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Physiological effects of major up-regulated *Alnus glutinosa* peptides on *Frankia* sp. ACN14a

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Alnus glutinosa has been shown previously to synthesize, in response to nodulation by *Frankia* sp. ACN14a, an array of peptides called *Alnus* symbiotic up-regulated peptides (ASUPs). In a previous study one peptide (Ag5) was shown to bind to *Frankia* nitrogen-fixing vesicles and to modify their porosity. Here we analyse four other ASUPs, alongside Ag5, to determine whether they have different physiological effects on *in vitro* grown *Frankia* sp. ACN14a. The five studied peptides were shown to have different effects on nitrogen fixation, respiration, growth, the release of ions and amino acids, as well as on cell clumping and cell lysis. The mRNA abundance for all five peptides was quantified in symbiotic nodules and one (Ag11) was found to be more abundant in the meristem part of the nodule. These findings point to some peptides having complementary effects on *Frankia* cells.

INTRODUCTION

Black alder (*Alnus glutinosa*) is a tree which belongs to the actinorhizal group of plants (Torrey & Tjepkema, 1979). These plants have the ability to establish a mutualistic relationship with nitrogen-fixing actinobacteria, classified in the genus *Frankia* (Normand & Benson, 2015). This capacity has attracted a lot of attention but despite this it is still difficult to isolate and cultivate *Frankia* strains (Gtari *et al.*, 2015). Another difficulty is the absence of a transformation assay for *Frankia* (Kucho *et al.*, 2010) despite several attempts with protoplast regeneration (Normand *et al.*, 1987; Tisa & Ensign, 1987), electroporation (Cournoyer & Normand, 1992; Myers & Tisa, 2003) and conjugation (unpublished data from our laboratory).

However, several physiological characteristics unique to *Frankia* may play a role in the molecular dialogue whereby only symbionts are allowed to gain access to host plant tissues, such as the presence of the specific sugar, 2-*O*-methyl-D-mannose (Mort *et al.*, 1983), the specific lipid, bacteriohopanetetrol phenylacetate monoester (Berry *et al.*, 1993), with auxin activity that induces nodule-like structures (Hammad *et al.*, 2003), or the presence of lectins which in some strains are known to improve nodulation (Pujic *et al.*, 2012), but currently there is no way to demonstrate a role for these compounds due to the absence of a transformation system. New '-omics' technologies are improving our understanding of host–bacteria relationships, in particular the *Alnus–Frankia* symbiosis. Genome descriptions (Normand *et al.*, 2007; Gtari *et al.*, 2014), transcriptomics (Alloisio *et al.*, 2010) and proteomics (Hammad *et al.*, 2001; Alloisio *et al.*, 2007) have yielded many potential symbiotic determinants in recent years that are being studied by various complementary biochemical and genetic approaches to assess their role in symbiosis.

The molecular dialogue between partners which is the key to the symbiotic establishment has been the focus of many recent studies. Early symbiotic steps have been investigated through expressed sequence tag (EST) analyses to detect genes up-regulated upon nodulation (Hocher *et al.*, 2011). One of the most up-regulated genes encodes a peptide (Ag5) that is homologous to class A3 defence proteins that contain eight cysteines, shown later to modify the physiology of *Frankia* and hence play a role in trophic exchanges (Carro

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Abbreviations: ARA, acetylene reduction assay; ASUP, *Alnus* symbiotic up-regulated peptide; dpi, days post-inoculation; EST, expressed sequence tag; PI, propidium iodide; qRT-PCR, quantitative reverse transcriptase PCR; SEM, scanning electron microscopy.

Four supplementary figures and three supplementary tables are available with the online Supplementary Material.

et al., 2015). The production of antimicrobial peptides in nodules has also been shown for *Datisca* (Demina *et al.*, 2013).

Defence peptides in plants and animals have a strong and specific activity against microbes (Steiner *et al.*, 1981) and are now considered the main effectors of innate immunity (Brogden, 2005). The activities of these peptides range from modification of membrane porosity (Yamaguchi & Ouchi, 2012) to intracellular enzymatic activity (Spencer *et al.*, 2013). Such peptides have been found to play a role in symbiotic systems such as in *Sinorhizobium*/*Medicago* where endoreduplication results in the formation of bacteroids (Van de Velde *et al.*, 2010) or in the cereal weevil *Sitophilus* association where an up-regulated peptide, Coleoptericin A, maintains the microbial symbionts under control (Login *et al.*, 2011).

This study was designed to determine the effects on *Frankia* of additional up-regulated peptides in *Alnus* tissues and to study their origin.

METHODS

Bacterial strains and growth conditions. To achieve rapid cellular growth, *Frankia* sp. ACN14a (Normand & Lalonde, 1982) was stirred (200 r.p.m.) in defined (BAP) broth with 5 mM ammonium chloride (N-replete condition) or without (N-fixing condition) as previously described (Alloisio *et al.*, 2010).

Plant growth conditions. Seeds from an *A. glutinosa* tree growing on the banks of the river in Lyon were surface sterilized, planted in a soil/vermiculite substrate (1 : 1, v/v) and grown for 5–10 weeks in a glasshouse under fluorescent lighting with a 16 h/8 h nycthemeral regime at temperatures of 21/25 °C. The seedlings were transferred to a nitrogen-free solution (Broughton & Dilworth, 1971) in plastic pots and grown for 4–9 weeks with 0.5 g KNO_3 per litre followed by growth for 1 week without KNO_3 prior to inoculation (7.5 ml of bacterial culture for 1-litre pots) with a culture of *Frankia* sp. ACN14a derived from log-phase cultures which had been syringed (pulled inside the syringe and pushed outside five times through a 21G needle to fragment mycelium).

Nodule harvesting. Three month old root nodules (three or fewer lobes) were harvested and segments perpendicular to the long axis of some nodules were cut with a sterile scalpel to obtain sections from the meristem, fixation and senescence regions. Complete nodules and segments were ground with a mortar and pestle with liquid nitrogen for subsequent mRNA extraction.

