into pGEM3Zf (+) (Promega) using T4 DNA ligase (New England Biolabs, Ipswich, MA, USA) and sequenced on a CEQ 8000 Genetic Analysis System (Beckman Coulter, Madrid, Spain) to obtain the 5′ UTR of the *PpAMP1* and *PpAMP2* genes. From the 5′ UTR, the AMP1C 5′CACCTTCCTCATCCGAATTCC-3′ and AMP2C 5′-AGAGGCTTTATCCCGTGAACGC-3′ oligonucleotides were designed to clone the *PpAMP1* and *PpAMP2* full-length sequences, respectively, using the 3′ UTR protocol of the FirstChoice RLM-RACE Kit (Ambion).

Sequence and structural analyses

Amino acid sequences homologous to maritime pine AMP were identified in GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) and Phytozome (http://www.phytozome.net/) databases; these were aligned using TCoffee software (Notredame, Higgins & Heringa 2000). Jalview 2 software (Waterhouse *et al*. 2009) was used to edit the alignment. The presence of a signal peptide and the theoretical cleavage site were determined with Signal IP 3.0 software (Emanuelsson *et al*. 2007). Seaview 4.0 software (Gouy, Guindon & Gascuel 2010) was used to construct a maximum-likelihood phylogenetic tree. The molecular weight and theoretical pI were calculated with ProtParam (http://www.expasy.ch/tools/protparam.html).

The protein structure homology-modelling server Phyre (Kelley & Sternberg 2009) was used to generate the structural model of PpAMP1 and PpAMP2 using as an MiAMP1 model as a template (McManus *et al*. 1999). From these models, the surface electrostatic potentials were calculated using the PBEQ-Solver software (Jo *et al*. 2008).

Real-time quantitative PCR (qPCR)

cDNA synthesis was performed with the PrimeScript RT Reagent Kit (Takara, Saint-Germain-en-Laye, France) using total RNA as template (500 ng) in the presence of oligo(dT). Real-time PCR was carried out on an Mx3000P system (Stratagene, Madrid, Spain) with SYBR Premix Ex Taq (Takara) under the following conditions: 95 °C for 30 s (one cycle); 95 °C for 5 s, 55 °C for 10 s and 72 °C for 15 s (40 cycles). Relative mRNA levels were determined and normalized using a geometric mean of three reference genes (ACT, 40 SRP and EF1) according to the method described by Canales *et al*. (2010).

Protein analysis

Total protein was extracted from 1 g of ground maritime pine samples in 0.5 mL of extraction buffer [5% (w/v)

sodium dodecyl sulphate (SDS), 50 mM Tris pH 8.0, 2 mM EDTA, 10% (v/v) glycerol and 4% (v/v) β-mercaptoethanol]. Because of the presence of high concentrations of SDS and β-mercaptoethanol in the extraction buffer, protein quantification was performed using the method described by Ekramoddoullah & Davidson (1995). Total proteins were separated on a 10% (w/v) Tris–tricine gel following the protocol described by Schägger (2006). Western blots were conducted as described by Gallagher *et al*. (2008) using a Trans-Blot SD Semi-Dry transfer cell (Bio-Rad) for electrotransfer of the proteins to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). A rabbit-generated antibody against *Pinus monticola* AMP1 was used as the primary antibody (Ekramoddoullah *et al*. 2006). Immobilized immunocomplexes were probed by a peroxidase-linked goat anti-rabbit IgG antibody (Vector Labs, Burlingame CA, USA), and then visualised with 4-chloro-1-naphthol (Sigma-Aldrich Quimica S.A., Madrid, Spain) as a chromogenic substrate.

Recombinant protein expression, purification and assay of antimicrobial activity

The PpAMP1 cDNA coding region without the signal peptide was cloned into the expression vector pDEST17 (Invitrogen S.A., Barcelona, Spain) for production of His-tag fusion peptides in *Escherichia coli* BL21-AI cells. An Erlenmeyer flask containing LB and ampicillin was inoculated with an *E. coli* overnight culture, and then cells were grown at 37 °C until the culture reached an absorbance of 0.4 at 600 nm. Expression of the peptide was induced with 0.2% (w/v) L-arabinose (Sigma) at 25 °C for 6 h. Most of the recombinant peptide was recovered in the pellet resulting from centrifugation of the whole-cell extract, suggesting that it predominantly accumulated in bacterial inclusion bodies. The pellet was solubilized with 8 M urea (Panreac Química S.A.U., Barcelona, Spain), and recombinant PpAMP1 was subsequently purified by immobilized metal ion affinity chromatography with Protino Ni-IDA2000-packed columns (Macherey-Nagel, Düren, Germany). Then, 6–7 mg of recombinant PpAMP1 was routinely obtained from 1 L of culture after 6 h of induction. A rapid 100-fold dilution with 20 mM MES (Sigma), pH 6.0, was then performed to refold the denatured peptide. Finally, the PpAMP1 preparation was concentrated by ultrafiltration using Vivaspin 20 centrifugal tubes (Sartorius, Madrid, Spain), and dilution/concentration was repeated to desalt the samples thoroughly. The concentration of purified PpAMP1 was determined by the Bradford's (1976) procedure.

The antimicrobial activity assay was performed as described by Marcus *et al*. (1997), growing *Saccharomyces cerevisiae* in the presence of increasing concentrations of purified recombinant PpAMP1. The percentage of yeast growth inhibition was calculated as: $[(Abs_{600}\text{control} - Abs_{600}\text{sample})/Abs_{600}\text{control}] \times 100$.

