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(Article begins on next page)

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Fungal and plant gene expression in the *Tulasnella calospora* - *Serapias vomeracea* symbiosis provides cues on N pathways in orchid mycorrhiza

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Summary

• Orchids are highly dependent on their mycorrhizal fungal partners for nutrient supply, especially during early developmental stages. In addition to organic carbon, nitrogen (N) is likely a major nutrient transferred to the plant because orchid tissues are highly N-enriched. We know almost nothing on the N form preferentially transferred to the plant and on the key molecular determinants required for N uptake and transfer.

• We identified, in the genome of the orchid mycorrhizal fungus *Tulasnella calospora*, two functional ammonium transporters and several amino acid transporters but no evidence of a nitrate assimilation system, in agreement with N preference of the free living mycelium grown on different N sources.

• Differential expression in symbiosis of a repertoire of fungal and plant genes involved in transport and metabolism of N compounds suggests that organic N may be the main form transferred to the orchid host and that ammonium is taken up by the intracellular fungus from the apoplatic symbiotic interface.

• This is the first study addressing the genetic determinants of N uptake and transport in orchid mycorrhiza, and provides a model for nutrient exchanges at the symbiotic interface, which may guide future experiments.

Keywords: orchid mycorrhiza, *Tulasnella*, *Serapias*, nitrogen

Introduction

Like the majority of terrestrial plants, orchids form mycorrhizal associations with soil fungi that provide them with essential nutrients. However, orchids are peculiar because seed germination and early development in nature fully depend on the mycobionts (Rasmussen, 1995; Smith & Read, 2008), that provide the embryo with organic carbon (C) and other essential nutrients. Following seed germination, orchids form the protocorm, a heterotrophic structure that precedes seedling development and continue to rely on their mycobionts for organic C supply, a strategy known as mycoheterotrophy (Leake, 2004). Some orchid species remain achlorophyllous or with inefficient

photosynthesis at adulthood (Selosse & Roy, 2009; Hynson *et al.,* 2013), whereas most orchids develop photosynthetic leaves and become fully autotrophic. These photosynthetic orchid species usually associate with saprotrophic fungi belonging to the anamorphic form-genus *Rhizoctonia*, featuring basidiomycete members in the Ceratobasidiaceae, Tulasnellaceae and Sebacinales (Taylor *et al.,* 2002; Weiß *et al.,* 2004).

Given the fascinating mycoheterotrophic strategy of orchids as mycorrhizal plant parasites, most experiments on nutrient transfer in orchid mycorrhiza (ORM) have focussed on the acquisition and transfer of organic C, whereas little is known about other nutrients, such as nitrogen. Nitrogen (N) is often a limiting factor for plant growth in natural ecosystems (Vitousek & Howarth, 1991). Particularly high concentrations of total N have been reported in the tissues of many orchids (see Hynson *et al.,* 2013) and measurements of stable isotope natural abundance, commonly used to identify the source and direction of nutrient flow in ecological systems (Dawson *et al.,* 2002), provide evidence that terrestrial orchids receive N from their mycobionts (Gebauer & Meyer, 2003; Hynson *et al.,* 2013; Stöckel *et al.,* 2014).

Nitrogen occurs in soil as inorganic forms, such as ammonium ($NH4^+$) and nitrate ($NO3^-$), or as organic N compounds. Fungal uptake and transfer of soil-derived N to the host plant has been extensively investigated in arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) fungi (see references in Chalot *et al.,* 2006; Martin *et al.,* 2007; Müller *et al.,* 2007; Koegel *et al.,* 2015; Bücking & Kafle, 2015). Nitrate transporters and genes responsible for nitrate utilization have been identified mainly in AM fungi (Kaldorf *et al.,* 1998; Tian *et al.,* 2010; Koegel *et al.,* 2015), and nitrophilous ECM fungi (Plassard *et al.,* 2000; Jargeat *et al.,* 2003; Montanini *et al.,* 2006). Genes coding for high- and low-affinity ammonium transporters have been identified and characterized in both AM (López-Pedrosa *et al.,* 2006; Perez-Tienda *et al.,* 2011; Calabrese *et al.,* 2016) and ECM fungi (Montanini *et al.,* 2002; Javelle *et al.,* 2001; Willmann *et al.,* 2007). Amino acid permeases and peptide transporters, that allow for organic N uptake, have been identified and characterized mainly in ECM fungi (Nehls *et al.,* 1999; Wipf *et al.,* 2002a; Guidot *et al.,* 2005; Benjdia *et al.,* 2006; Shah *et al.,* 2013) but also in AM fungi (Cappellazzo *et al.,* 2008; Belmondo *et al.,* 2014), although the ability of AM fungi to utilize organic N sources is considered to be relatively low. Ammonium is thought to be the main N form transferred to the host plant in AM (Koegel *et al.,* 2015; Bücking & Kafle, 2015), whereas N transfer across the ECM interface is not fully clear and may involve both organic (i.e. amino acids) and inorganic nitrogen forms (Chalot *et al.,* 2006; Couturier *et al.,* 2007; Müller *et al.,* 2007).

Compared to ECM and AM fungi, very little is known on N metabolism in orchid mycorrhiza. Some ORM fungi belonging to *Ceratobasidium*, *Tulasnella* and *Sebacina* can grow on organic N as well as on ammonium (Hadley & Ong, 1978; Nurfadilah *et al.*, 2013). In addition, a *Ceratobasidium* spp. isolate also grew on nitrate (Nurfadilah *et al.*, 2013). Uptake of N from inorganic sources by a *Ceratobasidium* isolate and transfer to the host protocorm was demonstrated by Kuga *et al.* (2014) after feeding the fungus with ^{15}N-labelled NH_4NO_3. Cameron *et al.* (2006) demonstrated uptake of double-labelled [^{13}C-^{15}N] glycine by *C. cornigerum* and transfer of labelled ^{15}N to the adult photosynthetic host *Goodyera repens*. However, the mechanisms underlying fungal N uptake and transfer to the plant, as well as the form of N transferred to the orchid host, remain unknown.

Here, we used the ORM *Tulasnella calospora* isolate AL13/4D, either as free-living mycelium (FLM) or in symbiosis with achlorophyllous protocorms of the photosynthetic orchid host *Serapias vomeracea*, as a model system to explore the genetic bases of N uptake and transfer to the ORM host plant. We show that *T. calospora* lacks a nitrate uptake system but, like other mycorrhizal fungi, has functional ammonium transporters (AMTs). Two AMT coding genes were identified in the *T. calospora* genome (*TcAMT1* and *TcAMT2*) and were characterized by functional complementation in yeast. The expression pattern of these fungal AMTs, together with the expression of additional *T. calospora* and *S. vomeracea* genes potentially involved in N uptake and transfer, allowed us to formulate hypotheses on the N pathway in ORM.

Materials and methods

Growth of the free-living mycelium (FLM)

Tulasnella calospora AL13/4D was isolated from mycorrhizal roots of *Anacamptis laxiflora* in Northern Italy (Girlanda *et al.*, 2011) and deposited in the mycological collection of the University of Turin (MUT4182). FLM was maintained on solid 2% Malt Extract Agar at 25°C. To evaluate growth on different N sources, 8 hyphal plugs (6 mm diameter) of 20-days-old *T. calospora* mycelia were transferred into flasks containing 50 ml of modified synthetic Pachlewski P5 liquid medium (Kemppainen & Pardo, 2011) with L-glutamine, monosodium glutamate, ammonium tartrate, sodium nitrate or ammonium sulphate, each added as 0.49g of N. Inoculated flasks were maintained at 25°C under constant shaking (120 rpm). After 20 days, the mycelium was recovered by filtration, washed with distilled water, weighted and dried to obtain biomass. Three replicate flasks were used for each N source.

Modified P5 solid medium with the same N sources, as well as oat agar medium (0.3% milled oats, 1% agar), were used to grow FLM for RNA extraction and expression studies. In this case, *T. calospora* was inoculated onto a sterilized cellophane membrane placed on top of the agar medium (Schumann *et al.,* 2013) and kept until the plate was fully colonised (about 20 days). The mycelium was then collected from the cellophane membrane, immediately frozen in liquid nitrogen and stored at -80°C.

Symbiotic and asymbiotic germination of S. vomeracea seeds

Symbiotic germination was obtained by co-inoculation of mycorrhizal fungi and orchid seeds in 9 cm Petri dishes, as described in Ercole *et al.* (2013). Seeds of *S. vomeracea* were surface sterilized in 1% sodium hypochlorite and 0.1% Tween-20 for 20 minutes on a vortex, followed by three 5-minutes rinses in sterile distilled water. Seeds were re-suspended in sterile water and dropped on strips of autoclaved filter paper (1.5 x 3 cm) positioned on solid oat medium (0.3% milled oats, 1% agar). Plates were inoculated with a plug of actively growing *T. calospora* mycelium and were incubated at 20°C in full darkness. Asymbiotic seed germination was obtained on modified BM culture media (Van Waes & Deberg, 1986) at 20°C in darkness. Symbiotic and asymbiotic achlorophyllous protocorms collected at stage P2 (Otero *et al.,* 2004) were either frozen immediately in liquid nitrogen and stored at -80 °C for RNA extraction, or fixed and embedded in paraffin for laser microdissection or in resin for microscopy.