RNA extraction for quantitative real-time PCR (qRT-PCR). Total RNA was purified from the roots and nodules using an RNeasy plant mini kit (Qiagen) (Alloisio *et al.*, 2010). Residual DNA was removed from the RNA samples using a Turbo DNA-free kit (Ambion, Life Technologies SAS). RNA was quantified using a Nano-Drop spectrophotometer (Thermo Fisher Scientific, Life Technologies SAS), and qualitatively assessed by agarose gel electrophoresis. Primers targeted at the five *Alnus* symbiotic up-regulated peptide (ASUP) genes (Table S1, available in the online Supplementary Material) were used to quantify RNA transcripts, as described previously (Carro *et al.*, 2015). The housekeeping *ubi* gene that encodes ubiquitin was used as standard as described previously (Hocher *et al.*, 2011).

***In silico* analysis.** All the ASUP genes were analysed for the presence of translation start and stop sites (http://insilico.ehu.es/translate/), and for the presence of a signal peptide (SignalP, http://www.cbs.dtu.dk/services/SignalP/) by Carro *et al.* (2015). Peptide characteristics were determined using the protein peptide calculator of LifeTein (http://www.lifetein.com/peptide-analysis-tool.html). All the sequences were aligned using CLUSTAL X (Thompson *et al.*, 1997) then compared with homologous sequences retrieved from GenBank. The five peptides (including Ag5) analysed here were those whose genes showed the highest differential expression as described in Carro *et al.* (2015).

Peptide production. The five selected mature peptides (without the signal peptides predicted by SignalP) were synthesized by Proteogenix and purified to 96 % purity by HPLC.

PI/SYTO9 analysis. Microscopic observations of *Frankia* cells were done after 24 h of incubation at 28 °C with and without the peptides. Membrane porosity was assessed by the addition of a mixture of dyes [30 μM propidium iodide (PI)/20 μM SYTO9] to the cultures. After 15 min of incubation in the presence of the dyes, the cells were observed by using a Zeiss Axioskop fluorescence microscope (Zeiss France), as previously described (Carro *et al.*, 2015).

Cell conditions for *in vitro* treatment with peptides. Cells for amino acid, cation, respiration, acetylene reduction assay (ARA) and microscopy analysis were obtained from syringed cultures (as above) and diluted with BAP media to give OD_{600} values of 0.03 or 0.20. These cells (200 μl for microscopy analysis and 1 ml for the other analyses) were then exposed to the five ASUPs either individually or as a equimolar mixture of all five peptides (where, for instance, '1 μM' indicates that the mix contained 1 μM Ag1, 1 μM Ag5, 1 μM Ag6, 1 μM Ag9 and 1 μM Ag11) at six different concentrations (0.1, 0.3, 1, 3, 10 and 100 μM) and then incubated for 1–7 days at 28 °C. Previously analysed physiological effects of Ag5 (Carro *et al.*, 2015) were used as reference. Calculations (means, standard errors, histograms, Student's *t*-test) were performed using Excel. Each measurement was taken in at least three biological replicates.

Determination of cation release. Peptides were added at different concentrations to *Frankia* sp. ACN14a cells which were then cultured for 7 days, centrifuged at 12 000 ***g*** for 10 min and 450 μl of cell-free supernatants analysed by ion chromatography using a DioneX ICS-900 ion chromatogrpahy system (Thermo Fisher Scientific) with a Dionex IonPac CS12A column, a flow rate of 0.5 ml min^{-1} and a conductivity detector.

Amino acid analysis. For the determination of amino acids present in *Frankia* cells, 1 ml dilutions of OD_{600} suspensions of 0.03 were prepared in PBS, and left for 7 days in contact with the peptides prior to harvesting the cells. Amino acid analysis was performed by HPLC (Agilent 1100; Agilent Technologies) filled with a guard cartridge and a reversed-phase C18 column (Zorbax Eclipse-AAA 3.5 μm, 150×4.6 mm, Agilent Technologies), according to the procedure specifically developed for this system (Henderson *et al.*, 2000), as described earlier (Carro *et al.*, 2015).

Nitrogenase activity and respiration analyses. Determination of nitrogenase activity of *Frankia* cells treated or untreated with ASUPs was made using the ARA (Stewart *et al.*, 1967) with a 10 % (v/v) acetylene concentration. Measurements of ethylene production after 4 days of growth in the presence of acetylene (five biological replicates) were made using conditions previously described for *Frankia* cells by Carro *et al.* (2015). We used a gas chromatograph (Girdel series 30), with a J&W PORA-Plot Q (Agilent) column with an H_2 pressure of 1.2 bar, a dinitrogen pressure of 2.6 bar and an air pressure of 2.7 bar at a temperature of 45 °C, and equipped with a flame ionization detector. Respiration was evaluated as the amount of CO_2 (in p.p.m.) liberated by cells treated or untreated with the five ASUPs either individually or as a mixture to estimate the metabolically active biomass using a gas chromatograph (P200

Table 1. Major theoretical properties and expression data of the five ASUPs tested compared with non-inoculated plants

	Ag1	Ag5	Ag6	Ag9	Ag11
Molecular weight (Da)	6923	8145	6911	8037	6276
Number of cysteines	6	8	8	8	8
Length (≠leader)*	58	68	58	67	57
pI	8.62	8.99	8.59	8.20	7.26
Charge (pH 7)	4	7.6	5.4	3.2	0.6
Hydrophobicity (%)	37	34	39	44	40
Up-regulation (fold change) at 7 days after inoculation†	19.15	39.17	1.40	1.45	30.92
Up-regulation (fold change) at 21 days†	429.13	155.02	199.54	28.75	46.10

*(≠leader), without peptide signal.
†Data from Carro *et al.* (2015).

MicroGC; Agilent Technology) with samples separated on a PPQ (Agilent) 6 m column heated to 55 °C, helium gas used as carrier and pressure adjusted to 20.1 p.s.i. Respiration was also assayed by the tetrazolium/formazan (INTF) assay (Prin *et al.*, 1990). The mix of peptides used includes the five peptides added at the same concentrations of 0, 0.1, 0.3, 1, 3 and 10 μM with cumulative final concentrations of 0, 0.5, 1.5, 5, 15 and 50 μM.

Microscopy. *Frankia* cultures were observed on a Zeiss Axioskop microscope fitted with 10× and 100× objectives. For scanning electron microscopy (SEM) cultures (24 h) of *Frankia* sp. ACN14a with and without 100 μM Ag5 peptide were used. Bacterial suspensions were fixed for 2 h in 2 % (v/v) glutaraldehyde solution in 0.1 M PBS at room temperature. After washing three times in 0.2 M PBS, bacteria were placed on thermanox coverslips with poly-L-lysine and post-fixed in a 1 % osmium tetroxide solution. Dehydration was made in a graded series of ethanol followed by hexamethyldisilazane at room temperature. After drying, the samples were mounted on aluminium stubs, sputter-coated with a layer of gold–palladium using a MED020 sputter coater (Oerlikon-Balzers) and viewed under an S800 microscope (Hitachi) at 10 kV. SEM observations were made by the 'Centre Technologique des Microstructures – Plateforme de l'Université Claude Bernard Lyon 1'.