Nitrogen isotopic analysis

Roots from 1-month-old maritime pine seedlings were carefully excised in several sections of equal size. Root sections were incubated for 1 h at room temperature in a solution containing 20 mM MES, pH 6.0, and 500 μM ammonium-15N chloride (Sigma) in the absence (control samples) or in the presence (test samples) of different concentrations of recombinant PpAMP1. This concentration of ammonium-15N was selected to check whether PpAMP1 could affect the high-affinity transport system previously described in conifers (Kronzucker, Siddiqi & Glass 1996). Thirty seedlings were used for control samples and 30 for test samples in each experiment. Root samples were oven-dried at 70 °C for 48 h, and then homogenized with a mortar and pestle. $\delta^{15}N$ (‰) values were determined in triplicate at the Universidad Málaga Research Facilities (Unit of Atomic Spectrometry) using a Flash EA 1112 elemental analyser coupled to a Delta V Advantage isotope ratio mass spectrometer (Thermo Scientific, Waltham, MA, USA).

Ammonium influx was determined in pine roots as described by Ohlünd & Näsholm (2004). Atom % excess of 15N in seedlings was calculated by subtracting the mean 15N abundances of unlabelled roots from the atom % of each labelled sample. The mole excess of the isotopes was calculated by multiplying excess atom % N with the molar content of N of the root.

RESULTS

Molecular cloning and sequence analysis of two maritime pine cDNAs for AMPs

We performed a search in the GenBank nucleotide database and identified two different genes for AMPs of the β-barrelin family in the collection of FLcDNAs of Sitka spruce (*P. sitchensis*) (Ralph *et al*. 2008). Based on these data, we designed specific primers and obtained two full-length cDNA clones for maritime pine AMPs: AMP 1 (*PpAMP1*, accession number HM210085) and AMP 2 (*PpAMP2*, accession number HM210086). The *PpAMP1* sequence contained a 5′ untranslated region of 35 bp, an open reading frame of 318 bp and a 3′ non-coding region of 253 bp. The *PpAMP2* sequence contained a 5′ untranslated region of 107 bp, an open reading frame of 318 bp and a 3′ non-coding region of 395 bp.

Once the complete cDNAs were obtained, we conducted a detailed search of all β-barrelin AMP sequences available in the databases, including completely sequenced plant genomes. Table 1 contains a list of representative sequences for the β-barrelin AMP family. The amino acid sequences of *PpAMP1* and *PpAMP2* were compared to the primary structure of AMP polypeptides listed in Table 1. As shown in Fig. 1a, there is a considerable difference in the physicochemical characteristics of the amino acid residues of PpAMP1 and PpAMP2. The PpAMP2 sequence presents four negatively charged residues at the positions 7, 28, 49 and 66, while PpAMP1 has no charged residues at these positions. Furthermore, two positively charged amino acids

Table 1. List of representative sequences of the β-barrelin antimicrobial peptide (AMP) family in plants

Gene	Organism	Accession number	Net charge	Isoelectric point
CLASS 1				
ZaAMP1	*Zantedeschia aethiopica*	AJ703786	5	9.2
MiAMP1	*Macadamia integrifolia*	CAA71842	5	9.1
AtrAMP1	*Amborella trichopoda*	CK752394	5	9.1
PsoAMP1	*Papaver somniferum*	FE967323	4	8.9
PpAMP1	*Pinus pinaster*	HM210085	3	8.6
PtAMP1	*Pinus taeda*	CV034110	3	8.6
PsiAMP1	*Picea sitchensis*	ACN40103	3	8.6
PgAMP1	*Picea glauca*	BT101327	3	8.6
IpAMP1	*Illicium parviflorum*	CV190632	2	8.3
CLASS 2				
PgAMP2	*Picea glauca*	CO237362	0	6.7
SbAMP	*Sorghum bicolor*	XP_002446989	–2	6.1
ZmAMP	*Zea mays*	AW257904	–3	6.0
PsiAMP2	*Picea sitchensis*	ABR16715	–2	5.4
PtAMP2	*Pinus taeda*	CO163315	–2	5.0
PpAMP2	*Pinus pinaster*	HM210086	–2	5.0

in the PpAMP1 sequence (His-14 and Lys-20) are replaced with negatively charged amino acids in the PpAMP2 (Glu-14 and Glu-20). The multiple alignment also shows a high level of sequence conservation among β-barrelin family members, highlighting cysteine residues that are conserved in all sequences.

To investigate the evolutionary relationships among different β-barrelin AMP family members, a phylogenetic tree was constructed with AMP sequences (Fig. 1b). The phylogenetic analysis with maximum-likelihood criterion revealed the existence of three major clusters, including a group of conifer sequences, the AMPs of early angiosperms and the monocot AMP polypeptides. Interestingly, AMP1 and AMP2 formed clearly differentiated branches in the conifer cluster, suggesting that they have different biological functions. Figure 1b also shows that conifer sequences are closely related to early angiosperm sequences. The absence of β-barrelin AMP homologs in the sequenced plant genomes of *Populus* and *Arabidopsis* is notable.

Molecular and structural analyses of PpAMP1 and PpAMP2

The open reading frames of *PpAMP1* and *PpAMP2* cDNAs encode polypeptides of identical size (105 residues) with putative secretory signal peptides of 26 and 27 amino acids, respectively. The predicted molecular mass of the two mature peptides is approximately 8.5 kDa. The theoretical isoelectric point (pI) of PpAMP1 is 8.6 and that of PpAMP2 is 5.0, so the net charge of the two polypeptides at pH 7.0 would be positive for PpAMP1 and negative for PpAMP2. This difference is also present in other members of the β-barrelin AMP family, as shown in Table 1, such that two classes of AMPs can be distinguished: class 1 AMPs with a pI >8 and class 2 AMPs with a pI <7.

The significant difference in the pI of both classes of AMPs led us to conduct an homology modelling of their three-dimensional structures, from which we calculated the surface electrostatic potential. As previously described, MiAMP1 is an amphipathic molecule in which most of the positive charges are concentrated on one side of the peptide (McManus *et al*. 1999). The result of the three-dimensional modelling indicates that PpAMP1 also has most of its positive charges concentrated on one side of the peptide and that it is structurally similar to MiAMP1 (Fig. 2). Interestingly, on the front side of MiAMP1, there is a small region with negative charge (Glu-16) surrounded by a large positively charged region. However, in PpAMP1, this negative zone does not exist because Glu-16 is replaced by a neutral Ala residue.

