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ORIGINAL ARTICLE

Alnus peptides modify membrane porosity and induce the release of nitrogen-rich metabolites from nitrogen-fixing *Frankia*

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Actinorhizal plant growth in pioneer ecosystems depends on the symbiosis with the nitrogen-fixing actinobacterium *Frankia* cells that are housed in special root organs called nodules. Nitrogen fixation occurs in differentiated *Frankia* cells known as vesicles. Vesicles lack a pathway for assimilating ammonia beyond the glutamine stage and are supposed to transfer reduced nitrogen to the plant host cells. However, a mechanism for the transfer of nitrogen-fixation products to the plant cells remains elusive. Here, new elements for this metabolic exchange are described. We show that *Alnus glutinosa* nodules express defensin-like peptides, and one of these, Ag5, was found to target *Frankia* vesicles. *In vitro* and *in vivo* analyses showed that Ag5 induces drastic physiological changes in *Frankia*, including an increased permeability of vesicle membranes. A significant release of nitrogen-containing metabolites, mainly glutamine and glutamate, was found in N_2-fixing cultures treated with Ag5. This work demonstrates that the Ag5 peptide is central for *Frankia* physiology in nodules and uncovers a novel cellular function for this large and widespread defensin peptide family.

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Introduction

Most plants rely for their nutrition on inorganic nitrogen (ammonium and nitrate), a macronutrient found to be limiting in most terrestrial ecosystems (Chapin *et al.*, 2002). Legumes and actinorhizal plants have acquired the ability to establish a root nodule symbiosis with nitrogen-fixing bacteria known as rhizobia and frankiae, respectively. N_2-fixing root nodule symbioses by actinorhizal plants are important contributors to ecosystems worldwide. Phylogenetically, this symbiosis occurs among eight plant families and three orders that have diverse global distributions (Benson and Silvester, 1993). Conversely, most important crop plants such as cereals are unable to use atmospheric N_2 for their nutrition. Modern agriculture is therefore dependent on synthetic nitrogen fertilizers. The increasingly expensive fossil energy and the pervasive effects of atmospheric carbon dioxide associated with the industrial production of nitrogen fertilizer, together with the negative environmental impact of leached nitrogen fertilizers, have led to a renewed interest for biological nitrogen fixation.

How nitrogen fixation occurs has been studied in both free-living organisms and in symbioses for more than a century (Beijerinck, 1901; Wyss *et al.*, 1941; Berry *et al.*, 1990). However, key steps in the symbiotic process, including how fixed nitrogen is transferred from symbionts to host plants, are still only partially understood despite efforts made in this area (Benson and Silvester, 1993; Benson *et al.*, 2011; Oldroyd *et al.*, 2011; Pawlowski *et al.*, 2011). High-throughput sequencing of expressed sequence tags (ESTs) and DNA arrays are powerful tools to understand biological processes and have been widely used recently to study nitrogen-fixing symbiotic interactions (Ampe *et al.*, 2003; Alloisio *et al.*, 2010; Hocher *et al.*, 2011; Heath *et al.*, 2012). Nevertheless, there is presently no obvious candidate gene identified in either rhizobia or *Frankia* to account for release of nitrogen compounds to plants.

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Although both symbiotic systems have some common features, both the bacteria and the plants involved are very different, and hence it is expected that different mechanisms have evolved for similar purposes in legumes and actinorhizal plants.

Frankia bacteria are able to fix nitrogen both as free-living organisms and in symbiosis. In both conditions, *Frankia* develops special cell types called vesicles for nitrogen fixation. These structures are formed at the tip of hyphae, are spherical with a diameter from 2 to 5 μm and are covered by several layers of hopanoid lipids to protect the nitrogenase enzyme from oxygen (Benson and Silvester, 1993; Berry *et al.*, 1993). *In vitro*, growth in nitrogen-free medium (BAP^-) has been found to induce the formation of these structures (Murry *et al.*, 1984).

Defensin peptides, generally described as antimicrobial peptides, are effectors of innate immunity in plants and animals and thus permit to control growth of pathogens. In plants, members of the A family of defensins (A1, A2, A3 and A4) are known to be able to inhibit growth of a broad range of fungi without adverse effects on plant or mammalian cells. The A3 subfamily is known to cause membrane permeabilization and also hyphal branching (Fant *et al.*, 1999; Thevissen *et al.*, 1999). More recent studies have shown that antimicrobial peptides are also important in symbiotic relationships. An antimicrobial peptide expressed by insects has been found to control symbiotic bacteria (Login *et al.*, 2011), and an extremely large gene family from *Medicago truncatula* that encodes polypeptides related to defensins was shown to have a nodule-specific expression and to be implicated in the terminal differentiation of bacterial symbionts (Mergaert *et al.*, 2003; Van de Velde *et al.*, 2010).

In actinorhizal symbiosis, a collection of *Alnus glutinosa* and *Casuarina glauca* ESTs has been sequenced from roots and 3-week-old nodules (Hocher *et al.*, 2011). These analyses showed that several genes related to carbon and nitrogen metabolism, defense against pathogens or stress resistance were strongly upregulated in nodules. More recently, similar results were found for another actinorhizal plant, *Datisca* (Demina *et al.*, 2013). Remarkably, all three actinorhizal plants induce a small family of defensin genes in their nodules (Hocher *et al.*, 2011; Demina *et al.*, 2013). In the present study, we sought to confirm the nodule-enhanced expression of defensin peptides produced by actinorhizal plants and to understand their role in the symbiotic process.

Materials and methods

Bacterial strains and growth conditions

Frankia alni ACN14a (Normand and Lalonde, 1982) was grown according to Schwencke (1991) at 28 °C in defined (BAP) medium containing 5 mM ammonium chloride (N-replete condition, BAP^+) or not (N-fixing condition, BAP^-) (Murry *et al.*, 1984) with some minor modifications (Alloisio *et al.*, 2010). The 5-day-old cultures were used for *in vitro* analyses.

Plant growth conditions and mRNA extraction

A. glutinosa seeds were harvested from an *A. glutinosa* specimen growing on the Rhône River banks in Lyon, France, as described previously (Alloisio *et al.*, 2010; Hocher *et al.*, 2011). Seeds were grown and inoculated with the compatible *F. alni* strain ACN14a (Normand and Lalonde, 1982) as described previously (Alloisio *et al.*, 2010). For RNA extraction, non-inoculated roots at 0 and 7 days post inoculation (d.p.i.), inoculated roots (7 d.p.i.) and nodules (4 weeks post inoculation) were collected and immediately frozen in liquid nitrogen as done by Hocher *et al.* (2011). Five biological replicates were used for each condition. Total RNA was purified from roots and nodules using the RNeasy plant mini kit (Qiagen, Courtaboeuf, France) (Alloisio *et al.*, 2010). Residual DNA was removed from RNA samples using the Turbo DNA free kit (Ambion, Aix-en-Provence, France). RNA was quantified using a Nano-Drop spectrophotometer (Thermo Fisher Scientific, Villebon sur Yvette, France) and qualitatively assessed using agarose gel electrophoresis and/or a Bioanalyzer 2100 according to the manufacturer's instructions (Agilent, Les Ulis, France).