Phylogeny of peptides. Sequences from *Alnus* peptides and homologous sequences retrieved by a BLASTP (Altschul *et al.*, 1990) search from GenBank were aligned using CLUSTAL X (Thompson *et al.*, 1997); their phylogeny was reconstructed using the neighbour-joining algorithm (Saitou & Nei, 1987) and the robustness of the resulting topology was assessed in a bootstrap analysis (Felsenstein, 1985) using MEGA version 6 software (Tamura *et al.*, 2013).

RESULTS

Physico-chemical characteristics of the ASUPs

A previous study of a collection of data from analysis of microarrays (Hocher *et al.*, 2011) led to the identification of 15 different ESTs that encoded defensin peptides which were up-regulated in nodules (Carro *et al.*, 2015). Peptides with higher expression values (Carro *et al.*, 2015) and with a signal peptide that allows them to be targeted to the secretory pathway – symbiotic bacteria or other specific structures involved in the symbiosis with *Frankia* – were selected for analysis: Ag1, Ag5, Ag6, Ag9 and Ag11. The predicted N-terminal signal peptide, charge, isoelectric point, hydrophobicity and molecular weight of these ASUPs were determined (Tables 1 and S2). These selected ASUPs have been analysed in order to understand their functions in the actinorhizal symbiosis.

Bioinformatics sequence analysis showed that all five ASUPs studied here contained a predicted N-terminal signal peptide of between 16 and 26 aa that was rich in phenylalanine and valine (Fig. S1). In general, the isoelectric points (pI) of the selected peptides were high, between 7.26 and 8.99, and their molecular weight varied from 6276 to 8145 Da. All were medium-length cationic peptides with a net charge at pH 7 ranging from 0.6 to 7.6. The defensin structure common to all of them except Ag1 was 'signal peptide-X_nCX_3 $SXTWX_3CX_5CX_3CX_3EXAX_2GXCX_nCXCX_nCX_n$', which is similar to the pattern found in class A3 defensins of plant peptides (Fant *et al.*, 1999). Ag1 was similar in structure to the other four ASUPs but lacked two of the cysteines found in the other peptides; this probably resulted in the formation of only three disulfide bridges in Ag1 compared with four in the other peptides.

Effects of ASUPs on the physiology of *Frankia* cells

To investigate the possible physiological function of the five ASUPs, cultures of *Frankia* were treated with each of these peptides. With the exception of Ag1, large aggregates of *Frankia* cells were formed after 24 h of treatment with the peptides (Fig. 1a, b). Cell aggregation was not seen with the untreated *Frankia*-grown cells in BAP media. A possible explanation of this effect was revealed by SEM (Fig. 1c) where extramatricial structures were found to bind *Frankia* hyphae together in the presence of Ag5. For Ag1 ASUP, no aggregation effects were observed but at high concentrations (100 μM), *Frankia* cells were completely disrupted into small particles (Fig. 1a). The only observable effect at this concentration for the other four peptides was still the aggregation of cells. In contrast, similar peptides produced by *Medicago sativa* when exposed to symbiotic *Sinorhizobium* strains (NCR035, NCR247 and NCR335) (Van de Velde