As shown in Fig. 2, the electrostatic potential of PpAMP2 is completely different from those of MiAMP1 and PpAMP1; most of the charges are negative rather than positive, and their distribution along the peptide surface is more homogeneous.

Expression profiles of *PpAMP1* and *PpAMP2* genes

To explore the expression patterns of *PpAMP1* and *PpAMP2* during tree development, total RNA was isolated from different organs of 2-month-old seedlings and 25-year-old trees to measure the mRNA levels of these two genes by qPCR. *PpAMP1* expression was much higher than *PpAMP2* expression in all samples examined, including seedlings and adult trees (Fig. 3a). For example, the levels of *PpAMP1* transcripts were about 100 times higher that those of *PpAMP2* in the cotyledons of the seedlings, and about 15 times higher in the apical buds of adult trees. These findings indicate that *PpAMP1* is the predominant AMP gene of the β-barrelin family that is expressed in maritime pine and suggest that it should have a more significant role than *PpAMP2*. Figure 3a also shows that *PpAMP1* transcripts were much more abundant in seedlings than in trees,

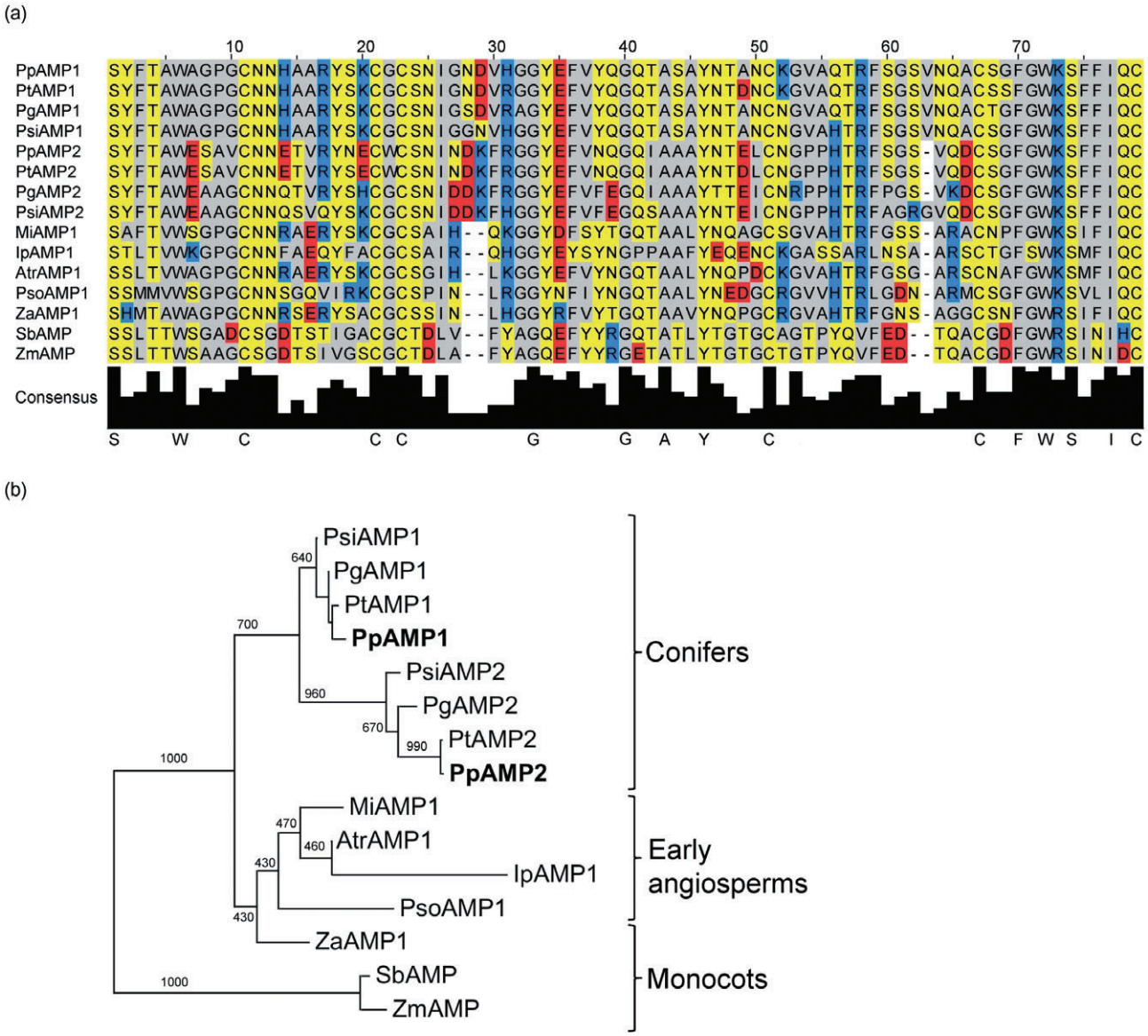

Figure 1. Sequence analysis of maritime pine *PpAMP1* and *PpAMP2* genes. (a) Alignment of the deduced amino acid sequences of the *β*-barrelin plant antimicrobial peptides (AMPs). The abbreviations for species and the gene accession numbers are given in Table 1. Multiple alignment of the sequences was performed with TCoffee software. The amino acids are coloured according to their physicochemical properties: hydrophilic in yellow, hydrophobic in grey, positively charged in red and negatively charged in blue. (b) Unrooted phylogenetic tree of the *β*-barrelin plant AMPs. The dendrogram was generated using Seaview 4 software by maximum-likelihood method. The numbers on the main branches indicate bootstrap values of 1000 replicates.

suggesting that *PpAMP1* expression is developmentally regulated. In contrast, *PpAMP2* transcript levels were quite similar in all samples tested.