Gene identification and phylogenetic analysis

Fungal genes coding for proteins possibly involved in N uptake and transfer were identified in the *T. calospora* genome database on the JGI fungal genome portal MycoCosm (http://genome.jgi.doe.gov/Tulca1/Tulca1.home.htm). Plant transcripts coding for proteins potentially involved in N uptake and transfer were selected from the RNASeq database obtained in this study as described below. Multiple protein alignments were done with MUSCLE (Edgar, 2004). Phylogenetic trees were constructed with the maximum likelihood method using the MEGA software, Version 7.0 (Kumar *et al.,* 2015); bootstrap analyses were conducted on the basis of 1,000 re-samplings of the sequence alignment.

Full-length TcAMT1 and TcAMT2 isolation

TcAMT1 (1467 bp) and *TcAMT2* (1611 bp) full length cDNA isolation was carried out through PCR amplification of *T. calospora* or mycorrhizal *S. vomeracea* protocorms cDNA with the full-length primers reported in Table S1. PCR reactions were performed in 50 µl final volume containing 10 µl of 5X Phusion HF Buffer, 1 µl of dNTPs (10mM stock each), 1.5 µl of each primer (10 mM stock), 0.5 µl of Phusion High-Fidelity DNA polymerase (NEB, USA), 34.5 µl of water and 1 µl of cDNA. PCR amplifications were carried out in a thermal cycler (Biometra) using the following program: 98 C for 60 s; 35 cycles of 98 C for 10 s, 57 C for 10 s and 72 C for 90 s; 72 C for 10 min. Amplicons were visualised on 1.2% agarose gel after electrophoresis in 0.5X TAE buffer, excised from the agarose gel and purified using Wizard® SV Gel and PCR Clean-Up System following the manufacturer's instruction. The purified DNA was eluted in 30 µl of Nuclease-Free Water and inserted into the p-GEM T plasmid (Promega) using a T4 DNA Ligase (Promega). Plasmids were transformed into *E. coli* chemically competent cells (Top10 cells, Invitrogen). Plasmids were then purified, from positive colonies, using QIAprep Spin Miniprep Kit (QIAGEN). Sequencing was performed at the sequencing service at Munich University (Sequencing Server, Biocenter of the LMU Munich, Germany).

Heterologous expression of TcAMT1 and TcAMT2 in yeast

Full-length *TcAMT1* and *TcAMT2* cDNAs were cloned into the yeast expression vector pFL61 under the control of the constitutive yeast phosphoglycerate kinase (PGK) promoter (Minet *et al.*, 1992). The *Saccharomyces cerevisiae* triple mep mutant 31019b (ΔΔΔmep1;2;3; Marini *et al.*, 1997), kindly provided by Nuria Ferrol (CSIC, Granada, Spain), was transformed with the empty pFL61 vector (negative control) or with the pFL61-*TcAMT1* and pFL61-*AMT2* constructs according to Gietz & Schiestl (2007). The coding sequence of *GintAMT1*, characterised in the AM fungus *Rhizophagus irregularis* (previously *Glomus intraradices*; López-Pedrosa *et al.*, 2006), was used as a positive control. Transformed yeasts were selected on solid N-free medium (2% Agar, 0.17% YNB w/o amino acids and ammonium sulphate) supplemented with 3% glucose and 0.1% arginine as the sole N source, buffered to pH 6.1 with 50 mM MES/Tris. For growth assays, yeast transformants were grown in liquid N-free medium plus arginine until OD600 reached 0.6–0.8. Cells were harvested, washed twice, resuspended in water to a final OD600 of 2, and 10 µl drops corresponding to a serial 10-fold dilutions were spotted on solid N-free medium supplemented with different NH_4Cl concentrations as the sole N source (0.1, 0.5, 1 and 5 mM). Yeast cells were also

spotted on 1 mM NH_4Cl-supplemented medium buffered at different pH (4.5, 5.5, 6.5, 7.5) with 50 mM Mes/Tris. Pictures of the plates were taken 3 days incubation at 30°C.

RNA-Seq experiment

Two different RNASeq experiments were carried out to investigate expression of *T. calospora* and *S. vomeracea* genes, and the experimental setup is illustrated in Figure S1. In the first experiment (RNASeq-A), *T. calospora* transcripts were investigated in FLM and in mycorrhizal *S. vomeracea* protocorms grown on solid oat medium (0.3% milled oats, 1% agar). Three independent libraries were prepared from three biological replicates. Preparation of libraries and 2x100bp Illumina HiSeq2000 mRNA sequencing (RNA-Seq) was performed at the IGA Technology Services facilities (Udine, Italy). The complete series was submitted to GEO (**GSE63869**).

For the second experiment (RNASeq-B), *T. calospora* FLM was grown on Modified Melin-Norkrans medium with two different N sources, ammonium and glutamine, each added as 0.49g of N. For each condition, three separate libraries were prepared from three biological replicates. To investigate fungal and plant gene expression in symbiosis, RNA was also extracted in a parallel experiment from symbiotic and asymbiotic *S. vomeracea* protocorms obtained as described above. Independent libraries were prepared from three replicates for each condition. Illumina HiSEq2500 sequencing (2x150bp) was performed at the Joint Genome Institute (JGI, Walnut Creek, USA). The complete series was submitted to GEO (**XXXX**).

De novo assembly of *S. vomeracea*: Filtered fastq files of plant-only samples were used as input for *de novo* assembly of *S. vomeracea* RNA contigs (Fig. S1). Reads were assembled into consensus sequences using Trinity (ver. 2.1.1) (Grabherr *et al.* 2011). Trinity partitions the sequence data into many individual de Bruijn graphs, each representing the transcriptional complexity at a given gene or locus, and then processes each graph independently to extract full-length splicing isoforms and to tease apart transcripts derived from paralogous genes. Trinity was run with the --normalize_reads (In-silico normalization routine) and --jaccard_clip (Minimizing fusion transcripts derived from gene dense genomes) options. Contigs were annotated following BlastX searches against the *Arabidopsis thaliana* (TAIR) and the *Phalaenopsis equestris* (Cai *et al.*, 2015) proteome.

Reads Alignments: Reads were aligned either to the *T. calospora* reference transcripts (http://genome.jgi-psf.org) or to the *S. vomeracea de novo* assembly using CLC Genomics Workbench. For mapping, the minimum length fraction was 0.9, the minimum similarity fraction 0.8 and the maximum number of hits for a read was set to 10. The unique and total mapped reads number for each transcript were determined, and then normalized to RPKM (Reads Per Kilobase of

exon model per Million mapped reads). The Baggerly et al.'s Test (Baggerly *et al.,* 2003) implemented in CLC Genomic workbench was applied to the data. In addition, Benjamini & Hochberg multiple-hypothesis testing corrections with False Discovery Rate (FDR) were used. In our analysis, transcripts were considered to be up-regulated when FC≥2.5 and FDR<0.05, and down-regulated when FC≤0.5 and FDR<0.05.

Real Time quantitative PCR (RT-qPCR) analyses

Total RNA for RT-qPCR was extracted from symbiotic and asymbiotic *S. vomeracea* protocorms and from *T. calospora* FLM following the method of Chang *et al.* (1993). Genomic DNA was removed using the Turbo DNA-free TM reagent (Ambion, Austin, TX, USA), according to the manufacturer's instructions. RNA was then quantified using spectrophotometry (NanoDrop 1000, BioRad) and subjected to reverse-transcription PCR (RT-PCR) to exclude DNA contamination, using the One Step RT-PCR kit (Qiagen), before cDNA synthesis. SuperScriptII Reverse Transcriptase (Invitrogen) was used to synthesize cDNA starting from 500 ng of total RNA for each sample, following the manufacturer's instructions. At the end of the reaction, cDNA was diluted 1:5 for quantitative gene expression analysis (RT-qPCR). Primers for RT-qPCR (Table S1) were designed using Primer3plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) and tested for their specificity with Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Reactions were carried out in a StepOnePlus™ RT-qPCR System (Applied Biosystems), following the SYBR Green method (Power SYBR® Green PCR Master Mix, Applied Biosystems) as described by Perrone *et al.* (2012). Thermal cycling conditions were as follows: initial denaturation phase at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Expression of target transcripts was quantified after normalisation to the geometric mean of the endogenous control genes, Elongation Factors (*TcEF-1α, SvEF-1α*). Gene expression data were calculated as expression ratios (relative quantity, RQ) to controls (FLM on oat medium). All reactions were performed with three biological and three technical replicates.

Laser Microdissection (LMD) analysis

Symbiotic and asymbiotic *S. vomeracea* protocorms were collected in RNase-free tubes containing freshly prepared Farmer's fixative (absolute ethanol/glacial acetic acid 3:1). Samples were dehydrated and embedded in paraffin as described in Perez-Tienda *et al.* (2011) with minor modifications. Sections (12 μm) cut with a rotary microtome were transferred onto Leica RNase-

free PEN foil slides (Leica Microsystem, Inc., Bensheim, Germany), dried at 40 °C in a warming plate, stored at 4 °C and used within 1 day. Specific cell types were collected from paraffin sections of *S. vomeracea* symbiotic protocorms with a Leica LMD 6500 system (Leica Microsystem, Inc.), as described by Balestrini *et al.* (2007). Approximately 1000-1500 cells for each type were collected, with three independent biological replicates. RNA was extracted from LMD cells following the Pico Pure kit (Arcturus Engineering) protocol, with some modifications. In particular, DNase treatment was not performed on the kit column, but RNA was treated with Turbo DNA-free (Ambion, Austin, TX, USA), according to the manufacturer's instructions.