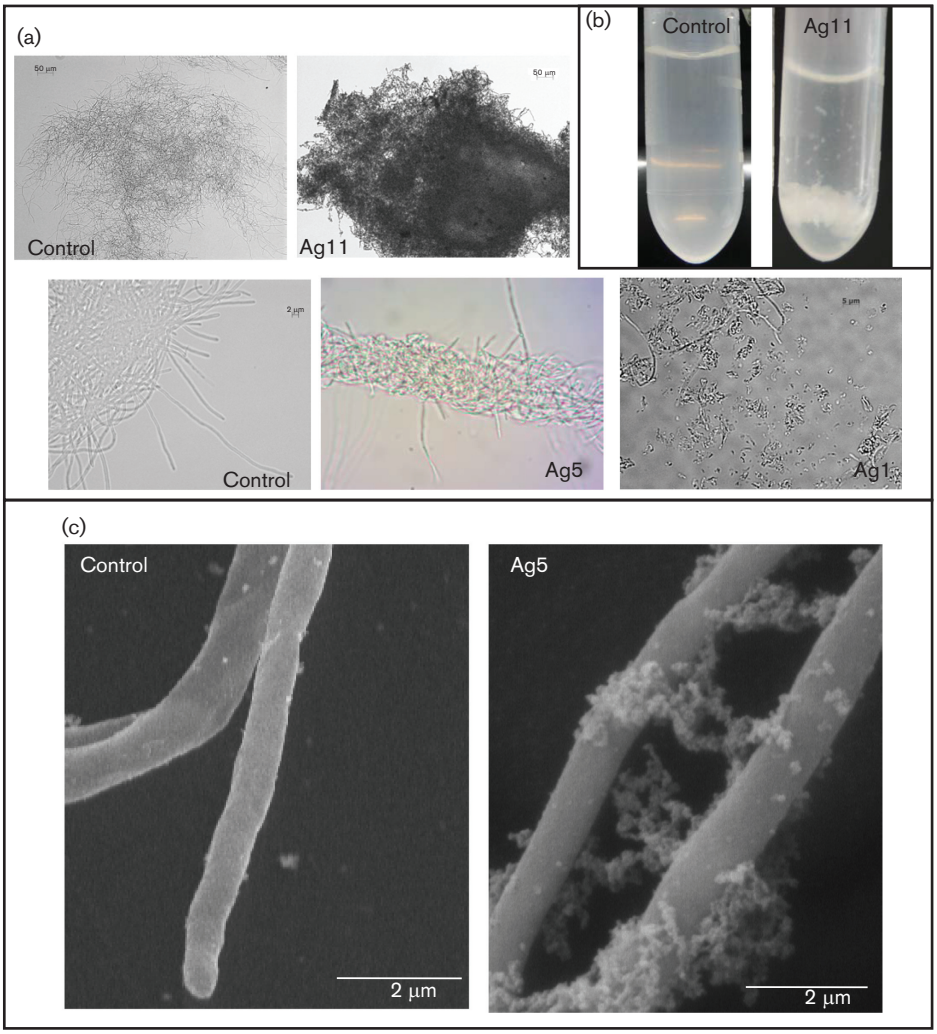

Fig. 1. Morphological changes in *Frankia* sp. ACN14a cells treated with different ASUPs. (a) Optical microphotograph at 10× of control cells (without peptide) and Ag11 peptide (100 μM) inducing cell aggregation (top) and at 100× of control cells (without peptide), Ag5 peptide inducing cell aggregation and Ag1 peptide (100 μM) inducing cell lysis (bottom). (b) Photograph of the strong clumping effect of Ag11 peptide (100 μM) over *Frankia* cells in a 1 ml culture in BAP medium after 1 h. (c) Scanning electron micrograph of control cells (without peptide) and with Ag5 peptide (100 μM) inducing cell clumping.

et al., 2010) have no detectable effects on *Frankia* sp. ACN14a (Fig. S2).

qRT-PCR analysis of peptide transcripts in nodules

Expression of the five ASUPs induced after inoculation with *Frankia* in roots and subsequently in nodules was confirmed by qRT-PCR normalized using *ubi* gene values. For *ag5* and *ag11* genes, expression was detected in uninoculated roots, albeit at a very low level compared with that in nodules. Conversely, expression of the three other defensin peptide genes was only detected in 7 days post-inoculation (dpi) roots and in 21 dpi nodules (Table 2). The *ag5* and *ag11* genes showed the highest differential expression after 7 dpi, which was maintained in nodules, while up-regulation of *ag1*, *ag6* and *ag9* genes was significantly higher in nodules than in 7 dpi samples.

To determine if defensin gene expression was specifically found in different areas of the nodules, qRT-PCR of three sections of the nodules (meristem area, fixation area and senescence area) and the complete nodules were made (Fig. 2). The expression level in the fixation area was similar for all the defensin genes. However, some differences were observed in other areas; in the meristem, the most important expression was found for the *ag11* gene and the lowest for the *ag9* gene, whilst in the senescence area, *ag9* showed the highest expression (Fig. 2).

Table 2. Transcript levels and relative quantification in 7 dpi roots and nodules of selected ESTs

ND, No detectable expression in the absence of bacteria (out of range); –, not determined; Nd, in root nodule 21 days after inoculation; T0, in root 0 days before inoculation; T7, in root 7 dpi.

	EST-TIK name	Microarrays*			qRT-PCR			
		T7/T0	Nd/T0	Nd/T7	T7/T0	*P*-value†	Nd/T7	*P*-value†
Ag1	CL1464Contig1	19.15	429.13	4.21	ND	–	72	0.0000
Ag5	AG-N01f_007_J18	39.17	155.02	3.96	6	0.0013	6	0.0019
Ag6	AG-N01f_016_P23	1.4	199.54	142.53	ND	–	610	0.0001
Ag9	AG-R01f_027_J16	1.45	28.75	19.83	ND	–	282	0.0013
Ag11	AG-R01f_030_E08	30.92	46.1	1.49	9	0.0001	0.9	0.8931

*Data from Carro *et al.* (2015).
†*P*-value for Student's *t*-test.

Membrane permeability and metabolite release

Permeability of *Frankia* cells after Ag5 peptide treatment has been described for some amino acids and for ammonium (Carro *et al.*, 2015), so determination of this capacity was evaluated for the other selected peptides. The analysis of membrane permeabilization was evaluated using two dyes, PI and SYTO9. At 1 μM concentration of each peptide, only Ag6 showed similar effects on permeability of vesicles to those observed for Ag5, with an increase in the number of vesicles staining red (PI). For the other three peptides, Ag1, Ag9 and Ag11, most vesicles were stained green (SYTO9), similar to the negative controls without peptides (Fig. 3). When higher concentrations of these peptides were added (>10 μM), the number of vesicles and hyphae staining red increased correspondingly (data not shown).

Amino acids present in cells of *Frankia* sp. ACN14a treated with the selected ASUPs at different concentrations (0, 0.1, 0.3, 1, 3, 10 μM) were evaluated (Fig. 4 and Fig. S3). Cells treated with Ag1 showed a decrease in glutamine (Gln) concentration only at ≥3 μM concentrations of this peptide. This profile was quite similar for cells treated with Ag11, with only a small decrease in Gln concentration at 1 μM. Ag6 and Ag9 had a profile more similar to Ag5, with a decrease in Gln concentration at lower concentrations of the peptides. For glutamate (Glu) concentration, only cells treated with 1 μM Ag6 showed a decrease. The other peptides only showed differences at concentrations of 3 μM or more. The profile of Ag6 was