To further examine the expression of AMP genes in maritime pine, the relative abundance of AMP protein was determined by Western blot analysis in the same samples for which *PpAMP1* and *PpAMP2* expression levels were determined by qPCR (Fig. 3b). The presence of AMP protein in maritime pine protein extracts was shown using an antibody raised against an AMP of *P. monticola* (Ekramoddoullah *et al*. 2006), the amino acid sequence of which was 89% identical to PpAMP1. A major protein band of about 11 kDa in size was identified in protein extracts from maritime pine seedlings, and its relative abundance was particularly high in hypocotyls and cotyledons of the seedlings. Figure 3b also shows that a weak signal for a protein of the same size was detected in extracts from the apical bud and female strobili of adult trees. These data for AMP protein accumulation in maritime pine correspond well to the observed expression pattern of *PpAMP1* mRNA, the predominant AMP gene expressed in the tree.

As the maximal levels of AMP expression were observed in the seedlings, we were particularly interested to know in which region of each organ of the seedling *PpAMP1* and

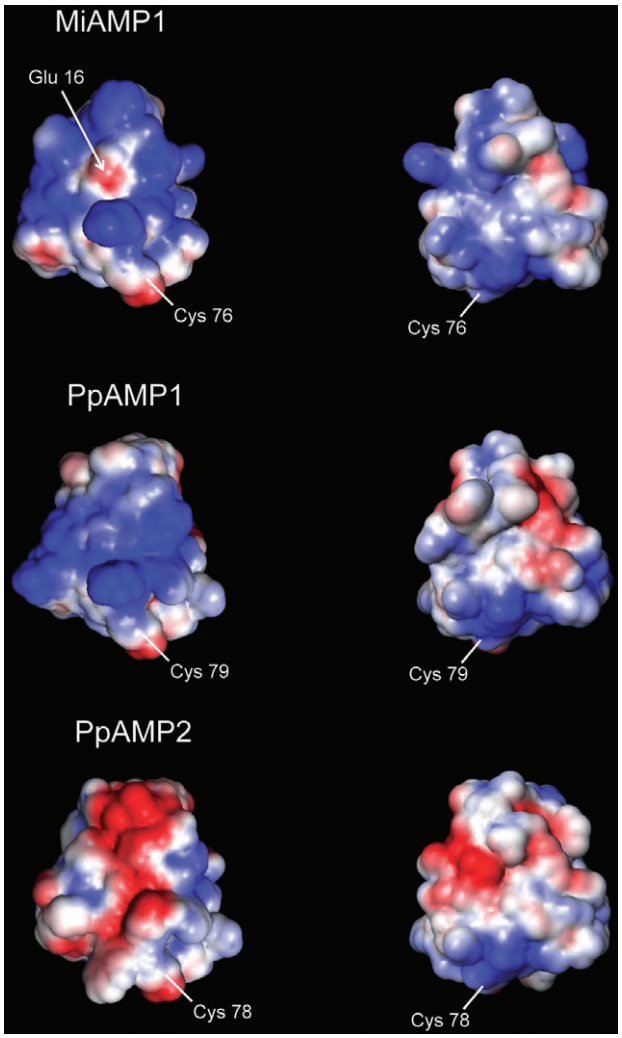

Figure 2. Three-dimensional structural models of MiAMP1, PpAMP1 and PpAMP2 peptides with the surface electrostatic potentials [+1 kcal/(mol.*e*) in blue to −1 kcal/(mol.*e*) in red]. Frontal and opposite views of each peptide are shown. Glu-16 in MiAMP1 and conserved Cys residues in the three peptides are marked.

PpAMP2 genes are expressed. To carry out this study, we dissected the three principal organs of seedlings in several fragments (Fig. 4a), and then extracted total RNA. The qPCR analysis (Fig. 4b) indicated that the expression of *PpAMP1* was located mostly in the root tip (R3), the hypocotyl apical region (H2) and the cotyledons (C). In contrast, *PpAMP2* expression was homogeneous in all regions of the root analysed, and their mRNAs were mainly located in needles.

Regulation of AMP expression by ammonium availability

In a previous study, we have reported that pine seedlings grown with excess ammonium had higher levels of *PpAMP1* mRNA in the roots and suggested that PpAMP1 may have a role in the response to increased ammonium availability (Canales *et al*. 2010). We were interested in determining whether *PpAMP2* expression is also regulated by ammonium availability. Increases in the ammonium supply induced the accumulation of *PpAMP1* transcripts in maritime pine roots, but did not affect the levels of *PpAMP2* transcripts (Fig. 5). To further characterize the regulation of PpAMP1, we next examined whether the accumulation of *PpAMP1* transcripts in response to the ammonium supply was uniform or localized in specific areas of the seedling. *PpAMP1* expression levels were determined in different sections of pine seedlings that had been treated with excess (10 mM) or sufficient (3 mM) ammonium. Figure 6 shows that *PpAMP1* expression was significantly induced by ammonium only in the root tip and the lateral roots.

Functional studies of PpAMP1

To gain further insights into the function of PpAMP1 in maritime pine, our next experiments were designed to answer two questions: (1) Does PpAMP1 have antimicrobial activity? and (2) Does PpAMP1 have a role in the ammonium uptake of maritime pine roots? The first necessary step was to produce enough amount of PpAMP1 for functional studies by recombinant expression in *E. coli* (Fig. 7a). The availability of recombinant protein allowed us to assay the antimicrobial activity of PpAMP1. Marcus *et al*. (1997) showed that *S. cerevisiae* is one of the microorganisms most sensitive to MiAMP1 AMP, which is homologous to PpAMP1. Therefore, we decided to test the antimicrobial activity of PpAMP1 recombinant peptide against *S. cerevisiae* cells; the results are presented in Fig. 7b. PpAMP1 exhibited a strong antimicrobial activity: a concentration of 10 μg mL^{-1} of the peptide was sufficient to inhibit 50% of yeast growth, and 40 μg mL^{-1} completely inhibited yeast growth.