The One Step RT-PCR kit (Qiagen, Valencia, CA, USA) was used to amplify transcripts from three biological replicates. Samples were incubated for 30 min at 50°C, followed by 15 min of incubation at 95°C. Amplification reactions were run for 40 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 40 s using the same *T. calospora* and *S. vomeracea* specific primers used for RT-qPCR (Table S1). DNA contamination in the RNA samples was evaluated with primers for the plant (*SvEF1α*) and the fungal (*TcEF1α*) elongation factor by omitting the RT step at 50°C (Fig. S2). PCR products were separated on a 1.4% agarose gel.

Microscopy

Symbiotic *S. vomeracea* protocorms were fixed in 2.5% (v/v) glutaraldehyde, post-fixed in 1% (w/v) osmium tetroxide and embedded in LR White resin (Polysciences, Warrington, PA, USA) as described in Perotto *et al.* (2014). Semi-thin sections (1 μm) were stained with 1% (w/v) toluidine blue for morphological observations. Thin sections (0.05-0.07 μm) were post-stained with uranyl acetate and lead citrate before being observed under a Philips CM10 transmission electron microscope (Philips, Eindhoven, The Netherlands).

Statistical analysis

Significant differences among treatments (*T. calospora* biomass and RT-qPCR experiments) were tested by a one-way analyses of variance (ANOVA) test, and Tukey's HSD test was used for mean separation when ANOVA results were significant ($P<0.05$). Significant differences of pairwise comparisons were assessed by Student's *t*-test. The SPSS statistical software package (version 23.0; SPSS Inc., Cary, NC, USA) was used to run statistical analyses.

Results

Growth of Tulasnella calospora on different nitrogen sources

After 20 days of culture on modified Pachlewski P5 medium containing several defined organic and inorganic N sources, the highest fungal biomass (as dry weight) was on glutamine and the lowest one on nitrate (Fig. 1). Growth was intermediate on glutamate and ammonium tartrate and slightly but significantly lower ($P<0.05$) on ammonium sulphate (Fig. 1).

Identification of T. calospora genes involved in the uptake of inorganic N forms

The complete genome sequence of *T. calospora* (Kohler *et al.,* 2015) is available on the Mycocosm portal (http://genome.jgi.doe.gov/Tulca1/Tulca1.home.html) and was searched for fungal genes potentially involved in the uptake of inorganic N forms. Genes corresponding to nitrate uptake and assimilation (nitrate and nitrite reductases) could not be identified in the *T. calospora* genome. By contrast, three gene models coding for ammonium transporters (AMTs) were identified (corresponding to protein IDs 241632, 186135 and 10772). Only the first two, respectively named *TcAMT1* and *TcAMT2*, contained a signal peptide, whereas the third, shorter sequence showed only partial homology with AMTs from other fungi and was not investigated further. Searches in the protein sequence databases indicated for *TcAMT1* and *TcAMT2* a high similarity to AMTs proteins identified in other mycorrhizal basidiomycetes, such as *Hebeloma cilindrosporum* (76% and 69% identity with TcAMT1 and TcAMT2, respectively) or *Laccaria bicolor* (74% and 65% identity with TcMT1 and TcAMT2, respectively). Phylogenetic comparison with functionally characterized transporters from other fungi (Perez-Tienda *et al.,* 2011) showed that *TcAMT1* clustered with high-affinity transporters, whereas *TcAMT2* clustered with low-affinity transporters (Fig. 2).

Heterologous expression of TcAMT1 and TcAMT2 in a yeast mep mutant

To verify that *TcAMT1* and *TcAMT2* encode functional ammonium transporters, the corresponding cDNAs were constitutively expressed in the yeast triple *mep* mutant 31019b (Marini *et al.,* 1997). This strain is unable to grow on medium containing <5 mM NH_4^+ as the sole N source because is defective in all three endogenous Mep ammonium transporters. Both *TcAMT1* and *TcAMT2* were able to complement the growth defect of the mutant yeast strain in the presence of NH_4^+ (from 0.1 to 1mM) as the sole N source, demonstrating that they encode functional AMTs (Fig. 3). To assess if external pH affects their function, growth tests were performed at initial pH values ranging from

4.5 to 7.5 on minimal medium containing 1 mM NH_4^+ as the sole N source. Both transporters showed pH dependency, and growth promotion was best at acidic pH and strongly decreased at pH 7.5 (Fig. 3).

Expression of TcAMT1 and TcAMT2 on different N sources and in symbiosis

RNASeq and RT-qPCR experiments were used to investigate, both in the FLM and in symbiosis, expression of *TcAMT1* and *TcAMT2*, as well as of other *T. calospora* genes potentially involved in N uptake and metabolism. RNASeq expression data were derived from two separate experiments illustrated in (Fig. S1): RNASeq-A was run to identify *T. calospora* genes differentially expressed between symbiotic protocorms and free living mycelia (FLM), both obtained on oat meal. Another RNASeq experiment (RNASeq-B in Table 1) was run to investigate fungal gene expression patterns in FLM grown on two defined N sources, glutamine and ammonium. Gene expression in symbiosis was also measured in this second RNASeq experiment, in order to gain some insights on N regulation in the protocorm environment.

In the FLM, both *TcAMT* genes were expressed at a higher level on glutamine compared to ammonium, but the difference was statistically supported (FC<0.5) only for *TcAMT2* (RNASeq-B in Table 1). The results of RT-qPCR (Fig.4) on a wider range of N sources indicated a low level of *TcAMT1* and *TcAMT2* expression when *T. calospora* was grown on all N sources, with an increase on nitrate only significant for *TcAMT1* (P<0.05) (Fig. 4). Of the two *T. calospora* AMT genes, only *TcAMT2* was significantly upregulated in symbiosis (FC=3.6, P<0.05), whereas expression of *TcAMT1* was not significantly different from expression in the FLM (Table 1 and Fig. 4).

Fungal pelotons are thought to be key structures for nutrient exchanges in the symbiotic orchid protocorms (Fig. 5). Specific cell-type expression of the *TcAMT* genes in mycorrhizal *S. vomeracea* protocorms was analysed using laser microdissection (LMD), and transcripts corresponding to *TcAMT1* and *TcAMT2* were identified in LMD protocorm cells containing both younger (i.e. occupying the whole plant cell) and older (i.e. more condensed) fungal pelotons (Fig. S3).

Identification of T. calospora genes potentially involved in organic N uptake

In addition to inorganic nitrogen, the soil litter contains organic N forms such as amino acids, small peptides and proteins that can be absorbed by most fungi (Chalot & Brun, 1998). Genes coding for membrane proteins potentially involved in amino acid uptake were identified in *T. calospora*. Significant upregulation was recorded for only one amino acid transporter/permease gene (named *TcAAT9*) in FLM grown on glutamine (FC=3.8, P<0.05), as compared to ammonium (RNASeq –B

in Table 1). Using ammonium as the reference N-source, most of the other fungal amino acid transporters/permeases were not differentially expressed in the FLM grown on the two N sources, or they were down-regulated on glutamine (FC<0.5, P<0.05), like *TcAAT1* and *TcAAT11* (Table 1). Expression of some of the *T. calospora* amino acid transporters/permeases was regulated in symbiosis (RNASeq-A in Table 1). For example, *TcAAT1, TcAAT2* and *TcAAT6* were significantly upregulated in mycorrhizal protocorms (FC>2.5, $P<0.05$), as compared with FLM (Table 1). *TcAAT1* transcripts were also detected in colonized LMD protocorm cells (Fig.S3). The expression of the other amino acid transporter/permease coding genes identified in the transcriptome was unchanged, or even down-regulated in mycorrhizal protocorms (Table 1).

Nitrogen assimilation and pathways in T. calospora

Glutamine synthetase (GS) is an essential enzyme in N assimilation, and two genes coding for glutamine synthetase (named *TcGS1* and *TcGS2*) were identified in the *T. calospora* genome. Their expression in FLM did not change on ammonium or on glutamine containing media (Table 1), although *TcGS1* was more expressed (as raw reads number) and upregulated in symbiosis (FC=2.7; P<0.05). Both *TcGS1* and *TcGS2* transcripts could be amplified from LMD protocorm cells containing fungal pelotons (Fig. S3). The *T. calospora* gene coding for glutamate synthase, the other enzymes taking part to the GS/GOGAT nitrogen assimilation pathway, was also upregulated (FC=6.4, $P<0.05$) in mycorrhizal protocorms (Table 1).

As no information is currently available on the N pathways inside ORM, we investigated in *T. calospora* the expression of some genes identified in other mycorrhizal fungi. In particular, the urea cycle is a pathway reported for both AM (Tian *et al.*, 2010; Koegel *et al.*, 2015) and ECM fungi (Morel *et al.*, 2005; Wright *et al.*, 2005). Argininosuccinate lyase (ASL) is involved in arginine biosynthesis and is a key enzyme of the anabolic arm of the urea cycle; in the *T. calospora* FLM, the corresponding gene was found to be slightly induced by glutamine (FC=2.5, $P<0.05$), as compared to ammonium (Table 1). This gene was induced in symbiosis (FC=2.9, $P<0.05$). Arginase and urease are two enzymes of the catabolic arm of the urea cycle and are involved in arginine breakdown; in the current model of N pathway in AM, they are thought to release ammonium from arginine in the intraradical hyphae of AM fungi (Tian *et al.*, 2010). The expression of the corresponding *T. calospora* genes was investigated by RNASeq (Table 1). Only one of the three *T. calospora* gene models coding for arginase (*TcCAR*, protein ID 179058) was found in the transcriptome, but it was not differentially expressed on different N sources or in symbiosis. By

contrast, *TcURE*, coding for the *T. calospora* urease (protein ID 242909), showed a strong and significant down-regulation (FC=0.2, $P<0.05$) in symbiosis (Table 1).