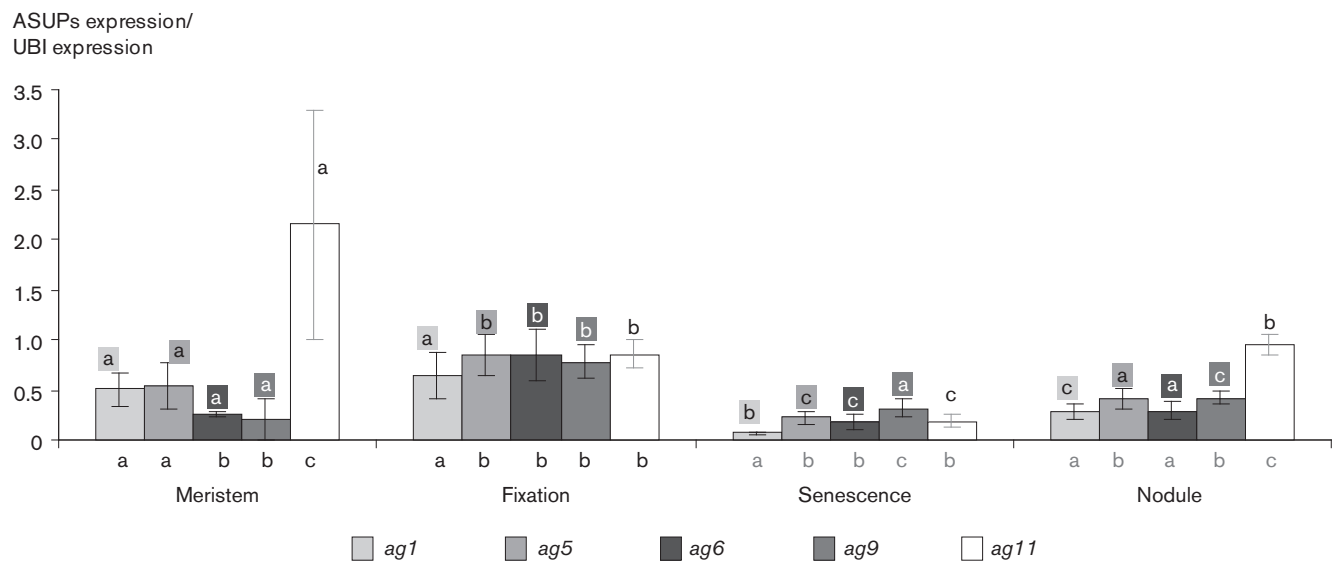

Fig. 2. Expression of defensin genes in different zones of nodules measured by qRT-PCR. Values are normalized with the *ubi* gene transcripts. Error bars are SD. Values with differences in *P*-values from Student's *t*-test <0.05 are classified in a different letter group: over each bar are shown differences for each peptide along the nodule axis; under each bar are shown differences between peptides in the respective area of the nodule (meristem, fixation, senescence or in the complete nodule).

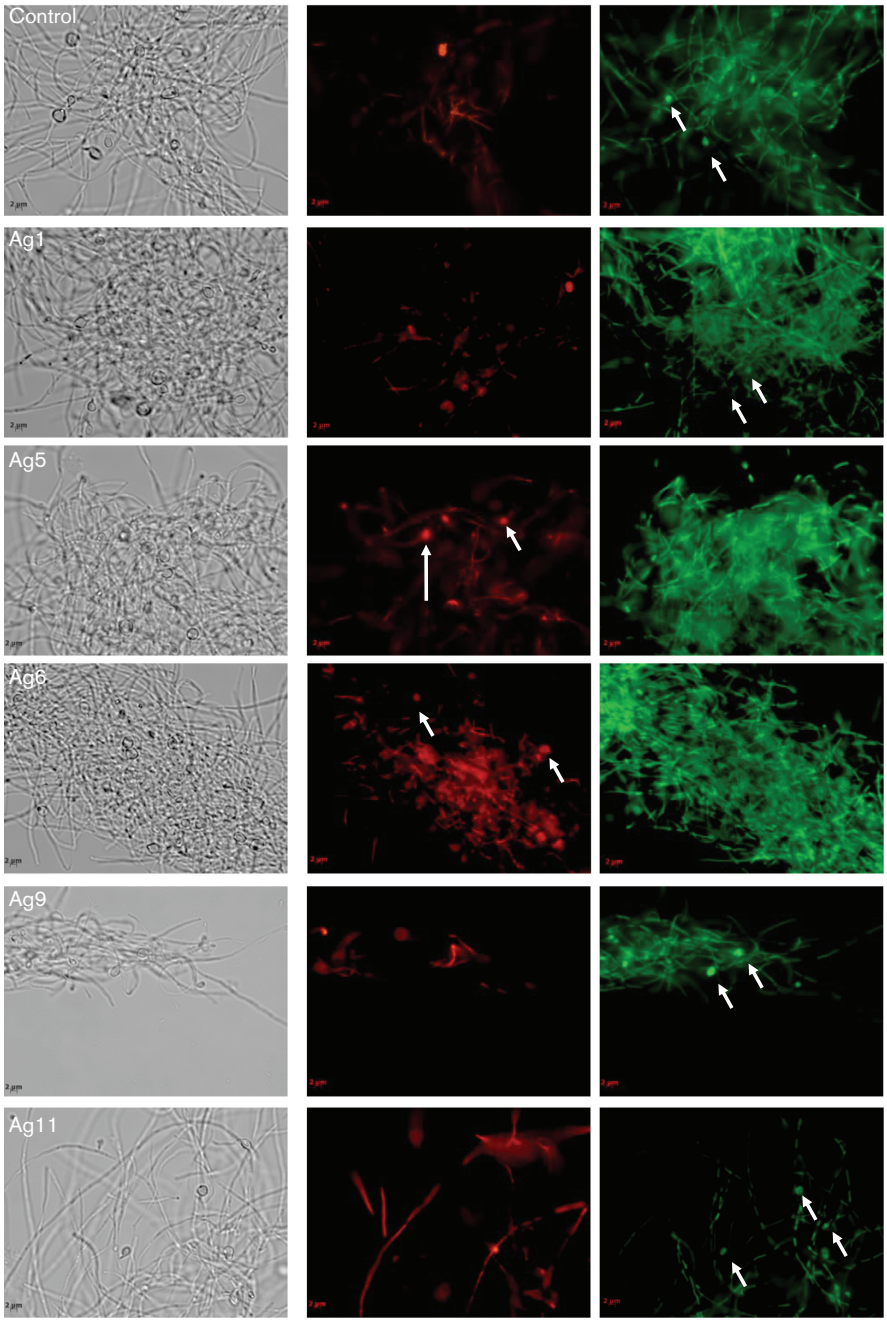

Fig. 3. Membrane permeability assessed with PI (red) – SYTO9 (green) staining in *Frankia* sp. ACN14a cultures after ASUP treatment at 1 μM concentration. Porosity in vesicles treated with Ag5 and Ag6 peptides was modified. First column: phase contrast images, second column: red fluorescence after PI treatment, third column: green fluorescence after SYTO9 treatment. Line 1: control cells (without peptide), line 2: Ag1 peptide, line 3: Ag5 peptide, line 4: Ag6 peptide, line 5: Ag9 peptide, line 6: Ag11 peptide. Arrows indicate vesicles.

most similar to Ag5, with some variation, albeit less pronounced in Glu and Gln at 1 μM. For other amino acid concentrations, significant differences were found only for serine (Ser) and glycine (Gly) following Ag5 and Ag11 treatments.