EST database and microarray expression analysis

The previously reported database of *A. glutinosa* ESTs and microarray analysis of these transcripts (Hocher *et al.*, 2011), which are available in the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo; accession number GSE24153), were analyzed. An extensive iterative BLAST search was made in order to find ESTs encoding defensin-homologous peptides. Then, differential expression of all recovered genes was determined using the microarray data set, according to Hocher *et al.* (2011). Briefly, Student's *t*-test was applied to compare nodules versus non-inoculated roots and inoculated roots (7 d.p.i.) versus non-inoculated roots and average fold changes were calculated.

RT-qPCR analysis

Reverse transcription-PCR (RT-PCR) and quantitative real-time reverse transcription PCR (RT-qPCR) were performed with the five biological replicates of nodules, inoculated roots and non-inoculated roots (as reference). RT was performed with 10 μg of total mRNA using Transcriptor Reverse Transcriptase and oligo $(dT)_{15}$ primer (Roche, Boulogne-Billancourt, France). RT-qPCR was run on a LightCycler 480 (Roche) using LightCycler 480 SYBR Green I Master (Roche) under the following conditions: 95 °C for 5 min; and 45 cycles of 95 °C for 20 s, 60 °C for 20 s

and 72 °C for 15 s. The primer set for the *Ag5* gene (07J18c 5′-AAACGCATCGAAAGGTGTAA-3′ and 07J18d 5′-CCCCGATATACGGAGAGT-3′) was designed using Primer 3 (Rozen and Skaletsky, 2000) and AmplifX version 1.5.4 (Nicolas Jullien; CNRS, Aix-Marseille Université, http://crn2m.univ-mrs.fr/pub/amplifx-dist) programs. Two RT-qPCRs were run for each biological replicate. Expression values were normalized using the expression level of the *ubi* gene (AgUbi 5′-AG GAGTCCACCCTCCATCTT-3′ and 5′-TCAGAACTC TCGACCTCCAA-3′) that encodes ubiquitin in *A. glutinosa* (Hocher *et al.*, 2011).

In silico analysis
All *Alnus* symbiotic upregulated peptide (ASUP) genes were analyzed for the presence of translation start and stop sites (http://insilico.ehu.es/translate/), and for signal peptides (SignalP, http://www.cbs.dtu.dk/services/SignalP/). All sequences were aligned using CLUSTAL_X (Thompson *et al.*, 1997).

Ag5 peptide production
The mature Ag5 peptide (the Ag5 peptide without the signal peptide as predicted by SignalP; Petersen *et al.*, 2011) was synthesized by Proteogenix (Schiltigheim, France). This chemically synthesized peptide was purified by high-performance liquid chromatography to a 96% level.

PI/SYTO9 analysis
Microscopic observations of bacteria were made after 24 h of incubation at 28 °C with and without Ag5 peptide (1 μM). Membrane porosity was assessed by addition of 30 μM propidium iodide (PI)/20 μM SYTO9 to cultures and 75 μM PI/50 μM SYTO9 in fresh nodule sections obtained by hand cutting. After 15-min incubation in the presence of the dyes, the cells were visualized with a Zeiss Axioskop fluorescence microscope (Marly-le-Roi, France).

In vitro treatments with Ag5 peptide
Treatments were done on cultures diluted to an OD_{600} of 0.03 or 0.10. These diluted cells (200 μl) were treated with Ag5 peptide at different concentrations (0.1, 0.3, 1, 3, 10 and 100 μM) and incubated from 1 to 7 days at 28 °C. For determination of cations and amino-acid analysis, 500 μl dilutions of OD_{600} of 0.03 were made in phosphate-buffered saline and were left for 7 days in contact with Ag5 before recovering supernatants and/or cells. Data handling (normalization and statistics) was performed using Excel. Each measurement was done in quintuplicate (biological repeats).

Colony-forming unit determination
A total of 50 μl of *F. alni* ACN14a cells treated or not with Ag5 1 μM for 24 h were deposited onto 6 cm Ø Petri dishes with BAP^- medium and spread out using glass beads. After 1 month of incubation at 28 °C, the number of colonies on each dish was determined.

Nitrogenase activity and respiration analyses
Determination of nitrogenase activity of cells treated or not with Ag5 ASUP, heated or not to 100 °C for 1 h, NCR035 peptide (from *Medicago*) and bovine serum albumin was made with the acetylene reduction assay using a 10% vol/vol acetylene concentration. Measures were made with a gas chromatograph (Girdel series 30, Suresne, France) after 4 days of growth in the presence of acetylene (five biological replicates). To estimate the metabolically active biomass, respiration was evaluated as the amount of CO_2 (in p.p.m.) liberated by the same cells using a gas chromatograph (P200 MicroGC, Agilent Technology, Massy, France) and by the tetrazolium/formazan assay (Prin *et al.*, 1990).

Determination of cation release
Ag5 peptide was added at different concentrations to *F. alni* ACN14a cells that were cultured for 7 days in phosphate-buffered saline. Supernatants were centrifuged at 12 000 *g* for 10 min to remove cells. Then, 250 μl of supernatants were diluted fourfold with sterile water and analyzed by ion chromatography.

Amino-acid analyses
Amino-acid analysis was performed by HPLC (Agilent 1100; Agilent Technologies) with a guard cartridge and a C18 reverse phase 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). Before injection, samples were buffered with borate at pH 10.2, and primary or secondary amino acids were derivatized with ortho-phthalaldehyde or 9-fluorenylmethyl chloroformate, respectively. The derivatization process, at room temperature, was automated using the Agilent 1313A autosampler. Separation was carried out at 40 °C, with a flow rate of 2 ml min^{-1}, using 40 mM NaH_2PO_4 (eluent A, pH 7.8, adjusted with NaOH) as the polar phase and an acetonitrile/methanol/water mixture (45:45:10, vol/vol/vol) as the non-polar phase (eluent B). A gradient was applied during chromatography, starting with 20% of B and increasing to 80% at the end. Detection was performed by a fluorescence detector set at 340 and 450 nm wavelengths for excitation and emission, respectively (266/305 nm for proline). These conditions do not allow detection and quantification of cysteine and tryptophan, and hence only 18 amino acids were quantified. For this quantification, norvaline was used as the internal standard and the response factor of each amino acid was determined using a 250 pmol μl^{-1} standard mix of amino acids. The software used was

the ChemStation for LC 3D Systems (Agilent Technologies).

$^{15}N/^{14}N$ measurement
To assess whether the Ag5 peptide was catabolized, *F. alni* ACN14a cultures were grown for three subsequent subcultures in BAP$^+$ medium with ^{15}N-labeled NH_4^+ to convert all cellular nitrogen to ^{15}N. A final subculture was made with and without ^{14}N Ag5 peptide. Amounts of ^{15}N (labeled cellular) and ^{14}N (peptide) were measured using 4 mg per sample. Each sample was placed in a tin capsule, pyrolyzed and then passed through a reduction column to convert all nitrogen to N_2. The N_2 was then analyzed by isotope ratio mass spectrometry (SCI, CNRS, Villeurbanne, France).