***Serapias vomeracea* gene expression in symbiotic and asymbiotic protocorms**

Since the genome of *S. vomeracea* is not yet sequenced, a *de novo* assembly of *S. vomeracea* transcripts was generated and contigs were annotated by BlastX searches against the *A. thaliana* (TAIR) and the *P. equestris* (Cai *et al.*, 2015) proteome. Although *de novo* assembly was highly fragmented and only included sequences derived from asymbiotic protocorms, it represented a useful tool to identify genetic functions that were then validated by RT-qPCR. A list of contigs that, based on their annotation in *A. thaliana*, may be related to N uptake in *S. vomeracea* and their level of expression in symbiotic and asymbiotic protocorms can be found in Table 2. Two contigs annotated as ammonium transporters, named respectively *SvAMT1* and *SvAMT2*, showed upregulation in symbiotic protocorms, but their induction was not statistically significant in the RNASeq experiment (Table 2). RT-qPCR demonstrated that *SvAMT1* was slightly but significantly upregulated (FC=2.5, $P<0.05$) in symbiotic protocorms, whereas *SvAMT2* was not regulated (Fig. 6). Phylogenetic comparison with plant AMTs (Guether *et al.*, 2009) confirmed that SvAMT1 and SvAMT2 cluster together with other members of the AMT1 and AMT2 subfamilies, respectively (Fig. S4).

Several contigs in the *S. vomeracea* transcriptome matched genes annotated as amino acid transporters/permeases in the *A. thaliana* and *P. equestris* genomes. Only contigs manually verified by BlastX and with FDR$\leq$ 0.05 are reported in Table 2. Some of them were validated by RT-qPCR, that confirmed the RNASeq results (Fig. 6). Two contigs coding for amino acid permeases (*SvAAP1* and *SvAAP2*) and a contig identified as a putative lysine histidine transporter (*SvLHT*) with very high identity with LHT1 transporters in Blastp searches, were the most upregulated in symbiotic protocorms (Table 2). Interestingly, the strong upregulation of *SvLHT* expression in symbiosis was mirrored by the upregulation of the biosynthetic pathways for lysine and histidine in the symbiotic fungus (RNASeq-A in Table 1). Histidinol dehydrogenase, the gene coding for the last steps in histidine biosynthesis, was significantly upregulated inside mycorrhizal protocorms (FC=5.7, $P<0.05$). One sequence corresponding to saccharopine dehydrogenase, the final enzyme responsible of lysine biosynthesis (protein ID 241089), was also upregulated (FC=4.4, $P<0.05$) in symbiosis (Table 1).

Because of the role of glutamine synthetase in N assimilation, we looked for the corresponding *S. vomeracea* contigs in the transcriptome (Table 2). Despite the expression level was not very high,

one contig (*SvGS)* was significantly upregulated (FC=245, $P<0.05$) in mycorrhizal protocorms (Table 2), a situation confirmed by RT-qPCR (Fig. 6).

Discussion

Nitrogen preference in orchid mycorrhizal fungi

Orchids are peculiar mycorrhizal partners because they acquires all nutrients through the fungal symbiont, including organic C, at least during the mycoheterotrophic life stages (Smith & Read, 2008; Selosse & Martos, 2014). For terrestrial orchids associated with *Tulasnella*, *Ceratobasidium* and *Sebacinales*, nutrients likely derive from C, N and P forms available in soil because these fungi are known saprotrophs that utilize soil nutrient sources for growth and survival (Waterman *et al.*, 2011; Smith & Read, 2008). Nurfadilah *et al.* (2013) reported different N source preference in ORM fungi because isolates in the genus *Tulasnella* could use ammonium but not nitrate as inorganic N forms, whereas isolates in the genus *Ceratobasidium* could use both ammonium and nitrate. Growth of *T. calospora* isolate AL13/4D on different N sources confirmed, like other cultivable ORM fungi (Hadley & Ong, 1978; Nurfadilah *et al.*, 2013), a preference for ammonium and organic N sources. The different ability of *Tulasnella* and *Ceratobasidium* to use inorganic N forms is likely explained by the fact that the *T. calospora* genome lacks genes involved in nitrate uptake and reduction, whereas these genes can be found in the recently sequenced genome of *Ceratobasidium*, available on the Mycocosm portal (http://genome.jgi.doe.gov/CerAGI/CerAGI.home.html). The ability of ORM fungi to use different N sources has important ecological implications because most photoautotrophic orchids host a diverse community of ORM fungi in their roots and protocorms, usually including both *Tulasnella* and *Ceratobasidium* species (see e.g. Girlanda *et al.*, 2011; Ercole *et al.*, 2015; Jacquemyn *et al.*, 2010). Co-occurrence of fungal symbionts able to utilize a wide variety of nutrient sources and to exploit different soil N forms would represent an advantage for the host plant because it could broaden the habitat range as well as the ability of the orchid to grow in a wide range of soil types (Nurfadilah *et al.*, 2013).

Nitrogen pathways in the fungal hyphae

In the current model proposed for AM, the best studied among mycorrhizal types, N taken up by the extraradical fungal mycelium as inorganic (Bago *et al.*, 1996; Govindarajulu *et al.*, 2005) or organic (Hawkins *et al.*, 2000; Cappellazzo *et al.*, 2008; Belmondo *et al.*, 2014) N forms is assimilated into

arginine *via* the biosynthetic arm of the urea cycle (Bago *et al.*, 2001). Arginine is then transported to the intraradical mycelium, where is broken down *via* the catabolic arm of the urea cycle to release ammonium (see Bücking & Kafle, 2015). The free ammonium is released into the plant-fungus interface, where it is taken up by the host plant thanks to local induction of plant ammonium transporters (Guether *et al.,* 2009; Gomez *et al.,* 2009; Kobae *et al*., 2010; Koegel *et al*., 2013). This inorganic N form is then assimilated in the plant cytoplasm thanks to the upregulation of the plant GS/GOGAT pathway (Bücking & Kafle, 2015). The urea cycle seems to be also involved in the N pathway of some ECM fungi, as urea was found to accumulate in the extraradical mycelium together with gene transcripts related to the urea cycle (Morel *et al.,* 2005; Wright *et al.,* 2005).

Although solely based on transcriptional evidence, it seems unlikely that *T. calospora* uses this N pathway to transfer ammonium to the orchid protocorm. Argininosuccinate lyase, a marker gene of arginine biosynthesis upregulated in the extraradical AM fungal mycelium (Koegel *et al.,* 2015), is upregulated in symbiosis in *T. calospora*. Moreover, the gene coding for urease, the main enzyme involved in arginine breakdown and ammonium release, is upregulated in the intraradical AM fungal mycelium (Koegel *et al.,* 2015) but was strongly down-regulated in *T. calospora* when inside ORM protocorms. It should be however noted that, also due to the obligate symbiotic nature of AM fungi, gene expression and enzymatic activities in AM fungi were assessed in two different but connected compartments, i.e. the extraradical and intraradical AM fungal mycelium (Gomez *et al.,* 2009; Tian *et al.,* 2010; Koegel *et al.,* 2015), whereas gene expression in *T. calospora* was measured separately in FLM and symbiotic conditions. The metabolic pathway and the form of N transferred inside the *T. calospora* hyphae that connect the substrate to the protocorm remains therefore to be understood.

Nitrogen transfer inside the mycorrhizal orchid protocorm

In the colonised protocorm cells, ORM fungi form coiled hyphae, known as pelotons (Smith & Read, 2008), surrounded by a plant-derived membrane and by an apoplastic plant-fungus interface (Peterson *et al.*, 1996). Similarly to the AM fungal arbuscules, ORM fungal pelotons are ephemeral structures rhythmically digested inside the host cell (Smith & Read, 2008). Based on this observation, Rasmussen (1995) proposed fungal lysis as the main mechanism underlying nutrient transfer in orchid mycorrhiza. Although this mechanism may explain some of the nutrient uptake by the plant (Kuga *et al.,* 2014), other authors (see in Smith & Read, 2008; Cameron *et al*., 2006, 2008; Kuga *et al.,* 2014) have provided convincing evidence that nutrient transfer takes place across intact membranes, thus requiring membrane transporters.

One way to elucidate the N source delivered by the mycorrhizal fungus in symbiosis is to investigate the plant import systems. Ammonium transfer by AM fungi is suggested by the high and localized upregulation of plant AMTs in arbuscule-containing cells (Guether *et al.*, 2009; Gomez *et al.*, 2009; Kobae *et al.*, 2010; Koegel *et al.*, 2013). In *L. japonicus*, *LjAMT2;2* was the most upregulated gene in mycorrhizal roots (Guether *et al.*, 2009). In our ORM system, by contrast, the importance of ammonium transfer to the plant remains unclear because the two *S. vomeracea* *SvAMT1* and *SvAMT2* genes were lowly expressed (as raw reads number) and not strongly upregulated in mycorrhizal protocorms.