We next tested whether the recombinant exogenous PpAMP1 was able to alter the incorporation of ammonium into maritime pine roots. To perform this test, the roots of the seedlings were cut into small pieces (about 2 mm in length), and then incubated for 1 h in a solution containing ammonium ^{15}N and increasing concentrations of PpAMP1. Nitrogen uptake rate was 4.4 ± 0.6 μmol g^{-1} of dry weight h^{-1}, which is in the range of the previously described values in pine roots (Ohlünd & Näsholm 2001, 2004). The δ^{15}N values of the ground roots were determined with an isotope ratio mass spectrometer, and the results are presented in Fig. 8. The samples treated with 10 or more micrograms per millilitre PpAMP1 had significantly lower δ^{15}N (around 25%) than control samples, while below this threshold no significant changes in the ammonium incorporation were observed. These results indicate that PpAMP1 inhibits ammonium uptake and that a peptide concentration of 10 μg mL^{-1} is necessary to carry out this function. Higher amounts of PpAMP1 did not increase the observed inhibitory effect.

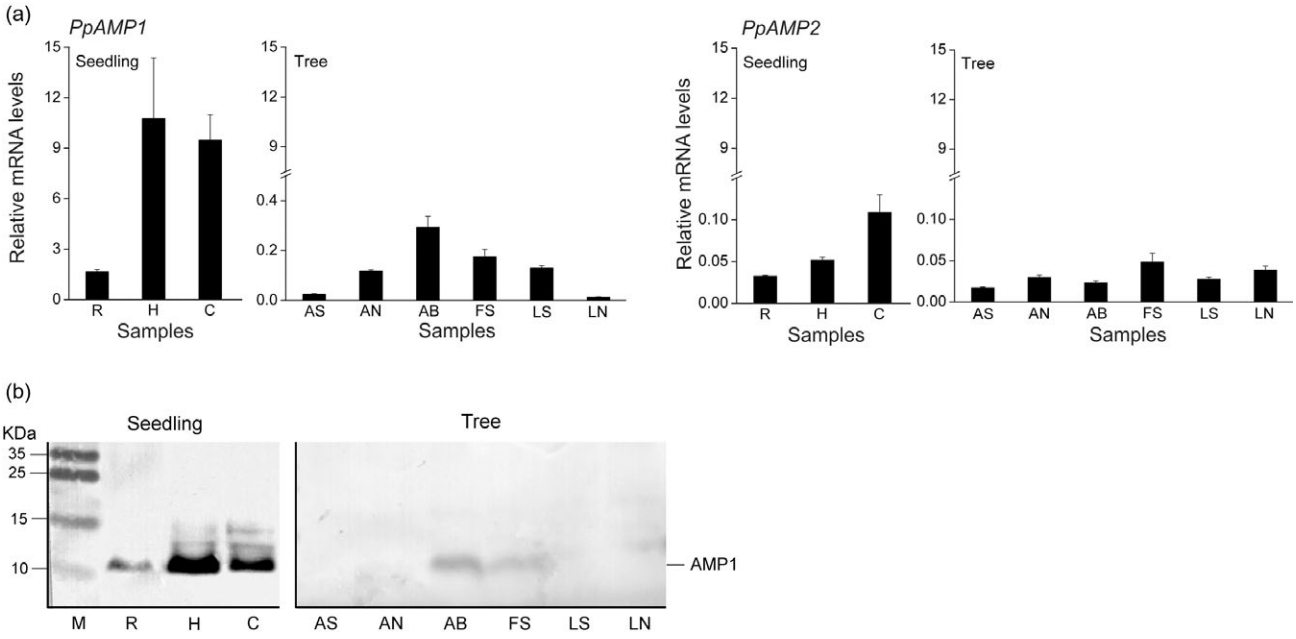

Figure 3. Expression profiles of *PpAMP1* and *PpAMP2* genes in maritime pine. Total RNA and proteins were extracted from different samples harvested from 2-month-old seedlings and 25-year-old trees. Roots (R), hypocotyls (H), cotyledons (C), apical shoots (AP), apical needles (AN), apical buds (AB), lateral shoots (LS) and lateral needles (LN). (a) Transcript levels of *PpAMP1* and *PpAMP2* genes determined by real-time quantitative PCR (qPCR) analysis as described in Materials and methods. (b) Western blot analysis of antimicrobial peptides (AMPs). Twenty micrograms of proteins per lane was separated by Tris–tricine sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), and then transferred to a nitrocellulose membrane, where the proteins were probed using a specific antibody developed against Western white pine AMP1 (Ekramoddoullah *et al.* 2006). Immunocomplexes were probed by peroxidase goat anti-rabbit IgG antibody, and then visualized with 4-chloro-1-naphthol as chromogenic enzyme substrate.