Antibodies
The chemically synthesized Ag5 was used to develop a polyclonal anti-Ag5 antibody in rabbits by standard intravenous injection using a commercial service (Covalab, Villeurbanne, France; http://www.covalab.com).

Microscopy
For light microscopy, samples were observed using a $\times 10$ objective on a Zeiss Axioskop microscope. For fluorescent microscopy, nodules and roots were dipped into a paraformaldehyde 4% (vol/vol) fixative solution in phosphate-buffered saline. After 1 week at 4 °C, the fixative was removed by rinsing several times with phosphate-buffered saline before embedding the tissue in 1.3% (wt/vol) agar. Subsequently, samples were dehydrated through a graded ethanol/H_2O series and transferred to 1-butanol, at 4 °C, for 1 week. Samples in agar were then impregnated and embedded in melted Paraplast resin (Hexcel Corp., Stanford, CT, USA). Wax blocks were kept dust free until sectioning. Tissue sections (5 μm thick) were obtained using a Thermo Scientific Microm HM340E microtome (Thermo Fisher Scientific Inc.,Waltham, MA, USA). Sections were placed on poly-lysine-coated slides, dried overnight in a 37 °C oven, and stored at 4 °C before immunostaining. Confocal microscopy was made at the Centre Technologique des Microstructures–Plateforme de l'Université Claude Bernard Lyon 1.

Immunolocalization
To determine the localization of Ag5 in *Alnus* tissues, immunolocalization was performed. The 4-week-old nodules were used for this analysis. The immunostaining and microscopy were made according to Login *et al.* (2011).

Results

Defensin genes are induced in symbiosis
In a previous expression microarray analysis (Hocher *et al.*, 2011), a gene encoding a defensin peptide was found to be present among the most highly upregulated genes in *A. glutinosa* nodules (3 weeks post inoculation). In the present study, an extensive screening of the *A. glutinosa* database (Hocher *et al.*, 2011) found 15 different ESTs coding for defensin-like peptides that were all upregulated in nodules (Table 1). Moreover some of these peptides were also upregulated as early as 7 d.p.i. during the early infection stage (Table 1).

To unravel the defensin peptide functions in the *A. glutinosa–Frankia* symbiosis, the Ag5 ASUP was

Table 1 ASUPs are highly upregulated during symbiosis

Gene name	*Clone name*	*EMBL accession*	*Fold change IR/NIR*	*Student's* t-*test,* P-*value*	*Fold change NOD/NIR*	*Student's* t-*test,* P-*value*
Ag2	AG-N01f_026_J04	FQ343405	**19.15**	0.15650	**429.13**	**0.00976**
Ag3	AG-N01f_002_C06	FQ334814	**32.86**	0.04267	**125.38**	**0.00074**
Ag4	AG-N01f_004_C12	FQ334620	1.34	—	**24.78**	**0.00190**
Ag5	AGCL3713Contig1	FQ334302, FQ334074	**39.17**	0.06507	**155.02**	**0.00124**
Ag6	AG-N01f_016_P23	FQ344294	1.4	—	**199.54**	**0.00007**
Ag7	AG-N01f_019_P24	FQ344001	**2.76**	0.23293	**13.35**	**0.00336**
Ag8	AG-N01f_044_D03	FQ341750	1.44	—	**242.85**	0.01931
Ag9	AG-R01f_027_J16	FQ351117	1.45	—	**28.75**	0.01055
Ag10	AG-N01f_014_F24	FQ344553	1.36	—	**85.16**	0.01672
Ag11	AG-R01f_030_E08	FQ350855	**30.92**	0.01261	**46.10**	0.01191
Ag15	AGCL684Contig1	[a]	**4.26**	0.10257	**341.10**	**0.00716**
Ag17	AG-N01f_001_N05	FQ333830	**11.53**	0.09855	**801.55**	**0.00067**
Ag19	AG-N01f_026_O01	FQ343374	1.48	—	**93.85**	0.01385
Ag21	AG-R01f_028_L14	FQ350936	1.21	—	**86.54**	0.05985
Ag22	AG-N01f_023_P01	FQ343655	1.28	—	**183.58**	0.01857

Abbreviations: ASUP, *Alnus* symbiotic upregulated peptide; EMBL, European Molecular Biology Laboratory; NIR, non-inoculated root; IR, 7 days post inoculation root; NOD, 21 days post inoculation nodule.
Microarray analyses in 7 days post inoculation (d.p.i.) roots and 21-day nodules. Microarray data are available in Gene Expression Omnibus (GEO) database, (www.ncbi.nlm.nih.gov/geo (GSE24153). Average fold change and Student's *t*-test, *P*-value were determined with three biological replicates of each condition. Fold change of >2 and $P<0.01$ are shown in bold. *P*-value was not considered when fold change was <2.
[a]FQ333688, FQ350809, FQ332425, FQ339124, FQ350970, FQ333167.

selected for an in-depth study because the *Ag5* gene displayed the highest differential expression at 7 d.p.i. and was ranked as the eleventh most upregulated gene in 3-week-old nodules (Hocher *et al.*, 2011) (Table 1). Early induction of *Ag5* was further confirmed by RT-qPCR, revealing a 6.15-fold upregulation ($P = 0.00783$).

Bioinformatics sequence analysis showed that Ag5 ASUP is 94 amino acids (aa) long and contains a predicted N-terminal signal peptide of 25 aa (Supplementary Figure S1), thus leaving a 69 aa sequence after removal of the peptide leader. It is a medium length cationic peptide with a net charge of 11, an isoelectric point of 8.99 and a molecular weight of 8145.26 Da. The defensin structure present in most ASUP, including Ag5, is: Signal peptide-$X_nCX_3SXTWX_3CX_5CX_3CX_3EXAX_2GXCX_nCXCX_nCX_n$, $(n = 1-30)$, and this is similar to the pattern found in class A3 defensins (Fant *et al.*, 1999) but differs from the one described for the NCR peptides of *Medicago* (Mergaert *et al.*, 2003). Other important differences from these NCR peptides are their size, 69 aa in Ag5 and 63 aa on the average for the other ASUPs, whereas NCR peptides only have a mean length of 40 aa. Similarly, the number of cysteine residues is 4 or 6 in NCR peptides and 8 for ASUPs.

Ag5 colocalizes with Frankia in nodules

Ag5 was strictly colocalized with *Frankia*-infected nodule cells (Figure 1) and no signal was observed in other nodule tissues or in roots. Guan *et al.* (1997) proposed four zones from nodule apex to base: the *Frankia*-free meristematic zone, the infection zone with hyphae, a nitrogen-fixation zone with mature vesicles and the senescence zone. In longitudinal nodule sections, a fluorescence intensity gradient was found, with no detectable signal at the apex, a moderate intensity in the infection zone, an intense fluorescence in the central section and a lowered intensity in the senescing zone (Figures 1b and d). Within the infected cells of the nitrogen-fixation zone, the fluorescence signal is strongly associated with the vesicles (Figure 1h), demonstrating that the Ag5 peptide is targeted to the *Frankia* endosymbionts.