Some putative *S. vomeracea* transporters strongly induced in mycorrhizal protocorms suggest transfer of organic N forms to the host plant in ORM. In addition to some amino acid transporters/permeases, *S. vomeracea* contigs coding for a putative lysine histidine transporter 1 (LHT1) were found to be very highly upregulated in mycorrhizal protocorms. Upregulation of genes coding for LHT1 in mycorrhizal roots have been also identified by deep-sequencing in the tropical orchid *Cymbidium hybridum* (Zhao *et al.*, 2014) and in *L. japonicus* AM roots (Guether *et al.*, 2011). LHT1 was first identified in *A. thaliana* and was demonstrated to be a particularly active and selective lysine and histidine transporter (Chen & Bush, 1997), whereas later studies showed a broader amino acid specificity (Hirner *et al.*, 2006).

Cameron *et al.* (2006) suggested amino acid transfer in ORM, based on incorporation of both ^{13}C and ^{15}N in mycorrhizal *G. repens* after feeding the symbiotic fungus with double-labelled [^{13}C-^{15}N]glycine. In Cameron *et al.*'s experiment, the ratio of assimilated ^{13}C:^{15}N recovered in the extraradical fungus and ORM roots was significantly lower than the ratio in the source glycine. As discussed by these authors, if cotransport of glycine-derived ^{15}N and ^{13}C occurred as amino acids with a higher N content, such as glutamine, the transamination could account for the change in ^{13}C:^{15}N ratio (Cameron *et al.*, 2006). Our data provide support to this hypothesis, and although specificity of the *S. vomeracea* LHTs remains to be established, they suggest that the amino acids preferentially transferred may be N-enriched amino acids such as lysine or histidine. RNASeq indicated that the biosynthetic pathways of these amino acids are upregulated in *T. calospora* when inside the mycorrhizal protocorm. LHTs can transport quite a broad range of amino acids (Hirner *et al.*, 2006) and a high affinity for proline was demonstrated for *LjLHT1.2* in *L. japonicus* (Guether *et al.*, 2011). However, *T. calospora* genes involved in proline biosynthesis were not upregulated inside symbiotic protocorms (data not shown).

In addition to transporters/permeases for single amino acids, several *S. vomeracea* contigs identified as putative oligopeptide transporters were very strongly upregulated in symbiotic protocorms (Table

2), similarly to what has been also observed in mycorrhizal roots of *Cymbidium hybridum* (Zhao *et al.,* 2014). However, the role of OPTs in N transport is unclear (Lubkowitz, 2011).

Nitrogen uptake by T. calospora inside mycorrhizal protocorm cells

Fungal gene expression in mycorrhizal protocorms suggests that ammonium is available in the apoplastic interface surrounding the pelotons and is actively taken up by the fungus. In particular, the strong upregulation of a low-affinity ammonium transporter (TcAMT2) and of the ammonium scavenging enzymes glutamine synthetase (TcGS1) suggests that ammonium is at high concentrations in the plant-fungus interface and is rapidly assimilated once taken up by the peloton. Fungal transcripts corresponding to high and low affinity AMTs in root colonized cells has been reported in AM (Perez-Tienda *et al.,* 2011; Calabrese *et al.,* 2016), where a role in ammonium retrieval from the apoplastic space surrounding the arbuscule has been suggested. As hypothesized for AM (Guether *et al.,* 2009; Calabrese *et al.,* 2016), the presence of both fungal and plant AMTs in the same colonized ORM cells may lead to a competition between the plant and the fungus for N present in the interfacial apoplast.

Conclusions

In the present study, we have identified for the first time some genetic determinants potentially involved in N uptake and trasfer in ORM. The *T. calospora* genome contains two genes coding for functional ammonium transporters and several amino acid transporters/permeases that allow this fungus to exploit inorganic and organic N sources (but not nitrate). Based on transcriptional evidence, we suggest that *T. calospora* mainly transfers organic N to the host plant, possibly in the form of N-rich amino acids. The transfer of amino acids with a high N:C ratio may explain the unusually high N content in orchid tissues (Hynson *et al.,* 2013).

Recent studies in AM interactions have demonstrated that reciprocal reward strategies guarantee a "fair trade" between the two mycorrhizal partners, where phosphorus and nitrogen from the fungus are exchanged against C from the plant (Kiers *et al.,* 2011; Fellbaum *et al.,* 2012). In particular, increased C supply to the mycorrhizal fungus by the host plant was found to stimulate the uptake and transfer of inorganic and organic N in the fungal partner (Fellbaum *et al.,* 2012). In this respect, orchids raise intriguing questions on the mechanisms controlling the nutrient flux, at least during the mycoheterotrophic stages (Selosse & Rousset, 2011). In fact, ORM fungi provide the plant not only with N (Cameron *et al.,* 2006; Kuga *et al.,* 2014) and P (Cameron *et al.,* 2007) but also with organic C, without an apparent reward (Selosse & Roy, 2009). What drives nutrient flow towards

the plant in the absence of a bidirectional exchange remains one of the most fascinating questions in ORM (Selosse & Rousset, 2011). The identification of the molecular components involved in this nutrient flow may provide some tools to start addressing this question.

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Author Contribution Statement

SP, RB and MG conceived and designed the research. VF and WC conducted all wet lab experiments. SV prepared the biological materials and extracted the RNA for the cDNA libraries. AK, VS, EL, KB, IVG, FM supervised and/or conducted the transcriptome sequencing and the bioinformatic analyses. SP and RB wrote the manuscript. All authors read and approved the manuscript.

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Table 1. Expression of *T. calospora* genes potentially involved in N metabolism in free living mycelium (FLM) and in symbiotic mycelium (SYM). In RNASeq-A, *T. calospora* gene expression was compared in FLM and in symbiotic protocorms grown on the same oat medium. In RNASeq-B, *T. calospora* gene expression was compared in FLM grown on two different N sources (Am=Ammonium; Gln=Glutamine) and in symbiosis. The experimental setup is illustrated in Fig. S1.