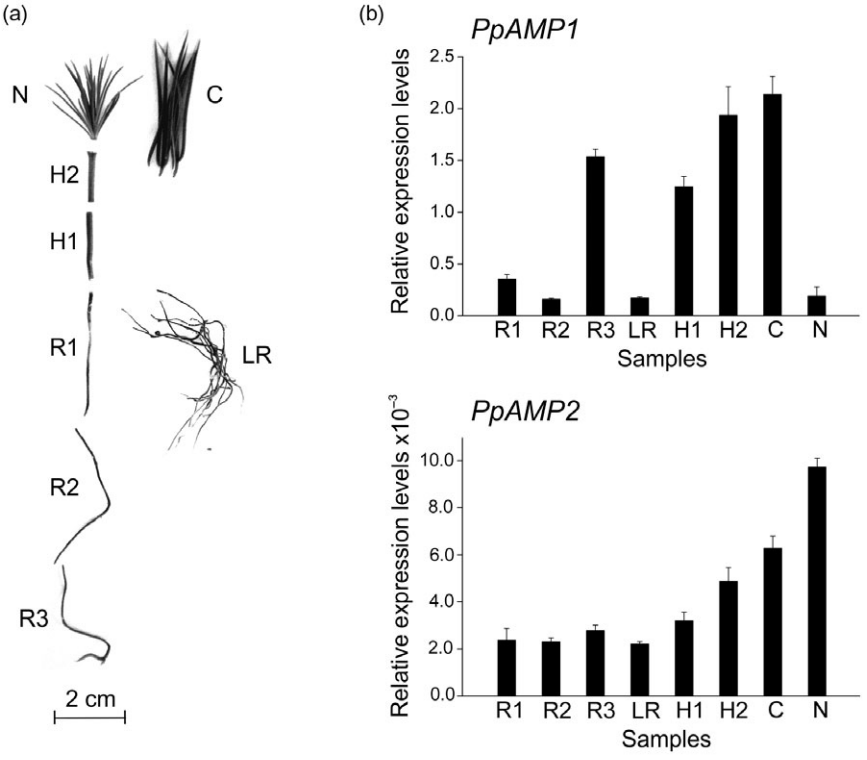

Figure 4. Specific distribution of *PpAMP1* and *PpAMP2* gene expression in maritime pine seedlings. (a) Two-month-old seedlings were dissected into eight sections: basal root (R1), central root (R2), apical root (R3), lateral roots (LR), basal hypocotyl (H1), apical hypocotyl (H2), cotyledons (C) and needles (N). (b) Transcript levels of *PpAMP1* and *PpAMP2* genes were determined by quantitative PCR (qPCR) analysis in the sections.

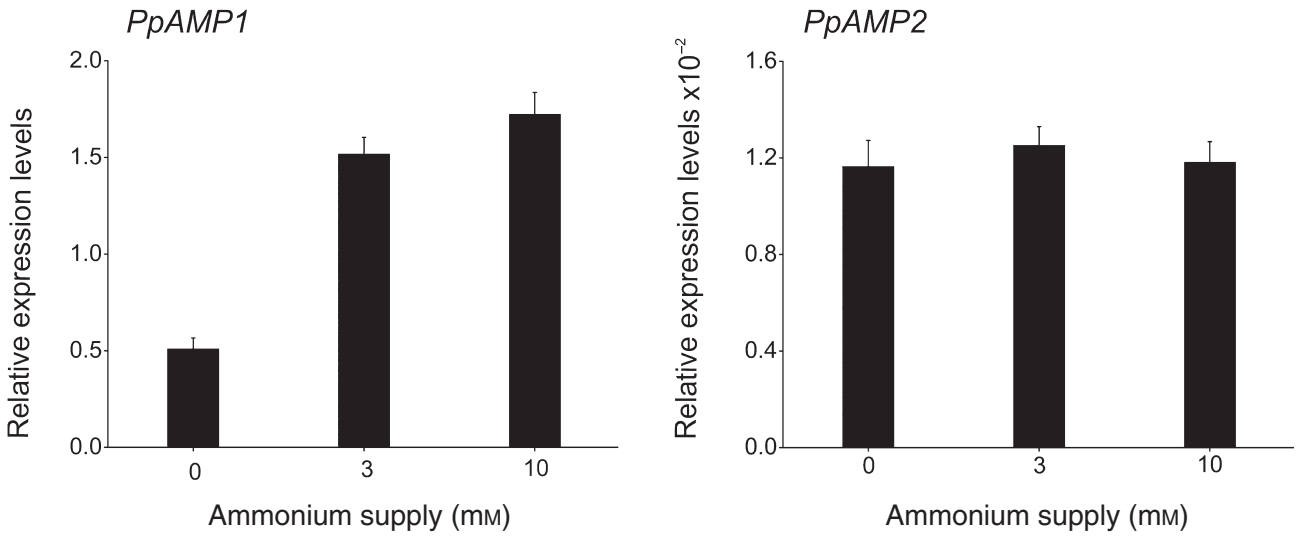

Figure 5. Effect of ammonium supply on the expression levels of *PpAMP1* and *PpAMP2* genes. Transcript levels in maritime pine roots were determined by quantitative PCR (qPCR) analysis as described in Materials and methods.

DISCUSSION

Analysis of the amino acid sequence presented in Fig. 1a shows that PpAMP1 belongs to the *β*-barrelin family of plant AMPs that is structurally characterized by the presence of eight *β*-strands arranged in two Greek key motifs (McManus *et al*. 1999). All *β*-barrelins described to date, as well as many other AMPs, are highly basic peptides with positive net charges that are necessary for their antimicrobial activity (Marcus *et al*. 1997). Previous studies have shown that cations, especially divalent cations such as Ca^{+2} and Mg^{+2}, greatly decrease the antimicrobial activities of such peptides, because their positive charges compete with AMPs for negatively charged surfaces of microorganisms (Carvalho Ade & Gomes 2009). Therefore, it was proposed that the positively charged faces of AMPs are important for electrostatic interactions with the opposites charges of the target microorganism surface (Marcus *et al*. 1997). However, in this study, we have identified a new member of the *β*-barrelin family, named PpAMP2, which presents a theoretically acidic pI and negative net charge (Table 1). This subfamily of *β*-barrelins in plants is represented by peptides from the conifers *P. pinaster* (PpAMP2), *Pinus taeda* (PtAMP2), *P. sitchensis* (PsiAMP2) and *Picea glauca* (PgAMP2), but also includes similar peptides identified in the monocots *Zea mays* (ZmAMP) and *Sorghum bicolor* (SbAMP). Therefore, according to these findings, we can distinguish two structural groups in the *β*-barrelin AMP family: class 1, characterized by a net positive charge and a pI >7.0, and class 2, characterized by a net negative charge and a pI <7.0. Class 1 and 2 AMP sequences in conifers are phylogenetically related to AMP sequences in early angiosperms, while the monocot AMP sequences clearly form a distinct cluster. The phylogenetic relationships among members of this AMP family are consistent with current models of plant phylogeny (Manners 2009). Members of the *β*-barrelin family of AMPs are present exclusively in angiosperms that diverged before the appearance of the subclade Pentapetalae (also named core eudicots). It is estimated that the major lineages of Pentapetalae evolved rapidly (in about 5 million years) and currently represent about 70% of angiosperms (Moore *et al*. 2010). Because this group of plants evolved and diverged rapidly (Soltis *et al*. 2008), it is possible that the small family of *β*-barrelins was displaced by larger families,