Ag5 binding in N-fixing cultures of ACN14a, treated or not with Ag5, was also analyzed in order to determine the localization of the peptide in the bacterial cells (Supplementary Figure S2). Ag5 was detected in both hyphae and vesicles but only at the periphery of the *Frankia* cells, suggesting binding of the peptides to the bacterial cell walls.

Effects of Ag5 on in vitro grown Frankia

To address the physiological function of Ag5, cultures of *F. alni* were treated with the Ag5 peptide *in vitro*. Evaluation of the response of *F. alni* ACN14a cells to increasing concentrations of peptide was made by measuring nitrogen-fixation activity and cellular respiration. Both analyses gave similar results with the maintenance of strong activities when the Ag5 peptide concentration was equal or inferior to 1 μM but with a sharp drop when the concentration was increased (Figure 2). However, a notable difference was observed, with cellular respiration gently decreasing at low concentrations of peptide, whereas nitrogen-fixation activity remained stable or indeed increased until after a 1 μM concentration was reached. Other peptides used as controls showed no significant differences, whereas heat-treated Ag5 showed similar effects to the nontreated one (Supplementary Figure S3). The ability of bacteria to grow on BAP^+ plates was also tested after treatment with 1 μM Ag5. Despite the high metabolic activity of the bacteria at this peptide concentration, we observed a decrease of two orders of magnitude in colony-forming ability (from 400 to 4 colony-forming units). The symbiotic function of *Frankia* inside the host is nitrogen fixation. Therefore, we used nitrogen-fixation activity as an indicator to determine the maximum tolerance to the Ag5 peptide and a 1 μM concentration of the peptide was used in further studies.

Ag5 increases membrane porosity

Defensin-type antimicrobial peptides are known to permeabilize bacterial membranes and this is considered a major mode of antimicrobial activity for these peptides (Brogden, 2005). To assess the effect of the Ag5 peptide on bacterial membrane structures *in vitro*, treated and nontreated *Frankia* cultures were stained with PI and SYTO9. SYTO9 is a freely diffusing DNA fluorescent stain, whereas the PI fluorescent DNA stain can only penetrate cells following lethal or nonlethal perturbations in membrane permeability (Boulos *et al.*, 1999). Untreated *Frankia* cells showed mostly SYTO9-stained (green) hyphae (Figures 3e, e′ and e″), whereas the proportion of PI-stained (red) hyphae was higher in cells treated with 1 μM Ag5 ASUP (Figures 3f, f′ and f″). However, the most important differences were observed in the nitrogen-fixing *Frankia* vesicles. The vesicles were mostly stained red with PI after addition of Ag5 ASUP to the medium (Figures 3f, f′ and f″), whereas they were mostly stained green with SYTO9 in untreated control cultures (Figures 3e, e′ and e″). Thus, the Ag5 peptide at 1 μM induces membrane permeabilization without affecting nitrogenase activity.

Next, we evaluated *Frankia* membrane porosity in nodules using the same SYTO9–PI staining procedure (Figures 3a–d). Confocal microscopy of nodule sections showed that hyphae were mostly stained green with SYTO9 (Figures 3b and d), suggesting membranes were not affected. In contrast, the vesicles were strongly stained red with PI, suggesting an increased porosity (Figures 3c and d). These results are in agreement with the *in vitro* activity of the Ag5 peptide and the *in vivo* localization of the