The liberation of cationic compounds such as NH_4^+, Na^+, K^+ and Ca^{2+} into the supernatant of *Frankia* cells was evaluated for the different concentrations of peptides by ion chromatography (Fig. S4). Some of the peptides caused significantly more abundant cation leakage than in non-

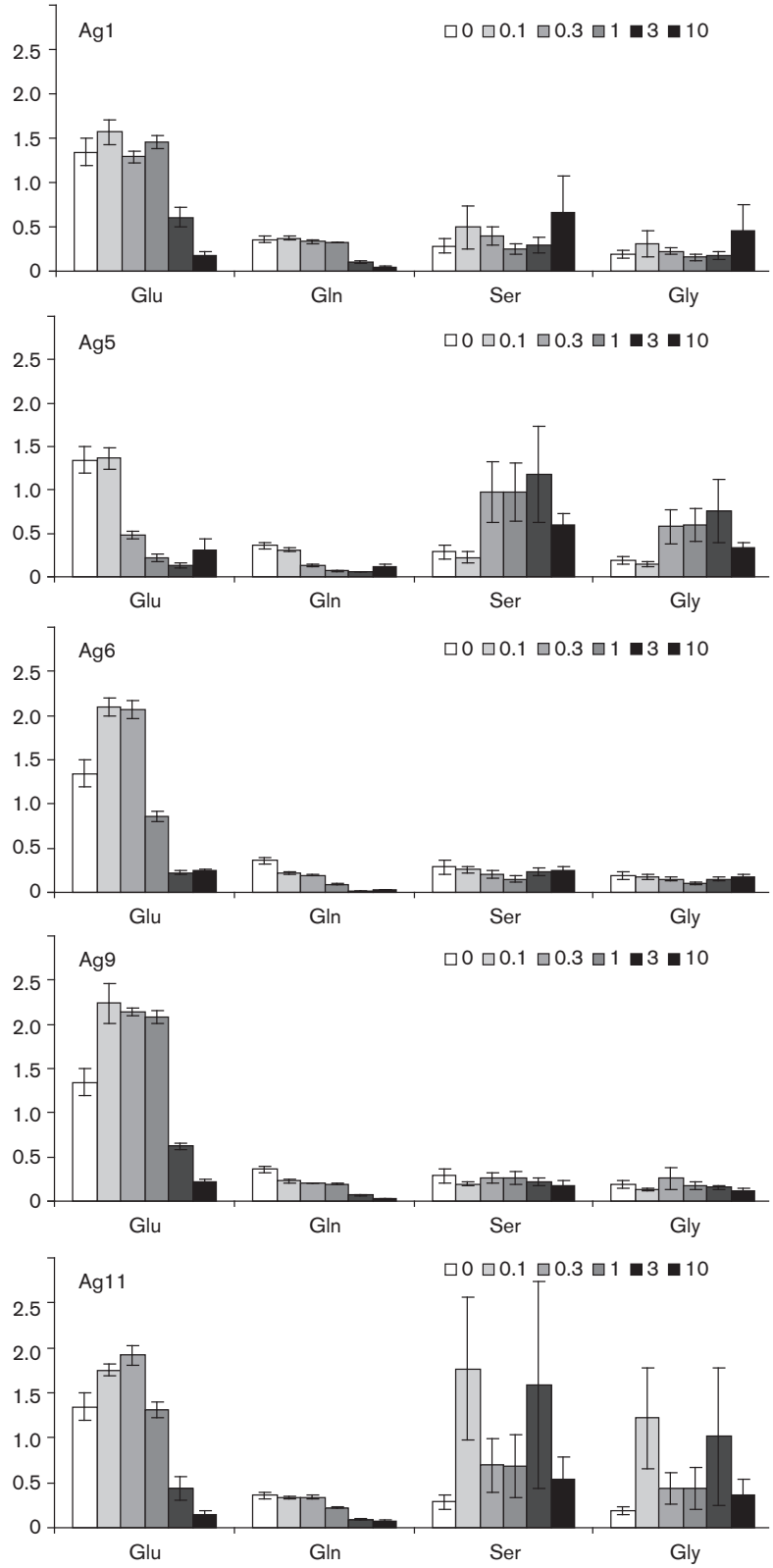

Fig. 4. Concentration of amino acids (nmol) in *Frankia* cells following peptide treatment for 7 days in PBS. From top to bottom: analysis of the peptides Ag1, Ag5, Ag6, Ag9 and Ag11. In all cases, the concentration zero bars correspond to control cells (without any peptide). Error bars indicate SD, n=5.