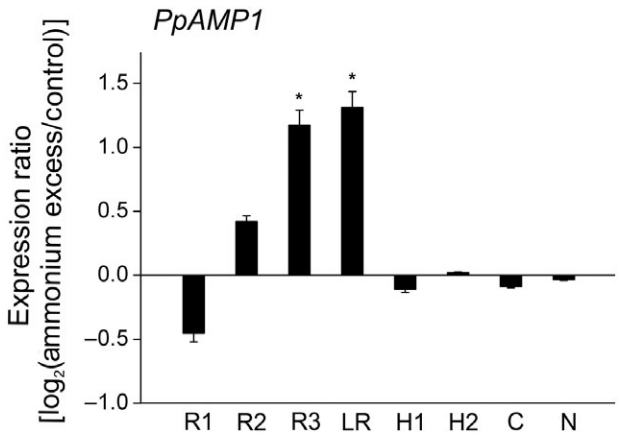

Figure 6. Gene expression analysis of *PpAMP1* following ammonium treatment. Maritime pine seedlings were grown in the presence of excess (10 mM) or sufficient (3 mM) ammonium for 30 d. Total RNA was isolated from the same eight sections described in Fig. 4: basal root (R1), central root (R2), apical root (R3), lateral roots (LR), basal hypocotyl (H1), apical hypocotyl (H2), cotyledons (C) and needles (N). The values represent the $\log_2$ of the ratios between the relative expression levels in ammonium excess and control samples. Significant changes are indicated with an asterisk. A *t*-test ($P < 0.05$) and changes of more than twofold in the expression ratio (represented by a $\log_2$ ratio of 1.0 or –1.0) were considered significant.

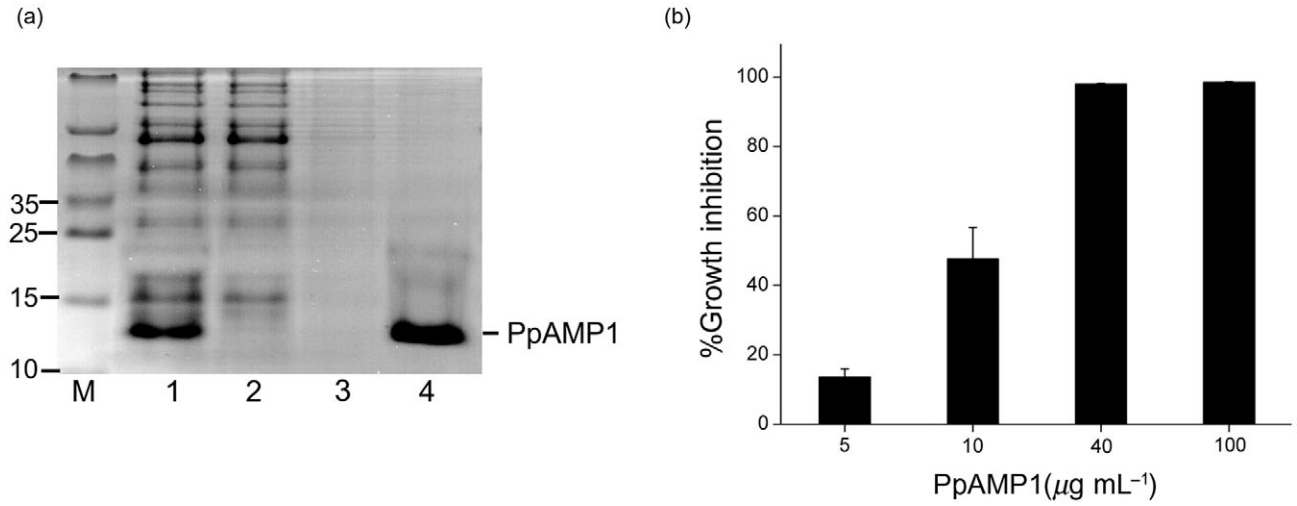

Figure 7. Purification and antimicrobial activity test of recombinant PpAMP1. (a) Purification process of the recombinant peptide analysed by tricine sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). PpAMP1 was expressed in *Escherichia coli* BL21-AI cells and insoluble proteins were loaded onto IMAC columns to purify recombinant peptides. M: protein molecular weight marker kDa; 1: insoluble proteins; 2: flow-through; 3: column washes; 4: elution. (b) Antimicrobial activity of PpAMP1against *Saccharomyces cerevisiae*, expressed as % of growth inhibition. Yeast growth inhibition was determined by measuring the absorbance at 600 nm after 24 h of growth in the presence of increasing concentrations of recombinant PpAMP.

such as defensins, or that their sequences diverged considerably (Manners 2009).

Our structural and phylogenetic data strongly suggest that AMP1 and AMP2 should have different functions in plants. The expression patterns of *PpAMP1* and *PpAMP2* (Figs 3 & 4) further support this hypothesis. *PpAMP1* expression was considerably higher in seedlings than in mature trees, and it was particularly localized in the apexes of roots and hypocotyls. In contrast, *PpAMP2* expression levels were similar in seedlings and trees, and in all samples tested. The accumulation of the PpAMP1 peptide, as determined by Western blot analysis, was consistent with the observed levels of *PpAMP1* mRNA, suggesting that the regulation of *PpAMP1* expression occurs at the transcriptional level. The apparent size of PpAMP1 observed in the maritime pine extracts was about 11 kDa, similar to the value reported for AMP1 in Western white pine (Davidson & Ekramoddoullah 1997). As the size predicted from the cDNA sequence is 8.5 kDa, the above data suggest that pine AMP1 may undergo post-translational modification.