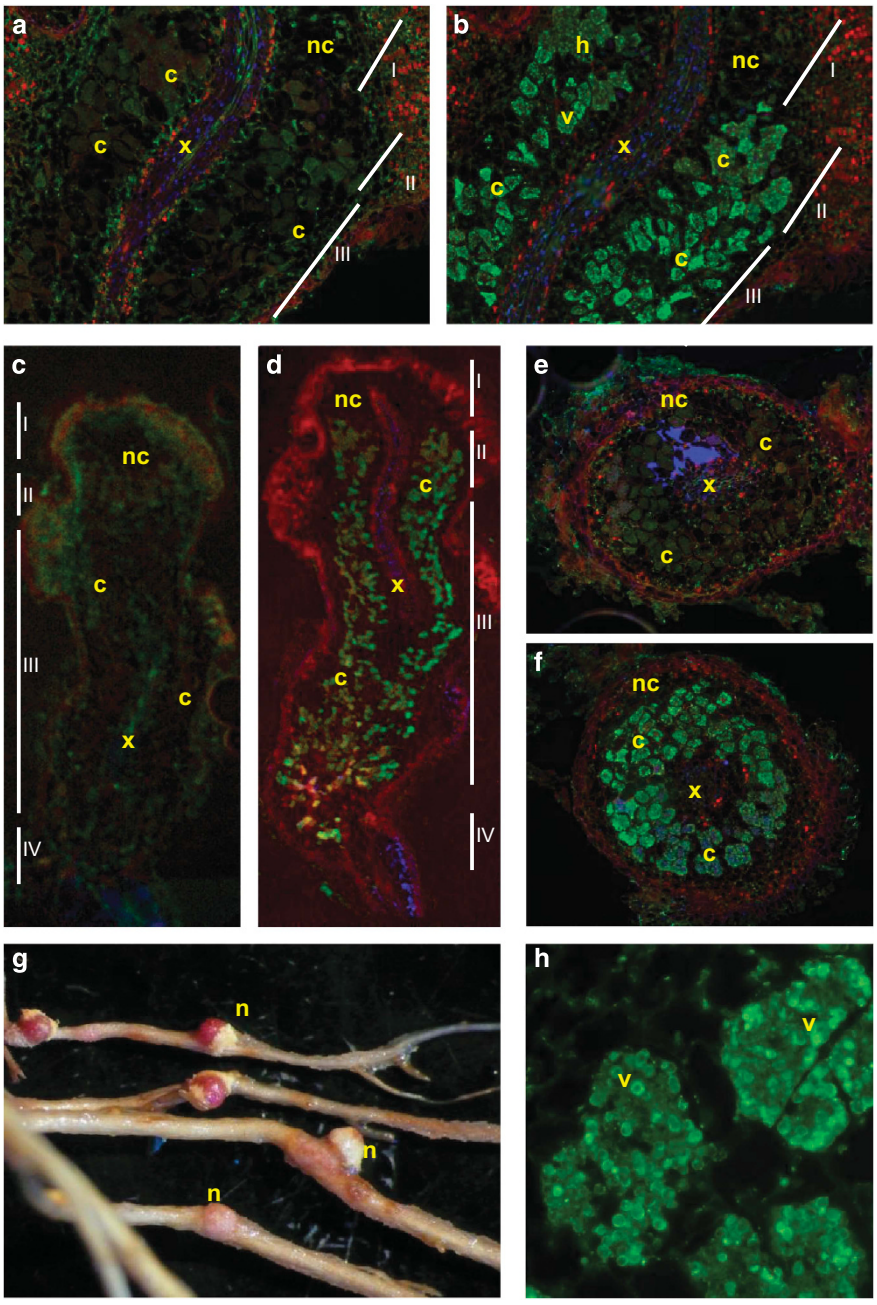

Figure 1 Ag5 ASUP colocalizes with *Frankia* in nodules of *Alnus glutinosa*. Negative control with rabbit serum before intramuscular injection of Ag5 (**a**, **c**, **e**) and immunofluorescence localization of Ag5 with anti-Ag5 antibody (**b**, **d**, **f**, **h**). Longitudinal (**a**, **b**, **c**, **d**) and cross-sections (**e**, **f**) of the nodule are shown. (**g**) *Alnus* roots carrying nodules infected with *F. alni* ACN14a. Blue: DAPI; red: autofluorescence; green: Alexa Fluor 488 dye anti-rabbit antibody (Life Technologies, Saint Aubin, France). c, infected cells; h, hyphae; nc, noninfected cells; v, vesicles; x, xylem. Zones in nodule: I, meristem zone (free of *Frankia*), II, infection zone (mostly hyphae of *Frankia*), III, nitrogen-fixation zone (mostly vesicles of *Frankia*) and IV, senescence zone.

peptide in the nodule cells, both suggesting that the peptide is affecting vesicles more than hyphae in live cultures.

Ag5 releases nitrogen-rich compounds from Frankia

We analyzed the possibility that the Ag5 peptide facilitates the release of nitrogen-containing metabolites from *Frankia* cells. First, we examined the release of amino acids from cultured cells. Significant differences were found between supernatants of Ag5-treated and untreated cells (Figure 4 and Supplementary Table S1). Remarkably, some amino acids were released whereas others were not. Glutamine (Gln) was 200 times and glutamic acid (Glu) was 30 times more abundant in supernatants of cells treated with 1 μM Ag5 grown under nitrogen-fixing condition, with a concentration increase of ~1 μM for Gln and 0.5 μM for Glu. Other amino acids such as arginine (Arg), tyrosine (Tyr), lysine (Lys),

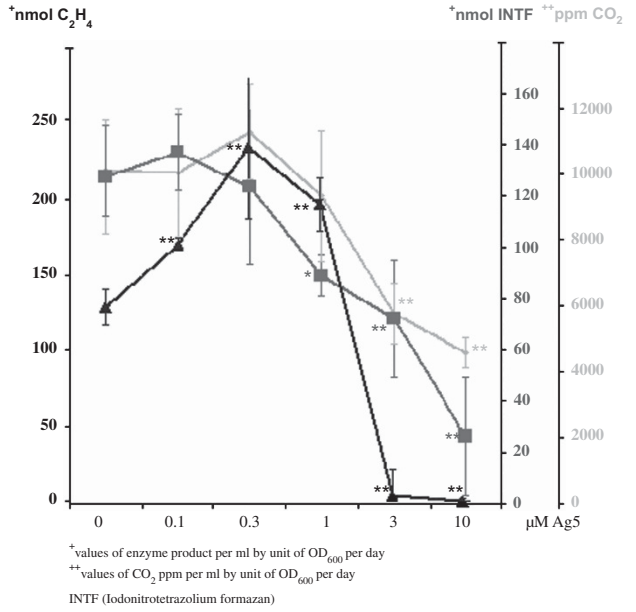

Figure 2 Strong activity of *Frankia* cells is detected when concentration of Ag5 ASUP is $\leq 1\,\mu M$. Nitrogen-fixation activity is measured by the acetylene reduction activity per µl of culture. Triangles, respiration analyses; diamonds, CO_2 p.p.m.; squares, tetrazolium/formazan $OD_{490\,nm}$.

methionine (Met), leucine (Leu) and asparagine (Asn) were also recovered in larger amounts in treated cell supernatants, but much less so than Gln and Glu. Amino acids present in *Frankia* cells of the same cultures were also analyzed (Figure 4 and Supplementary Table S1). The amino-acid abundance was generally not affected by the peptide treatment except for Glu whose concentration dropped strongly with peptide concentrations of 0.3 µM or higher. Thus together, these measurements indicate an important release of Gln in the spent medium of Ag5-treated cells, correlated with a drop of the cellular Glu level.

In addition, the release of other compounds from cells was also evaluated. Quantification of NH_4^+, Mg^{2+}, Ca^{2+} and K^+ levels in the culture supernatant was performed by ion chromatography (Supplementary Table S2). The quantity of these ions after addition of Ag5 ASUP was not affected at peptide concentrations up to 3 µM, indicating that at these peptide concentrations, there is no unspecific leakage out of the cells. Only when a high concentration of Ag5 (10 µM) was used, incompatible with physiological activities like N_2 fixation and respiration, was an increase in NH_4^+ observed in the cell supernatant.

To exclude the possibility that the amino-acid increase in the spent medium results from Ag5 peptide degradation, the stability of Ag5 was assessed in the presence of *Frankia*. The $^{15}N/^{14}N$ ratios were determined in a ^{15}N-labeled *F. alni* ACN14a culture with ^{14}N Ag5 added. All the nitrogen recovered from the supernatant was ^{15}N, indicating that the nitrogen originated only from the bacterial cells and not from degradation of the added Ag5 peptide.

Discussion

The *Ag5* gene displays strongly increased expression and was ranked as the eleventh most upregulated gene in 3-week-old *Alnus* nodules (Hocher *et al.*, 2011). This level of induction was comparable to that of known actinorhizal nodulins, suggesting a central role for this peptide in the host response to the infection with *Frankia* bacteria. Nevertheless, the upregulation of *Ag5* and the other ASUPs does not necessarily mean by itself that they play a role in symbiosis as these peptides could be generated as a generic response to microbial invasions. Interestingly, ASUP-like genes that were also upregulated in nodules of *Casuarina* plants (Hocher *et al.*, 2011) were not upregulated in mycorrhizae (Tromas *et al.*, 2012), supporting the possibility of a specific role for them in the *Frankia* symbiosis. In addition, immunolocalization showed that the Ag5 peptide specifically targets the *Frankia* cells in the nitrogen-fixation zone of the nodule. Ag5 ASUP was most abundant on the vesicle surfaces, suggesting a direct relationship with the symbiotic process, beyond the first steps of the interaction. This specific binding to vesicles could be related to the presence of the thick hopanoid lipidic external layers in vesicles that are not present on the hyphae (Berry *et al.*, 1993).