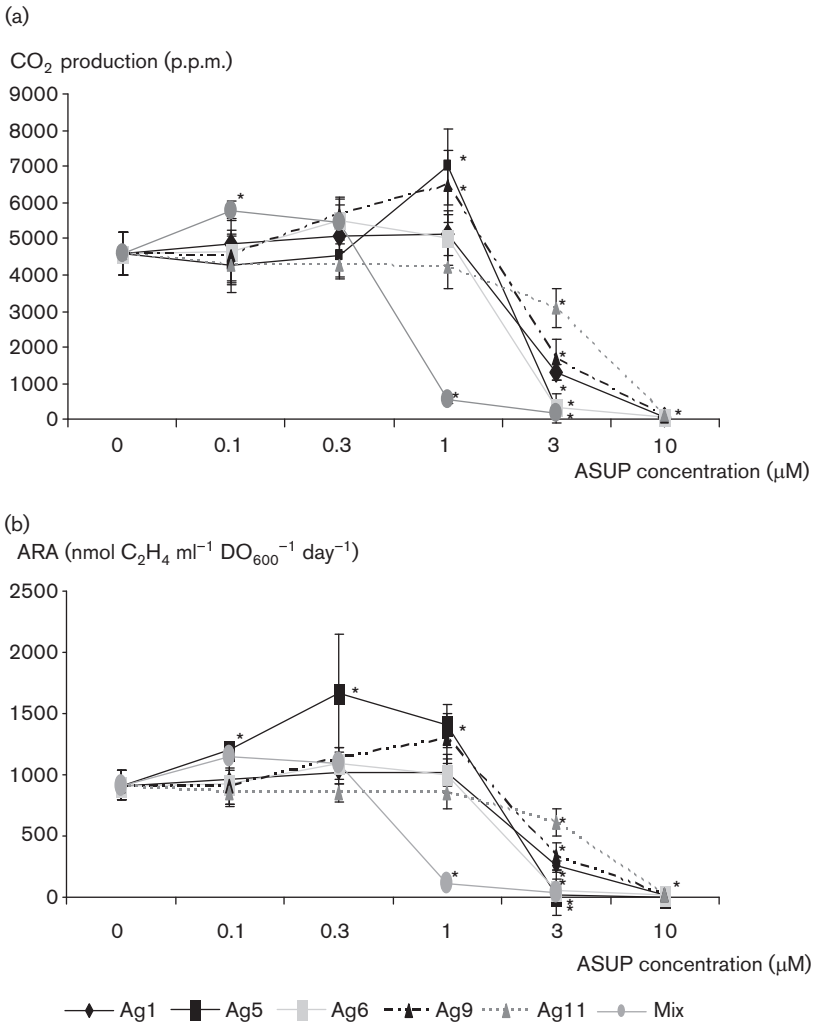

Fig. 5. Respiration and nitrogen fixation activities of *Frankia* sp. ACN14a cells treated with different ASUPs (Ag1, Ag5, Ag6, Ag9 and Ag11). (a) Respiration activity measured by CO_2 production by the cultures; (b) nitrogen-fixation activity was measured by the acetylene reduction activity assay per millilitre of culture. Error bars are SD, n=5. *Values with differences in P-values from Student's t-test <0.05. The concentrations of the mix indicated correspond to the concentration of individual peptides; the cumulative concentration of peptides was 0, 0.5, 1.5, 5, 15 and 50 μM.

treated cells at the different concentrations. For instance, cultures with high concentrations of Ag1, Ag5 and Ag11 showed more K^+ in their supernatants, while cells treated with Ag6 and Ag9 did not show any differences. Indeed, for cultures with Ag6 and Ag9 no changes were observed with any of the tested cations. Higher amounts of Ca^{2+} were detected when Ag1, Ag5 and Ag11 were added, while the most important differences for NH_4^+ were seen for Ag5. For Na^+, a decrease was observed for Ag1, Ag5 and Ag11 at the higher concentrations of these peptides.

Respiratory activity

The capacity of the *Frankia* sp. ACN14a cells to maintain homeostasis after the application of different concentrations of ASUPs was tested by measuring their ability to respire. For this, two different methods were applied, the test for conversion of iodonitrotetrazolium chloride to iodonitrotetrazolium formazan (INT) and the production of CO_2 (Fig. 5a). Both methods yielded similar results for the peptides analysed. The profiles obtained for Ag6 were most similar to those of Ag5, with values similar to those of the control cells up to a 1 μM concentration when a steep decrease at 3 μM and above was noted. However, for the other three peptides, Ag1, Ag9 and notably Ag11, the values obtained at 3 μM were different from those observed in the control cells but much less than in Ag5-treated cells. Consequently, *Frankia* sp. ACN14a is able to tolerate higher concentrations of these three peptides without detectable physiological changes.

A mix of the five peptides at equimolar concentrations was also tested to determine whether together they have a

cumulative or synergistic effect. In both tests, the equimolar mix of the five peptides caused a steep decrease in the respiration ability at a concentration of 1 μM of each peptide and above whereas the individual peptides caused a similar decrease only at 10 μM (Fig. 5a). This indicates a cumulative inhibitory effect that lowers the threshold below which ASUPs do not compromise function.

Nitrogen-fixing (ARA) activity

The main function of *Frankia* cells in actinorhizal symbiosis is the fixation of atmospheric nitrogen to make ammonium available to the plants. For this reason, the capacity of *Frankia* cells treated with the selected ASUPs for fixing nitrogen was evaluated using the ARA test. *Frankia* sp. ACN14a cells showed a nitrogen-fixation activity level close to that of the control with all the peptides tested when applied alone up to a 1 μM concentration, but this dropped to zero when the concentration of the peptides reached 10 μM (Fig. 5b). With a 3 μM peptide concentration, the most important variations were observed; *Frankia* was not able to fix nitrogen in the presence of peptides Ag5 and Ag6, but was seen to have one-third of the activity of the control with peptides Ag1 and Ag9 and two-thirds with the peptide Ag11 (Fig. 5b).

The same effect observed in respiration tests occurred with the nitrogen-fixation capacity for the mix of peptides; the ability of *Frankia* cells to fix nitrogen was only maintained up to a 0.3 μM concentration, where the cumulative concentration is 1.5 μM (Fig. 5b).

Phylogeny of peptides

The phylogeny of the peptides retrieved from the *Alnus* database (Table S3) shows they are interspersed with those present in *Casuarina* (Table S3) and *Datisca* (Fig. 6). All actinorhizal peptides were grouped together, and present a close relationship with the A3 group of plant defence-like peptides, such as those of *Vitis vinifera* (VITISV_040160) and *Pisum sativum* (DRR230-c). However, the NCR peptide (NCR001) from *Medicago sativa*, which is involved in symbiotic interaction with the nitrogen-fixing bacteria *Sinorhizobium meliloti*, is positioned outside this group.

DISCUSSION

The diversity of defence peptides in various eukaryotes is enormous and knowledge of these is increasing exponentially (Lehrer, 2004). They play contrasting roles in different organisms and this rapid discovery rate can be expected to increase with the application of different genome sequencing procedures (Belarmino & Benko-Iseppon, 2010; Gruber & Muttenthaler, 2012). It is difficult to establish the phylogeny of these genes due to their short length and the presence of numerous indels, yet there clearly are families of defensins that are evocative of evolutionary bursts (Seufi *et al.*, 2011). The localization of *Alnus* peptides within the phylogenetic tree suggests that the appearance and diversification of these peptides occurred before the appearance of *Datisca*, which is presumed to have taken place 65 million years ago (Bell *et al.*, 2010). Initially, research focused on their role in innate immunity against pathogens (Hazlett & Wu, 2011) but they also play pivotal roles in plant (Van de Velde *et al.*, 2010) and animal symbiosis (Login *et al.*, 2011). Defining the role of defensins is pivotal to understanding host–microbes relationships.

Changes in membrane porosity was the first physiological effect reported for defensins. Many mechanisms have been proposed to account for this, including the formation of toroidal pores, barrel staves or the carpet layer (Brogden, 2005). These result in varied changes in porosity and expected differences in the metabolites that will be leaked. Glutamate is the major amino acid present in *in vitro* grown *Frankia* (Berry *et al.*, 1990) and in symbiotic *Alnus* nodules (Lundquist *et al.*, 2003), an amino acid that is at the crossroads of several metabolic pathways. More specifically for symbiosis, it is used by glutamine synthetase to incorporate the reactive ammonium derived from nitrogenase into glutamine (Tsai & Benson, 1989), and from there to form all other nitrogen-containing metabolites.