				RNASeq-A				RNASeq-B									
Functional groups	**protein Id**	**Protein Length**	**Code**	**Mean raw read counts**		**Fold Change**		**Mean raw read counts**			**Fold Change**		**Fold Change**		**Fold Change**		
				FLM	SYM	**SYM vs FLM Oat**	***P*-value**	FLM Am	FLM Gln	SYM	**FLM Gln vs Am**	***P*-value**	**SYM vs FLM Am**	***P*-value**	**SYM vs FLM Gln**	***P*-value**	**Putative function**
Ammonia permeases	241632	489	*TcAMT1*	339.29	764.08	**2.25**	0.07128	380.84	195.35	329.02	**0.51**	0.044	0.86	0.6541	1.68	0.0008	Ammonium transporter
	186135	537	*TcAMT2*	200.13	724.54	**3.62**	0.01724	96.58	32.56	291.56	**0.34**	0.0015	3.02	0.0151	8.95	0.0002	Ammonium transporter
Amino acid transporters	29106	529	*TcAAT1*	59.22	378.40	**6.39**	0.00011	101.36	29.68	281.46	**0.29**	0.0248	2.78	0.0063	9.48	4E-07	Amino acid transporter/permease
	81514	516	*TcAAT2*	18.48	80.04	**4.33**	0.00147	24.10	24.45	87.63	**1.01**	1	3.64	0.0749	3.58	0.0594	Amino acid transporter/permease
	74421	238	*TcAAT3*	366.77	581.75	**1.59**	0.01275	1000.38	548.83	374.69	**0.55**	0.112	0.37	0.0331	0.68	0.0373	Amino acid transporter/permease
	74154	782	*TcAAT4*	55.72	80.85	**1.45**	0.00158	38.32	32.01	47.23	**0.84**	0.2896	1.23	0.6802	1.48	0.2303	Amino acid transporter/permease
	12255	411	*TcAAT5*	268.67	369.13	**1.37**	1.1E-06	715.87	379.32	209.47	**0.53**	0.001	0.29	4E-09	0.55	0.0047	Amino acid transporter/permease
	24746	512	*TcAAT6*	4.96	16.15	**3.25**	0.00736	4.70	5.53	12.45	**1.18**	0.9355	2.65	0.4287	2.25	0.4427	Amino acid transporter/permease
	150749	494	*TcAAT7*	70.81	127.59	**1.80**	8.7E-08	75.45	74.87	89.02	**0.99**	0.8505	1.18	0.9024	1.19	0.785	Amino acid transporter/permease
	33378	416	*TcAAT8*	23.66	40.98	**1.73**	0.00466	14.49	18.88	44.84	**1.30**	0.5262	3.10	2E-09	2.37	4E-07	Amino acid transporter/permease
	227	498	*TcAAT9*	8.90	19.23	**2.16**	0.00507	3.02	11.43	8.72	**3.78**	0.0152	2.88	0.5334	0.76	0.7975	Amino acid transporter/permease
	31587	453	*TcAAT10*	72.77	53.91	**0.74**	0.03185	15.82	9.22	17.94	**0.58**	0.0624	1.13	1	1.95	0.6214	Amino acid transporter/permease
	228655	449	*TcAAT11*	131.11	75.07	**0.57**	3.6E-05	19.85	9.33	40.36	**0.47**	0.0044	2.03	0.0782	4.32	0.0008	Amino acid transporter/permease
	155949	385	*TcAAT12*	31.08	15.91	**0.51**	0.00212	25.91	40.89	4.70	**1.58**	0.0903	0.18	0.0006	0.12	1E-14	Amino acid transporter/permease
	65140	551	*TcAAT13*	80.22	33.39	**0.42**	2.1E-08	30.31	19.83	13.40	**0.65**	0.1078	0.44	0.121	0.68	0.6195	Amino acid transporter/permease
	23211	586	*TcAAT14*	27.09	9.38	**0.35**	6.9E-06	8.49	6.71	11.25	**0.79**	0.6254	1.33	0.8797	1.68	0.6531	Amino acid transporter/permease
	13898	533	*TcAAT15*	124.13	30.88	**0.25**	2E-20	16.21	26.90	24.75	**1.66**	0.0433	1.53	0.5243	0.92	0.9589	Amino acid transporter/permease
Peptide transporters	231405	296		48.14	29.64	**0.62**	0.01195	35.30	24.47	10.93	**0.69**	0.3897	0.31	0.0683	0.45	0.1173	H+/oligopeptide symporter (PTR2)
	70976	682		0.35	4.38	**12.59**	0.00726	1.51	2.12	2.17	**1.40**	0.8669	1.44	0.9484	1.03	1	Oligopeptide transporter OPT superfamily
	6542	1664		3.66	10.93	**2.98**	0.00616	5.06	2.68	8.84	**0.53**	0.2972	1.75	0.338	3.30	0.0134	Oligopeptide transporter OPT superfamily
	73589	726		25.49	54.50	**2.14**	7E-05	15.74	16.75	41.61	**1.06**	1	2.64	0.0063	2.48	0.0061	Oligopeptide transporter OPT superfamily
	21934	989		29.03	17.51	**0.60**	0.01831	7.28	11.60	12.37	**1.59**	0.2914	1.70	0.6	1.07	1	Oligopeptide transporter OPT superfamily
	22904	1063		34.96	18.02	**0.52**	0.00047	16.11	11.56	27.46	**0.72**	0.2511	1.70	0.6462	2.38	0.3989	Oligopeptide transporter OPT superfamily
	209937	674		15.89	7.18	**0.45**	0.02306	10.66	7.40	5.43	**0.69**	0.3437	0.51	0.3178	0.73	0.79	Oligopeptide transporter OPT superfamily
	229195	805		12.25	4.18	**0.34**	0.00366	5.10	8.46	1.25	**1.66**	0.3447	0.25	0.0787	0.15	0.0018	Oligopeptide transporter OPT superfamily
	73703	710		14.96	4.94	**0.33**	0.00077	7.18	6.93	7.02	**0.96**	0.975	0.98	1	1.01	1	Oligopeptide transporter OPT superfamily
	71241	752		13.32	4.23	**0.32**	0.0013	1.83	3.58	0.86	**1.96**	0.4618	0.47	0.6798	0.24	0.1831	Oligopeptide transporter OPT superfamily
	229100	507		42.73	7.90	**0.18**	8.9E-16	4.28	7.65	1.61	**1.79**	0.3004	0.38	0.2249	0.21	0.0065	Oligopeptide transporter OPT superfamily
	21935	354		58.70	6.12	**0.10**	3.7E-27	2.89	6.05	15.10	**2.09**	0.2275	5.22	0.3862	2.49	0.5285	Oligopeptide transporter OPT superfamily
GS/GOGAT assimilation pathway	241239	314	*TcGS1*	421.07	1125.72	**2.67**	0.02795	281.58	222.61	714.11	**0.79**	0.0012	2.54	0.0533	3.21	0.0148	Glutamine synthetase
	183750	482	*TcGS2*	113.95	109.28	**0.96**	0.95391	52.68	75.84	83.63	**1.44**	0.041	1.59	0.3923	1.10	0.8607	Glutamine synthetase
	242592	2163	*TcGOGAT*	16.49	104.95	**6.36**	0.01265	20.47	27.70	95.05	**1.35**	0.4985	4.64	0	3.43	2E-12	Glutamate synthase
Urea Cycle	245827	466	*TcASL*	118.15	337.12	**2.85**	0	113.56	288.70	279.02	**2.54**	0.0048	2.46	6E-08	0.97	0.9927	Argininosuccinate lyase
	179058	331	*TcCAR*	50.95	70.18	**1.38**	0.05579	61.33	142.54	66.83	**2.32**	0.0007	1.09	0.9639	0.47	0.0006	Arginase
	242909	839	*TcURE*	409.82	82.77	**0.20**	1E-22	52.43	50.41	37.08	**0.96**	0.7455	0.71	0.0172	0.74	0.072	Urease

Lysine biosynthesis	241089	388		51.86	225.57	**4.35**	1.2E-06		134.11	353.32	307.68	**2.63**	0.0036	2.29	0.0571	0.87	0.7822	Saccharopine dehydrogenase
Histidine biosynthesis	141375	272		14.33	41.90	**2.92**	0.00507		19.12	35.78	54.22	**1.87**	0.0683	2.84	0.0015	1.52	0.0788	Phosphoribosylformimino-5-aminoimidazole carboxamide ribonucleotide isomerase
	77512	595		10.53	70.57	**6.70**	0		25.44	102.64	77.08	**4.03**	0.0144	3.03	0.0064	0.75	0.6169	Imidazoleglycerol-phosphate synthase
	242885	392		49.95	181.72	**3.64**	0		140.77	351.91	224.23	**2.50**	0.0043	1.59	0.4616	0.64	0.1164	Histidinol-phosphate transaminase
	75590	328		45.27	19.14	**0.42**	3.7E-05		55.14	36.68	17.44	**0.67**	0.0306	0.32	0.0011	0.48	0.2422	Histidinol-phosphatase
	73648	858		15.86	90.21	**5.69**	4E-05		34.61	89.00	74.79	**2.57**	0.0278	2.16	0.0289	0.84	0.7631	Histidinol dehydrogenase

N.B. Only transcripts with FDR≤0.05 in at least one sample comparison are listed in the table.

P-value: FDR *p*-value correction

873

Table 2. Expression of *S. vomeracea* contigs potentially involved in N metabolism, identified in a *de novo* assembly annotated by BlastX against the *A. thaliana* and *P. equestris* proteome. Expression of *S. vomeracea* contigs in mycorrhizal protocorms (SYM) was compared with expression in non-mycorrhizal protocorms at a similar developmental stage (ASYM).

Functional groups	Trunity Contig Name	Code	Mean raw read count		Fold Change	*P*-value	*A. thaliana* protein Id	Putative function in *A. thaliana*	score	e-value	percent identity	*P. equestris* protein Id	score	e-value	percent identity
			ASY	SYM											
Ammonia permeases	DN68801_c0_g1_i1	*SvAMT1*	7.69	21.75	**2.83**	0.896	AT1G64780.1	ammonium transporter 1;2	1613	0	76.7	PEQU_21149	1799	0	79.6
	DN77095_c1_g2_i1	*SvAMT2*	1.58	8.33	**5.26**	0.988	AT2G38290.1	ammonium transporter 2	193	5.00E-18	84.1	PEQU_10528	262	9.00E-28	86.2
Amino acid transporters/ permeases	DN71918_c0_g1_i1	*SvAAP1*	0.35	43.97	**125.27**	4E-05	AT1G77380.1	amino acid permease 3	1357	0	69.8	PEQU_10464	1445	0	72.5
	DN71918_c0_g2_i1	*SvAAP2*	0.93	49.83	**53.32**	2E-12	AT5G63850.1	amino acid permease 4	504	1.00E-60	75.2	PEQU_01321	532	7.00E-69	77.9
	DN77539_c1_g1_i1		4.17	46.89	**11.25**	5E-06	AT4G21120.1	amino acid transporter 1	1606	0	71.7	n.d.			
	DN74856_c3_g6_i1		1.67	17.03	**10.20**	7E-06	AT1G08230.2	transmembrane amino acid transporter family	1045	7.00E-137	55.8	n.d.			
	DN63460_c0_g1_i1	*SvLHT*	1.79	167.52	**93.43**	4E-05	AT5G40780.2	lysine histidine transporter 1	987	9.00E-133	87	n.d.			
	DN66338_c0_g1_i1		1.68	81.17	**48.21**	8E-09	AT5G40780.2	lysine histidine transporter 1	716	1.00E-89	76.7	PEQU_15531	843	5.00E-109	87.1
Oligopeptide transporters	DN49188_c0_g1_i1		0.03	149.13	**5357.71**	0.02	AT5G55930.1	oligopeptide transporter 1	239	2.00E-24	61.4	PEQU_16981	293	6.00E-32	72.9
	DN32096_c0_g1_i1		0.16	187.93	**1176.92**	0.002	AT5G55930.1	oligopeptide transporter 1	224	3.00E-22	57.3				
	DN1177_c0_g1_i1		0.85	381.43	**451.23**	0.011	AT5G55930.1	oligopeptide transporter 1	391	1.00E-43	60.5	PEQU_41210	525	9.00E-68	72
	DN61732_c1_g1_i1		0.11	35.83	**328.50**	2E-07	AT5G55930.1	oligopeptide transporter 1	551	3.00E-66	66.2	PEQU_29359	730	2.00E-92	90.5
	DN61732_c1_g2_i1		0.02	29.43	**1324.27**	2E-17	AT4G26590.1	oligopeptide transporter 5	283	5.00E-30	56	PEQU_40777	432	3.00E-55	89
	DN48315_c0_g1_i1		0.37	279.21	**750.97**	2E-10	AT4G26590.1	oligopeptide transporter 5	807	8.00E-101	58	PEQU_16981	1006	9.00E-131	71.3
	DN78718_c1_g1_i2		3.69	36.53	**9.90**	2E-11	AT4G26590.1	oligopeptide transporter 5	2138	0	59.8	n.d.			
	DN69777_c0_g1_i2		0.58	11.01	**19.02**	3E-05	AT3G54140.1	peptide transporter 1	2148	0	71.2	n.d.			
	DN75842_c0_g4_i1		9.36	41.38	**4.42**	5E-04	AT5G46050.1	peptide transporter 3	330	4.00E-35	56.3	PEQU_03726	441	1.00E-50	73.2
GS/GOGAT pathway	DN97391_c0_g1_i1	*SvGS*	0.12	28.27	**244.52**	0.032	AT5G35630.3	glutamine synthetase 2	925	3.00E-121	60.4	n.d.			