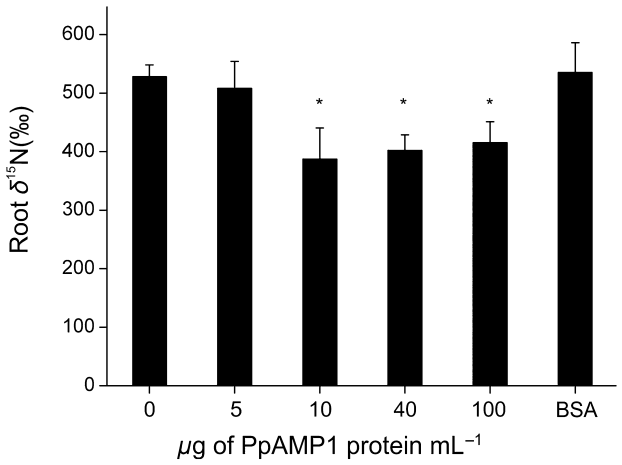

Figure 8. Effect of recombinant PpAMP1 on the ammonium uptake of maritime pine roots. The roots of 30 seedlings were sliced in small pieces and incubated for 1 h in a solution with ammonium-^{15}N (500 μM) and increasing concentrations of recombinant PpAMP1 (0, 10, 40 and 100 μg mL^{-1}) and bovine serum albumin (BSA, 100 μg mL^{-1}). The experiment was also performed with a boiled preparation of PpAMP1 (100 μg mL^{-1}) and no significant changes were observed (data not shown). Bars represent SEs of three independent experiments. Student's *t*-test was performed to test the significance of differences in ^{15}N content between peptide-treated and control samples ($P < 0.05$). Significant changes are indicated with an asterisk.

In this work, we also studied the regulation of *PpAMP1* and *PpAMP2* gene expression by ammonium availability. *PpAMP1* transcript levels increased with ammonium concentration, while *PpAMP2* expression remained unchanged (Fig. 5). Therefore, it is difficult to hypothesize what the function of *PpAMP2* could be, because it seems to be constitutively expressed and it does not have the typical physicochemical characteristics of the AMPs, as discussed earlier. In contrast, a possible role of AMP1 gene in the defence of conifers against fungal pathogens is well documented. For instance, AMP1 expression is activated during infection by the pathogenic fungus *Heterobasidion annosum* (Asiegbu *et al*. 2003), and recombinant Western white pine AMP1 inhibits the growth of the phytopathogenic fungus *Cronartium ribicola* (Ekramoddoullah 2005). Furthermore, in the present work, we have shown that recombinant maritime pine AMP1 strongly inhibits yeast growth (Fig. 7). The relative abundance of *PpAMP1* mRNAs at the apexes of

seedling roots and shoots, and in the apical buds of adult trees suggests that *PpAMP1* expression is correlated with development and/or growth of roots and stems. Although antimicrobial proteins in angiosperms were initially described as defence proteins, there are recent reports indicating that they are also involved in plant growth and development. Thus, three different defensins inhibit the growth of *Arabidopsis* roots (Allen *et al*. 2008), while the DEF2 peptide in tomato plants reduces pollen viability, seed production and growth (Stotz, Spence & Wang 2009).

The results presented here indicate a direct relationship between this gene and ammonium uptake. A recent study showed that ammonium uptake by conifer roots can vary significantly across different regions of the root and demonstrated that the greatest influx of ammonium occurs near the root tip (Hawkins, Boukcim & Plassard 2008). Because *PpAMP1* expression was mainly localized in the apex of the root and *PpAMP1* transcripts levels specifically increased in this region after ammonium treatment (Figs 4 & 6), a relationship between ammonium influx and the expression of *PpAMP1* could be established. Furthermore, we have shown that recombinant PpAMP1 alters the ammonium uptake of maritime pine roots (Fig. 8), which reinforces this hypothesis. The molecular mechanisms involved in the effect mediated by PpAMP1 remain unknown and require further research efforts. However, given that the specific increase in *PpAMP1* transcripts occurs in the same regions where some AMTs such as OsAMT1;2 (Sonoda *et al*. 2003) or AtAMT1;5 (Yuan *et al*. 2007) are localized, it is physically possible that the PpAMP1 peptide could regulate ammonium transport. AMP1 has been immunolocalized in the apoplast of the root cells of the conifers *Pseudotsuga menziesii* (Sturrock, Islam & Ekramoddoullah 2007) and *Pinus sylvestris* (Adomas *et al*. 2007); thus, the interaction between AMP1 and ammoniun transporters/sensors may be possible at the cellular level. The amount of PpAMP1 needed for the inhibition of ammonium uptake was the same as that needed for antimicrobial activity, indicating that a threshold concentration (10 μg mL^{-1}) of PpAMP1 is required to be biologically active (Figs 7 & 8). Moreover, the fact that higher concentrations of the recombinant peptide did not increase the inhibition of the ammonium uptake suggests that PpAMP1 is affecting a single transporter.

To our knowledge, this is the first work reporting the mediation of ammonium uptake by an AMP. However, there is increasing evidence that defensins may regulate ion fluxes in plants. Kushmerick *et al*. (1998) were the first to describe the inhibition of sodium channels by two γ-zeathionins isolated from maize kernels. More recently, it has been reported that isolated alfalfa MsDef1 is able to block a calcium channel (Spelbrink *et al*. 2004), and that a defensin-like gene in maize is able to open a potassium channel (Amien *et al*. 2010). Because members of the β-barrelin family of AMPs are not present in core eudicots, it is possible that defensins or other CRPs may undertake a similar function in these plants. In fact, it has been reported that the CRPs are an extraordinarily large and diverse family in *Arabidopsis* (Silverstein *et al*. 2007).

In summary, we have studied two different genes belonging to a small primitive family of AMPs in this work. Most of the already published work on AMPs indicates that these small proteins are involved in plant defence; however, there is recent evidence for a role of some of these proteins in the regulation of ion fluxes. Our work shows for the first time that an AMP of maritime pine (PpAMP1) is involved in ammonium uptake.

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