We demonstrate here that Ag5 peptide caused changes in the membrane properties of *Alnus*-infective *Frankia* strains and a release of cellular metabolites from cells. In the context of pathogenic interactions, such a leakage induced by antimicrobial peptides may help to weaken pathogens by decreasing or abolishing membrane potential, causing leakage of nutrients or releasing effectors that will boost host defenses. In symbiosis, the Ag5-induced membrane porosity may rather play a role in nutrient exchanges. The analysis of membrane porosity in nodule sections and *in vitro* cells suggests a specificity of the Ag5 action on nitrogen-fixing vesicles that were strongly permeable to PI whereas hyphae were not. This indicates an increased porosity of nitrogen-fixing vesicles relative to vegetative hyphae in host cells. This observation contrasts with those obtained from the *Rhizobium*–legume nodules, where the nitrogen-fixing *Rhizobium* bacteria were not stained with PI (Haag *et al.*, 2011). However, our observations confirm a previous hypothesis proposing that the permeability of vesicles in nodules is higher than that of *in vitro* vesicles (Tisa and Richards, 2004). The increased membrane permeability induced by Ag5 and possibly other ASUPs may affect the capacity of the *Frankia* cells to reproduce as indicated by the drop in colony-forming capability of *in vitro* grown cells treated with 1 µM Ag5 but this does not or hardly affect nitrogen-fixation and metabolic activity of those cells.

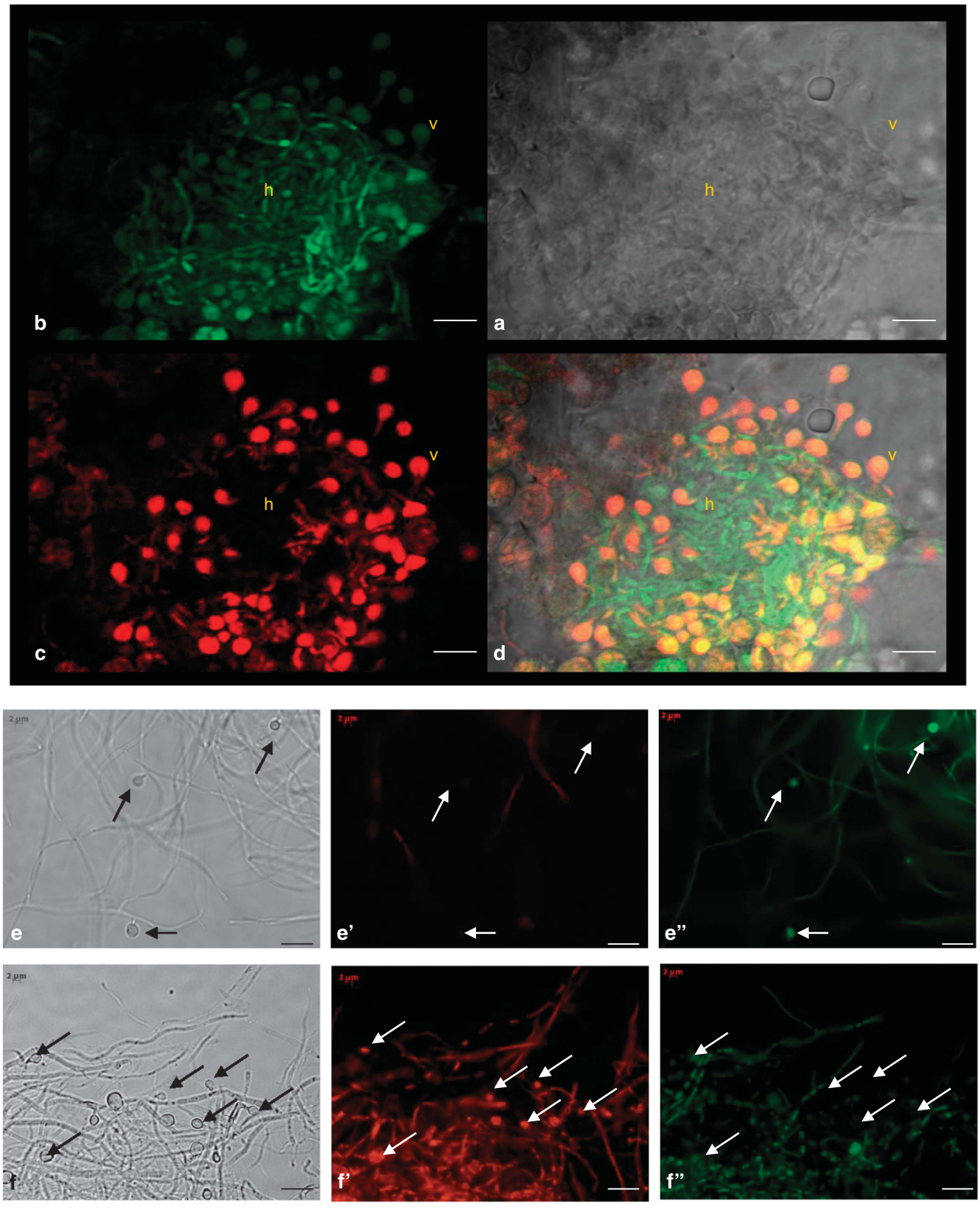

Figure 3 Vesicle permeability in nodules and *in vitro*. SYTO9–PI analysis shows porosity differences between *Frankia* hyphae and vesicles in fresh nodule sections (scale 5 µm) observed by confocal microscopy (**a**, **b**, **c**, **d**) and in *Frankia* cultures without Ag5 (**e**, **e′**, **e″**) and with 1 µM Ag5 (**f**, **f′**, **f″**) (scale 10 µm) observed by fluorescence microscopy. Phase-contrast (**a**, **e**, **f**); green fluorescence with SYTO9 (**b**, **e″**, **f″**); red fluorescence with PI (**c**, **e′**, **f′**); superimposed image (**d**). h, hyphae; v, vesicles. Arrows indicate vesicles.

Our results showed the release of amino acids from cultured *Frankia* cells to the media, induced by the presence of Ag5 ASUP. The major amino acid released from Ag5-treated *Frankia* is glutamine. Enzyme activity analyses of *Frankia* grown in N_2-free media have shown an important activity of