n.d: No match found

874 P-value: FDR p-value correction

875 **Figures legends**

876

877 **Figure 1.** Growth of *Tulasnella calospora* on different N sources. Biomass are expressed as mean
878 values of three replicates ± SD. ANOVA was performed comparing all five media, and values with
879 different letters above the bars differ significantly following Tukey's HSD test ($P<0.05$).

880

881 **Figure 2.** Phylogenetic tree of fungal ammonium transporters, based on amino acid deduced
882 sequences. The sequences were aligned using Muscle and a tree was constructed using the
883 maximum likelihood method. Numbers indicate bootstrap values, and are given only for ≥50 %.
884 Bootstrap tests were performed using 1,000 replicates. Sequences were obtained from the GenBank
885 database with the following accession numbers: *Glomus intraradices* (GintAMT1: CAI54276;
886 GintAMT2: CAX32490), *Hebeloma cylindrosporum* (HcAMT1: AAM21926, HcAMT2:
887 AAK82416, HcAMT3: AAK82417), *Tuber borchii* (TbAMT1: AAL11032), *Ustilago maydis*
888 (UmMEP1: AAL08424, UmMEP2: AAO42611), *Saccharomyces cerevisiae* (ScMEP1: P40260,
889 ScMEP2: P41948, ScMEP3: P53390), *Schizosaccharomyces pombe* (SpAMT1: NP_588424,
890 SpAMT2: NP_593462), *Aspergillus nidulans* (AnMEAA: AAL73117, AnMEPA: AAL73118),
891 *Fusarium fujikuroi* (FfMEPA: CAJ44733, FfMEPB: CAJ44734, FbMEPC: CAK55531),
892 *Cryptococcus neoformans* (CnAMT1: XP_566614, CnAMT2: XP_567361), *Synechocystis* sp.
893 (NP_442561). Arrows point to the *T. calospora* sequences TcAMT1 and TcAMT2.

894

895 **Figure 3.** Complementation of *mep* yeast mutant with *TcAMT1* and *TcAMT2*. The ammonium
896 uptake-deficient yeast 31019b (ΔΔΔmep1;2;3) was transformed with *TcAMT1*, *TcAMT2*, the
897 positive control *GintAMT* and the empty plasmid pFL61 as negative control. Serial dilutions of the
898 transformants were grown on ammonium as sole N source at different concentrations and pH or on
899 arginine as positive growth control.

900

901 **Figure 4.** Quantification by RT-qPCR of *TcAMT1* and *TcAMT2* expression in *T. calospora* FLM
902 grown on different organic and inorganic N sources and in symbiosis. Different lowercase letters
903 above the bars denote significant differences by two-tailed Student's *t* test ($P<0.05$).

904 **Figure 5.** Mycorrhizal protocorms of *S. vomeracea* 30 days after sowing with *T. calospora*. A.
905 Semi-thin section of a resin-embedded protocorm, showing the basal mycorrhizal region. Colonized
906 cells cell containing well-developed and collapsed (asterisks) fungal pelotons (coils) are visible. Bar

907 = 80 μm. B. At the electron microscope level, an interface space, based on membrane proliferation
908 (arrows), is evident around an intracellular hypha. Bar = 0.25 μm. C. A colonized cell containing
909 collapsed hyphae. cc, collapsed coil. Bar = 0.35 μm

910 **Figure 6.** RT-qPCR assessing expression of several *Serapias vomeracea* contigs in symbiotic and
911 asymbiotic protocorms. Different lowercase letters above the bars denote significant differences by
912 two-tailed Student's *t* test ($P<0.05$).
913
914
915 **List of supplementary material**
916
917 **Table S1.** List of primers used in this study
918
919 **Figure S1.** Diagram showing the experimental setup of the two RNASeq experiments that were
920 used to identify fungal and plant genes involved in N uptake and metabolism
921
922 **Figure S2.** Control experiment to verify the absence of DNA in the RNA extracted from LMD cells
923 and amplified by RT-PCR. C, cells containing coils occupying the whole plant cell; CC, cells with
924 older more condensed coils; NM, non-colonized cells; -, water. The signs + and – indicate presence
925 or absence of the RT step before PCR amplification with primers for the housekeeping genes
926 coding for the plant (*SvEF1α*) and the fungal (*TcEF1α*) elongation factor.
927
928 **Figure S3.** One-step RT-PCR analysis of *T. calospora* genes in three populations of laser
929 microdissected (LMD) cells: C, cells containing coils occupying the whole plant cell; CC, cells with
930 older and more condensed coils; NM, non-colonized cells; -, water. Fungal transcripts coding for
931 transporters and enzymes involved in N uptake and metabolism were amplified with the primers
932 listed in Table S1 and separated by agarose gel electrophoresis.
933
934 **Figure S4.** Phylogenetic tree of plant ammonium transporters, based on amino acid deduced
935 sequences. The sequences were aligned using Muscle and the unrooted tree was constructed using
936 the maximum likelihood method. Bootstrap tests were performed using 1,000 replicates. Numbers
937 indicate bootstrap values, and are given only for ≥50 %. Arrows point to the *S. vomeracea*
938 sequences SvAMT1 and SvAMT2.
939

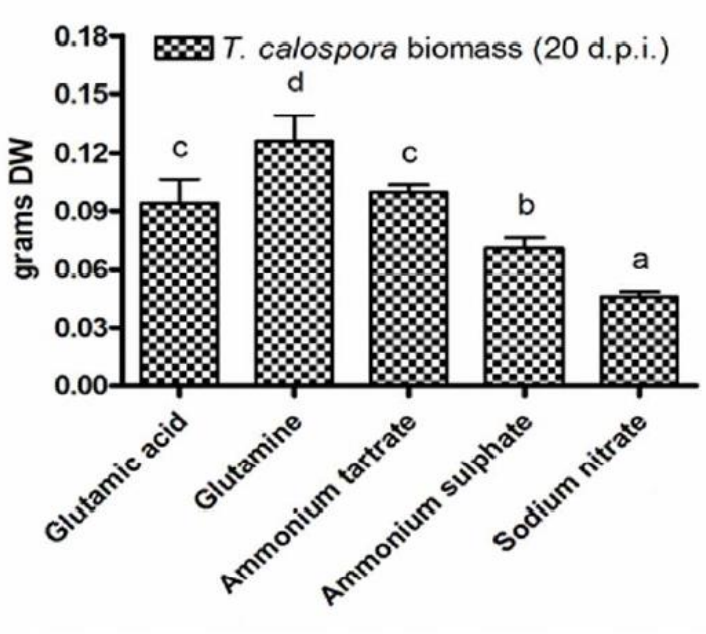
T. calospora biomass (20 d.p.i.)
grams DW
0.18
0.15
0.12
0.09
0.06
0.03
0.00
c
d
c
b
a
Glutamic acid
Glutamine
Ammonium tartrate
Ammonium sulphate
Sodium nitrate