Defensin-induced changes in porosity are expected to modify the activity of enzymes as well as the transcriptome. Defensins are known to interfere directly with intracellular constituents such as the cell wall, DNA, RNA or proteins (Brogden, 2005), for example the RNAse activity identified in kidney cells (Spencer *et al.*, 2013). We were thus not surprised to see contrasting effects on some key functions in *Frankia*. The results obtained showed the ability of ASUPs to react with *Frankia* cells; all the tested peptides induced changes in the morphology of *Frankia* sp. ACN14a, while other plant peptides, such as those produced by *Medicago sativa*, did not. Ag6 seems to be most similar to Ag5, inducing a similar increased permeability of vesicles at 1 μM concentration of the peptide and most similar profiles for respiration and nitrogen fixation. Although all of the ASUPs tested were expressed at their highest level in the fixation area of the nodules, where the nitrogen-fixing vesicles are located, some differences of expression were found in the other areas, suggesting this may be due to the plant regulating where *Frankia* accumulate and are active. The *ag11* gene showed highest expression in the meristem area, which could be related to the control of the position of the bacteria, taking into account that this peptide is also slightly expressed in roots, and is the peptide which induces the strongest and fastest aggregation of *Frankia* cells.

Some peptides that lack bactericidal activity have been found to have a range of activities such as binding to bacterial cells and forming self-assembled nanonets that prevent bacterial proliferation in host tissues (Chu *et al.*, 2012). Other defensins from plants have been shown to cause cell lysis by directly binding to the plasma membrane phospholipid phosphatidylinositol 4,5-bisphosphate (Poon *et al.*, 2014). The peptide Ag1 has been found to

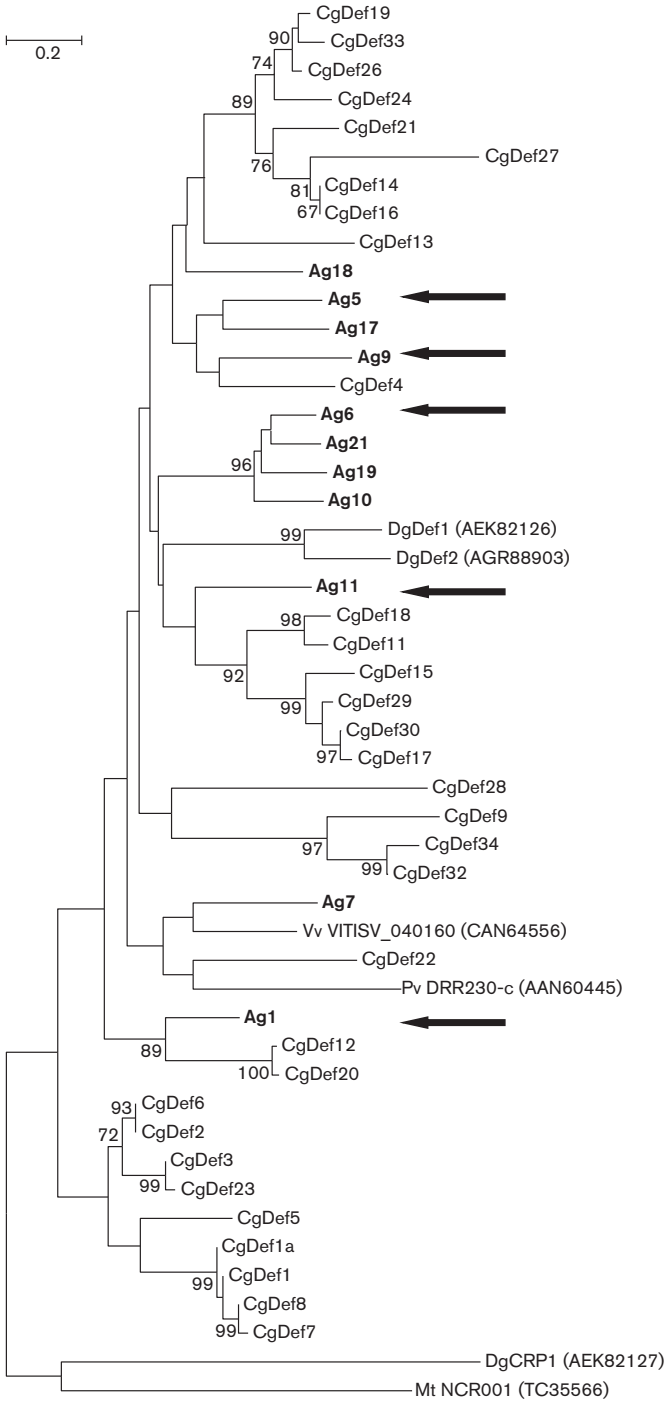

Fig. 6. Phylogenetic tree of *Alnus* defensin peptides and related peptides in *Casuarina* and other plants. Ag, *Alnus glutinosa*; Cg, *Casuarina glauca*; Dg, *Datisca glomerata*; Mt, *Medicago truncatula*; Ps, *Pisum sativum*; Vv, *Vitis vinifera*. Sequence details are given in Table S2.

have a drastic effect on *Frankia* at a concentration of 100 μM, a feature that could be useful for the host to scavenge and recycle nodular metabolites after the microsymbiont has ceased to be effective. However, this peptide, like all of the others assayed, was not up-regulated in the senescing part of the nodule as might be expected if its role was lysis of *Frankia* and scavenging of metabolites by the plant.

Beyond the very sensitive qRT-PCR performed in this work, other promising approaches could be tested such as

direct protein quantification by MALDI-TOF or ELISA, to investigate further the nodule localization of the different defensin peptides. Such *in situ* quantification should help define the precise identity and the function of the peptides involved in the symbiotic interaction.

Analyses of respiration and nitrogen fixation showed that each ASUP has a threshold below which it does not compromise these functions, and that the mixture of ASUPs lowered this threshold, which raises the question of the effective biological concentrations of these peptides in the host cells. ASUPs were seen to cause various effects besides increasing cell porosity, such as clumping and lysis, and thus evocative of plant control of the *Frankia* symbiont. Some differences were observed between the quantitative distribution of ASUPs in the different areas of the nodule, indicating a possible role in the structuring of *Frankia*. Their phylogeny indicated that they emerged a long time ago, as some peptides in *Alnus* are closer to those of *Casuarina* than to those of *Alnus*. Taken together, these results suggest that these peptides have evolved to play different symbiotic roles in the plant control of the symbiont.

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