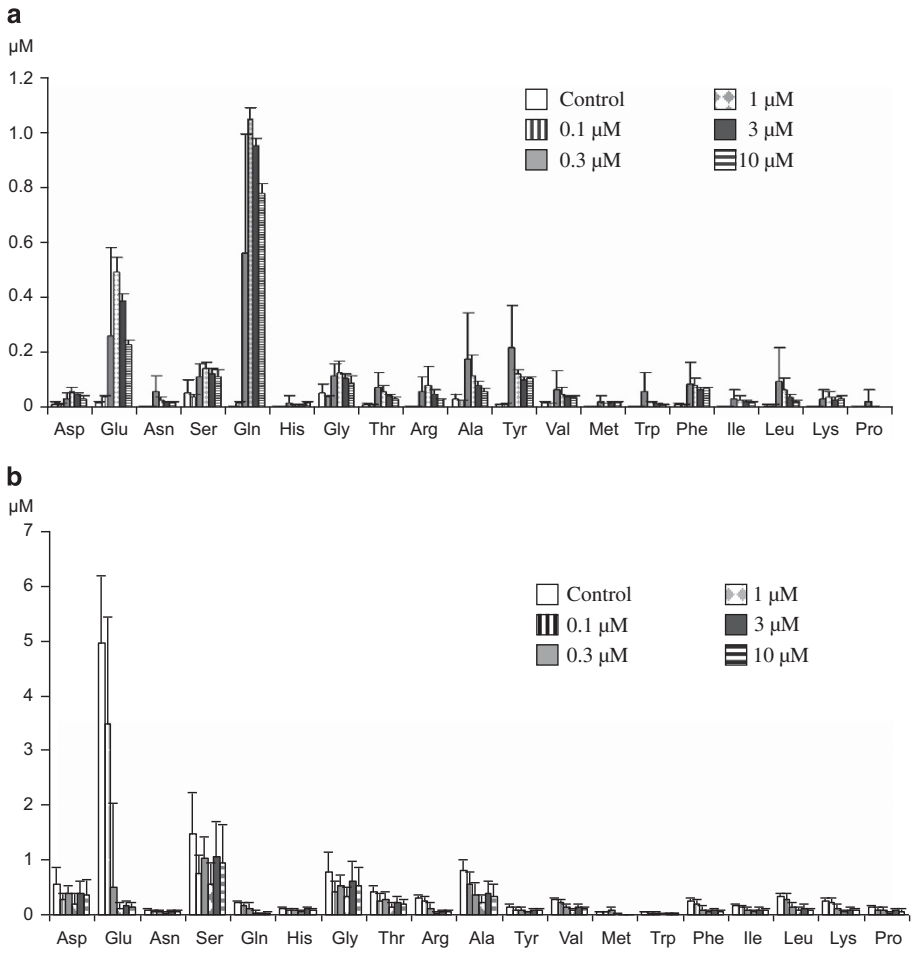

Figure 4 Release of amino acids from N-fixing *Frankia* cultures following treatment with Ag5 ASUP after 7 days in phosphate-buffered saline (PBS). (**a**) Amino acids in supernatants and (**b**) amino acids in *Frankia* cells. Error bars are s.d.

glutamine synthetase (GS) in vesicles and hyphae, whereas glutamine oxo-glutarate amino transferase (GOGAT), glutamate dehydrogenase (GDH) and alanine dehydrogenase (AlaDH) are restricted to hyphae (Schultz and Benson, 1990). Thus, the increased level of Gln in the supernatant agrees with this previous study and with the strong reduction in the cellular glutamic acid concentration after Ag5 treatment (Figure 4). However, the total amount of Gln in cells treated with Ag5 is higher than the amounts measured in control cells. This could be because of a change in enzymatic activities (for example, restriction of further assimilation for purine biosynthesis and so on) following Ag5 treatment. Another possibility is release of NH_4^+ to the supernatant, and this release could trigger the very fast formation of Gln from Glu inside the cells as shown by Berry *et al.* (1990). The efflux of glutamate from *in vitro* grown cells may be simply because of the fact that glutamate is the most abundant amino acid in *F. alni* cells (Berry *et al.*, 1990) linked to its position at the crossroads of many metabolic pathways (Kissen *et al.*, 2010). In symbiotic tissues, enzymatic activities related to ammonia assimilation are on several points different from those in pure cultures, with, in particular, a GS activity that is mostly restricted to the plant host cells (Hirel *et al.*, 1982; Guan *et al.*, 1996), resulting in a lowered Glu–Gln cycling in the *Frankia* vesicles and an expected NH_4^+ accumulation. Transcriptomic analyses also point out that in nodules *Frankia* GSII expression level is low compared with nitrogen-fixing condition in the free-living state (Alloisio *et al.*, 2010). Thus, ammonium could be the major nitrogen compound exuded from the bacteria to the host cells helped by the permeability increase because of Ag5 and possibly other ASUPs.

An ideal confirmation for our results would be the knockdown of *Ag5* expression by RNA interference mediated by *Agrobacterium* transformation of *Alnus* to determine its effect in nodules. However, the presence of several homologous peptides with probably related functions makes this approach unlikely to be successful. Moreover, previous *Alnus* transformation experiences have always failed or only transitorily transformed protoplasts were obtained (Mackay *et al.*, 1988; Franche *et al.*, 1998).

In recent years, reports about small secreted cysteine-rich peptides that control many aspects of plant signaling have emerged. Their known

functions range from the control of reproduction to defense against pathogens (Marshall *et al.*, 2011). The role of antimicrobial peptides has been well studied in the context of host–pathogens interactions (Maroti *et al.*, 2011), and more recently in the symbiosis between the legume *Medicago* and *Sinorhizobium* where the NCR peptides block division of endosymbiotic cells, resulting in endoreduplication of the intracellular rhizobia (Van de Velde *et al.*, 2010). Here we describe a new role for the antimicrobial peptide Ag5. Our work shows that the membrane porosity of *in vitro* nitrogen-fixing *Frankia* is modified by Ag5 to release Gln and Glu. This suggests that this peptide and possibly also the other ASUP peptides are implicated in trophic exchanges between the endosymbiont and the host. Our results suggest that these peptides act as host effectors that are plugged into the membrane of the endosymbionts and form specific channels permitting to release nutrients from the nitrogen-fixing *Frankia* vesicles. This work paves the way for the study of the *Frankia* symbiosis system, in which further studies of other ASUPs and their functions should be pursued.

Conflict of Interest

The authors declare no conflict of interest.

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References

Alloisio N, Queiroux C, Fournier P, Pujic P, Normand P, Vallenet D *et al.* (2010). The *Frankia alni* symbiotic transcriptome. *Mol Plant Microbe Interact* **23**: 593–607.

Ampe F, Kiss E, Sabourdy F, Batut J. (2003). Transcriptome analysis of *Sinorhizobium meliloti* during symbiosis. *Genome Biol* **4**: R15.

Beijerinck MW. (1901). Über oligonitrophile Mikroben. *Zentr Bakt Parasitenk Infektionskrank Hyg, Abt II* **7**: 561–582.

Benson DR, Silvester WB. (1993). Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. *Microbiol Rev* **57**: 293–319.

Benson DR, Brooks JM, Huang Y, Bickhart DM, Mastronunzio JE. (2011). The biology of *Frankia* sp. strains in the post-genome era. *Mol Plant Microbe Interact* **24**: 1310–1316.

Berry AM, Thayer JR, Enderlin CS, Jones AD. (1990). Patterns of (N-13) ammonium uptake and assimilation by *Frankia* HFPArI3. *Arch Microbiol* **154**: 510–513.

Berry AM, Harriott OT, Moreau RA, Osman SF, Benson DR, Jones AD. (1993). Hopanoid lipids compose the *Frankia* vesicle envelope, presumptive barrier of oxygen diffusion to nitrogenase. *Proc Natl Acad Sci USA* **90**: 6091–6094.

Boulos L, Prevost M, Barbeau B, Coallier J, Desjardins R. (1999). LIVE/DEAD BacLight: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *J Microbiol Methods* **37**: 77–86.

Brogden KA. (2005). Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat Rev Microbiol* **3**: 238–250.

Chapin IFS, Matson PA, Mooney HA. (2002). *Principles of Terrestial Ecosystem Ecology*. Springer.

Demina IV, Persson T, Santos P, Plaszczyca M, Pawlowski K. (2013). Comparison of the nodule vs. root transcriptome of the actinorhizal plant *Datisca glomerata*: actinorhizal nodules contain a specific class of defensins. *PLoS One* **8**: e72442.

Fant F, Vranken WF, Borremans FA. (1999). The three-dimensional solution structure of *Aesculus hippocastanum* antimicrobial protein 1 determined by 1H nuclear magnetic resonance. *Proteins* **37**: 388–403.

Franche C, Laplaze L, Duhoux E, Bogusz D. (1998). Actinorhizal symbioses: recent advances in plant molecular and genetic transformation studies. *Crit Rev Plant Sci* **17**: 1–28.

Guan C, Ribeiro A, Akkermans AD, Jing Y, van Kammen A, Bisseling T *et al.* (1996). Nitrogen metabolism in actinorhizal nodules of *Alnus glutinosa*: expression of glutamine synthetase and acetylornithine transaminase. *Plant Mol Biol* **32**: 1177–1184.

Guan C, Akkermans ADL, van Kammen A, Bisseling T, Pawlowski K. (1997). ag13 is expressed in *Alnus glutinosa* nodules in infected cells during endosymbiont degradation in the nodule pericycle. *Physiol Plant* **99**: 601–607.

Haag AF, Baloban M, Sani M, Kerscher B, Pierre O, Farkas A *et al.* (2011). Protection of *Sinorhizobium* against host cysteine-rich antimicrobial peptides is critical for symbiosis. *PLoS Biol* **9**: e1001169.

Heath K, Burke P, Stinchcombe J. (2012). Coevolutionary genetic variation in the legume-rhizobium transcriptome. *Mol Ecol* **21**: 4735–4747.

Henderson J, Ricker R, Bidlingmeyer B, Woodward C. (2000). Rapid, accurate, sensitive, and reproducible HPLC analysis of aminoacids. Amino acid analysis using Zorbax Eclipse-AAA columns and the Agilent 1100 HPLC. Agilent Technologies Inc.: Santa Clara, CA, USA, Publication Number **5980-1193E**: 1–10.

Hirel B, Perrot-Rechenmann C, Maudimas B, Gadal P. (1982). Glutamine synthetase in alder (*Alnus glutinosa*) root nodules. Purification, properties and cytoimmunochemical localization. *Physiol Plant* **55**: 197–203.

Hocher V, Alloisio N, Auguy F, Fournier P, Doumas P, Pujic P *et al.* (2011). Transcriptomics of actinorhizal symbioses reveals homologs of the whole common symbiotic signaling cascade. *Plant Physiol* **156**: 700–711.

Kissen R, Winge P, Tran DH, Jorstad TS, Storseth TR, Christensen T *et al.* (2010). Transcriptional profiling of an Fd-GOGAT1/GLU1 mutant in *Arabidopsis thaliana* reveals a multiple stress response and extensive reprogramming of the transcriptome. *BMC Genomics* **11**: 190.

Login FH, Balmand S, Vallier A, Vincent-Monegat C, Vigneron A, Weiss-Gayet M *et al.* (2011). Antimicrobial peptides keep insect endosymbionts under control. *Science* **334**: 362–365.
Mackay J, Seguin A, Lalonde M. (1988). Genetic transformation of 9 in vitro clones of *Alnus* and *Betula* by *Agrobacterium tumefaciens*. *Plant Cell Rep* **7**: 229–232.
Maroti G, Kereszt A, Kondorosi E, Mergaert P. (2011). Natural roles of antimicrobial peptides in microbes, plants and animals. *Res Microbiol* **162**: 363–374.
Marshall E, Costa LM, Gutierrez-Marcos J. (2011). Cysteine-rich peptides (CRPs) mediate diverse aspects of cell–cell communication in plant reproduction and development. *J Exp Bot* **62**: 1677–1686.
Mergaert P, Nikovics K, Kelemen Z, Maunoury N, Vaubert D, Kondorosi A *et al.* (2003). A novel family in *Medicago truncatula* consisting of more than 300 nodule-specific genes coding for small, secreted polypeptides with conserved cysteine motifs. *Plant Physiol* **132**: 161–173.
Murry M, Fontaine M, Torrey J. (1984). Growth kinetics and nitrogenase induction in *Frankia* sp. HFPArI3 grown in batch culture. *Plant Soil* **78**: 61–78.
Normand P, Lalonde M. (1982). Evaluation of *Frankia* strains isolated from provenances of two *Alnus* species. *Can J Microbiol* **28**: 1133–1142.
Oldroyd GE, Murray JD, Poole PS, Downie JA. (2011). The rules of engagement in the legume-rhizobial symbiosis. *Annu Rev Genet* **45**: 119–144.
Pawlowski K, Bogusz D, Ribeiro A, Berry AM. (2011). Progress on research on actinorhizal plants. *Funct Plant Biol* **38**: 633–638.
Petersen TN, Brunak S, von Heijne G, Nielsen H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat Methods* **8**: 785–786.
Prin Y, Neyra M, Diem HG. (1990). Estimation of *Frankia* growth using Bradford protein and INT reduction activity estimations: application to inoculum standardization. *FEMS Microbiol Lett* **69**: 91–95.
Rozen S, Skaletsky HJ. (2000). Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds). *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press: Totowa, NJ, pp 365–386.
Schultz NA, Benson DR. (1990). Enzymes of ammonia assimilation in hyphae and vesicles of *Frankia* sp. strain CpI1. *J Bacteriol* **172**: 1380–1384.
Schwencke J. (1991). Rapid, exponential growth and increased biomass yield of some *Frankia* strains in buffered and stirred mineral medium (BAP) with phosphatidyl choline. *Plant Soil* **137**: 37–41.
Thevissen K, Terras FRG, Broekaert WF. (1999). Permeabilization of fungal membranes by plant defensins inhibits fungal growth. *Appl Environ Microbiol* **65**: 5451–5458.
Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**: 4876–4882.
Tisa LS, Richards JW. (2004). Metabolic capacity of purified vesicles from *Frankia* EAN1pec. *Symbiosis* **37**: 13.
Tromas A, Parizot B, Diagne N, Champion A, Hocher V, Cissoko M *et al.* (2012). Heart of endosymbioses: transcriptomics reveals a conserved genetic program among arbuscular mycorrhizal, actinorhizal and legume-rhizobial symbioses. *PLoS One* **7**: e44742.
Van de Velde W, Zehirov G, Szatmari A, Debreczeny M, Ishihara H, Kevei Z *et al.* (2010). Plant peptides govern terminal differentiation of bacteria in symbiosis. *Science* **327**: 1122–1126.
Wyss O, Lind CJ, Wilson H, Wilson PW. (1941). Mechanism of biological nitrogen fixation. *J Biochem* **35**: 845–854.

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