Figure 1

940

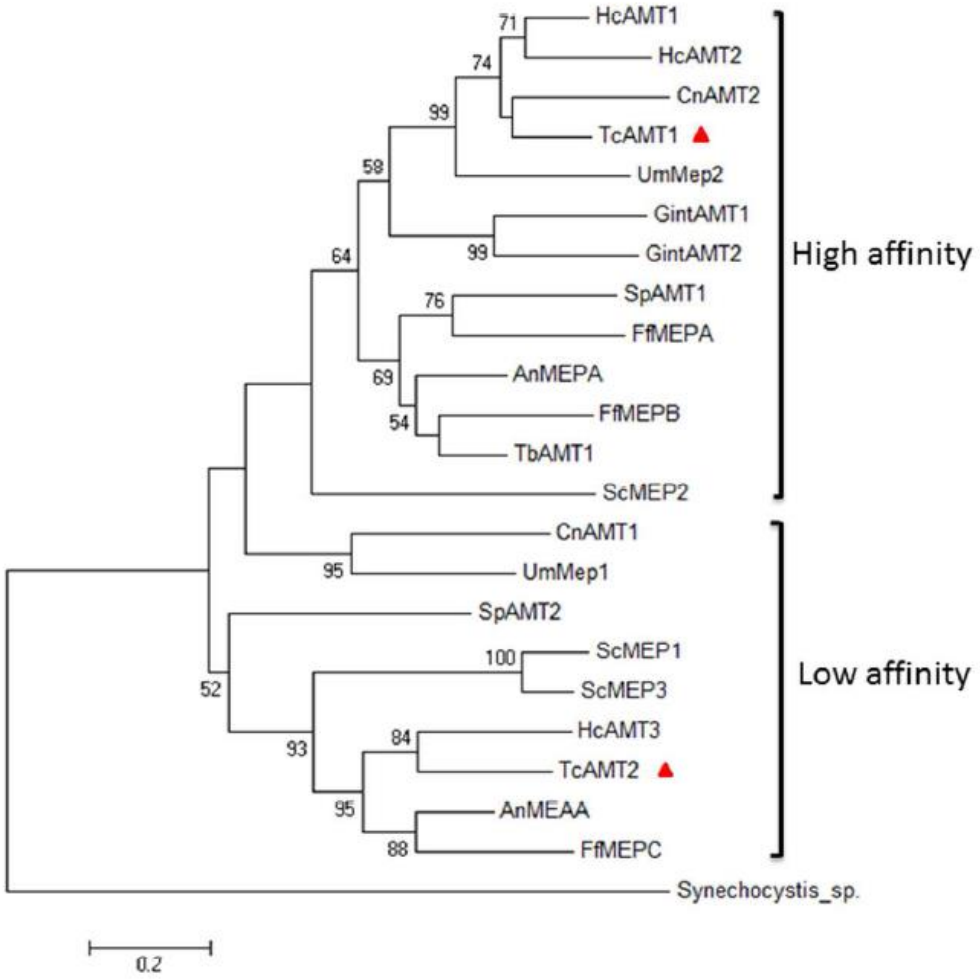
HcAMT1
HcAMT2
CnAMT2
TcAMT1
UmMep2
GintAMT1
GintAMT2
SpAMT1
FfMEPA
AnMEPA
FfMEPB
TbAMT1
ScMEP2
CnAMT1
UmMep1
SpAMT2
ScMEP1
ScMEP3
HcAMT3
TcAMT2
AnMEAA
FfMEPC
Synechocystis_sp.
71
74
99
58
99
64
76
69
54
95
100
52
93
84
95
88
High affinity
Low affinity
0.2

Figure 2

941

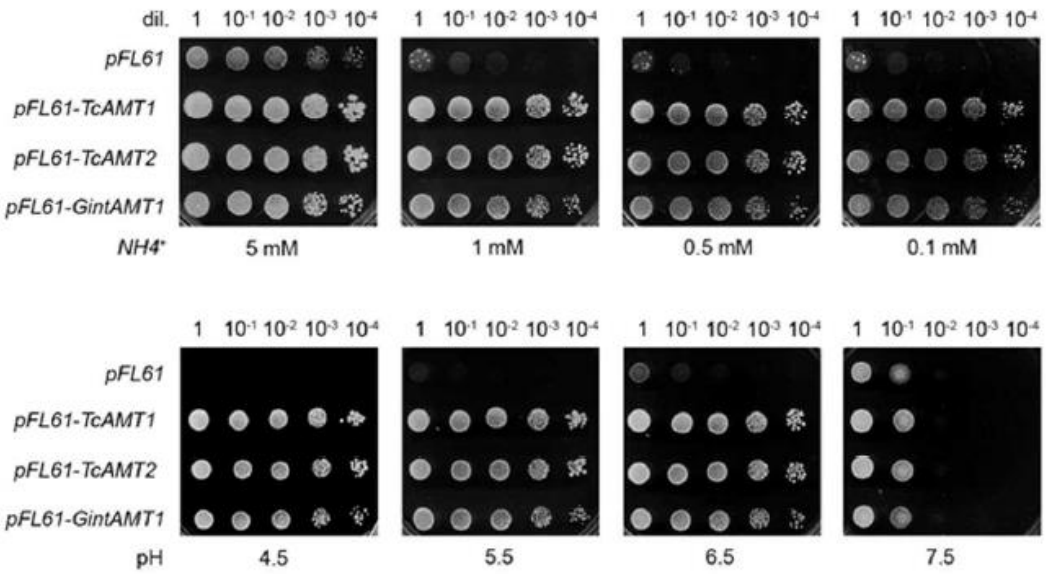
dil. 1 10-1 10-2 10-3 10-4
pFL61
pFL61-TcAMT1
pFL61-TcAMT2
pFL61-GintAMT1
NH4+
5 mM
1 mM
0.5 mM
0.1 mM
pH
4.5
5.5
6.5
7.5

Figure 3

942

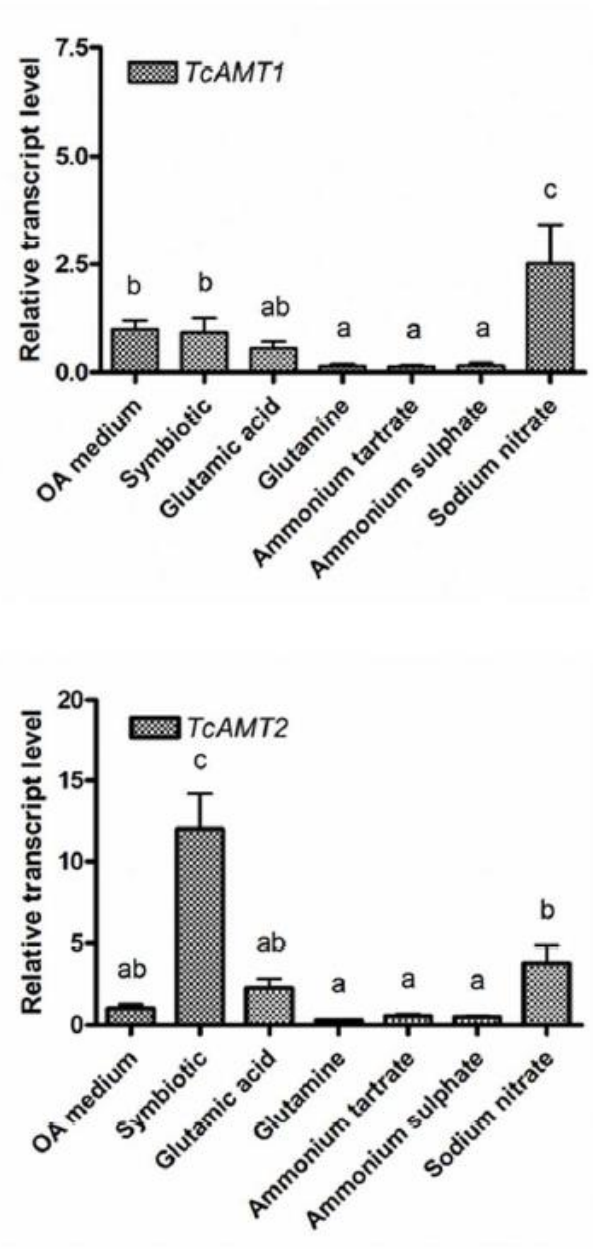
TcAMT1
Relative transcript level
7.5
5.0
2.5
0.0
b
b
ab
a
a
a
c
OA medium
Symbiotic
Glutamic acid
Glutamine
Ammonium tartrate
Ammonium sulphate
Sodium nitrate
TcAMT2
Relative transcript level
20
15
10
5
0
ab
c
ab
a
a
a
b
OA medium
Symbiotic
Glutamic acid
Glutamine
Ammonium tartrate
Ammonium sulphate
Sodium nitrate

Figure 4

943

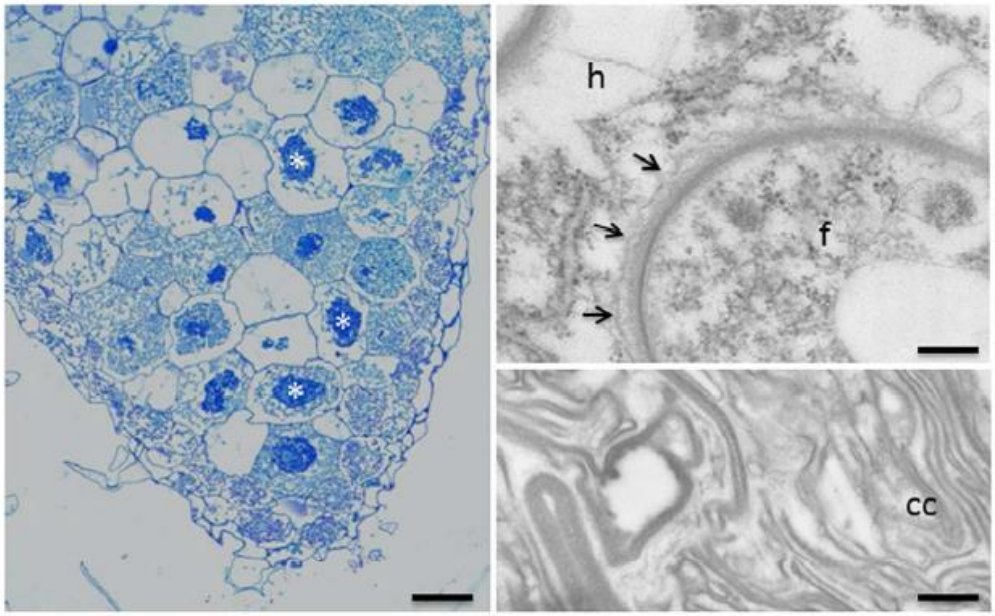

Figure 5

944

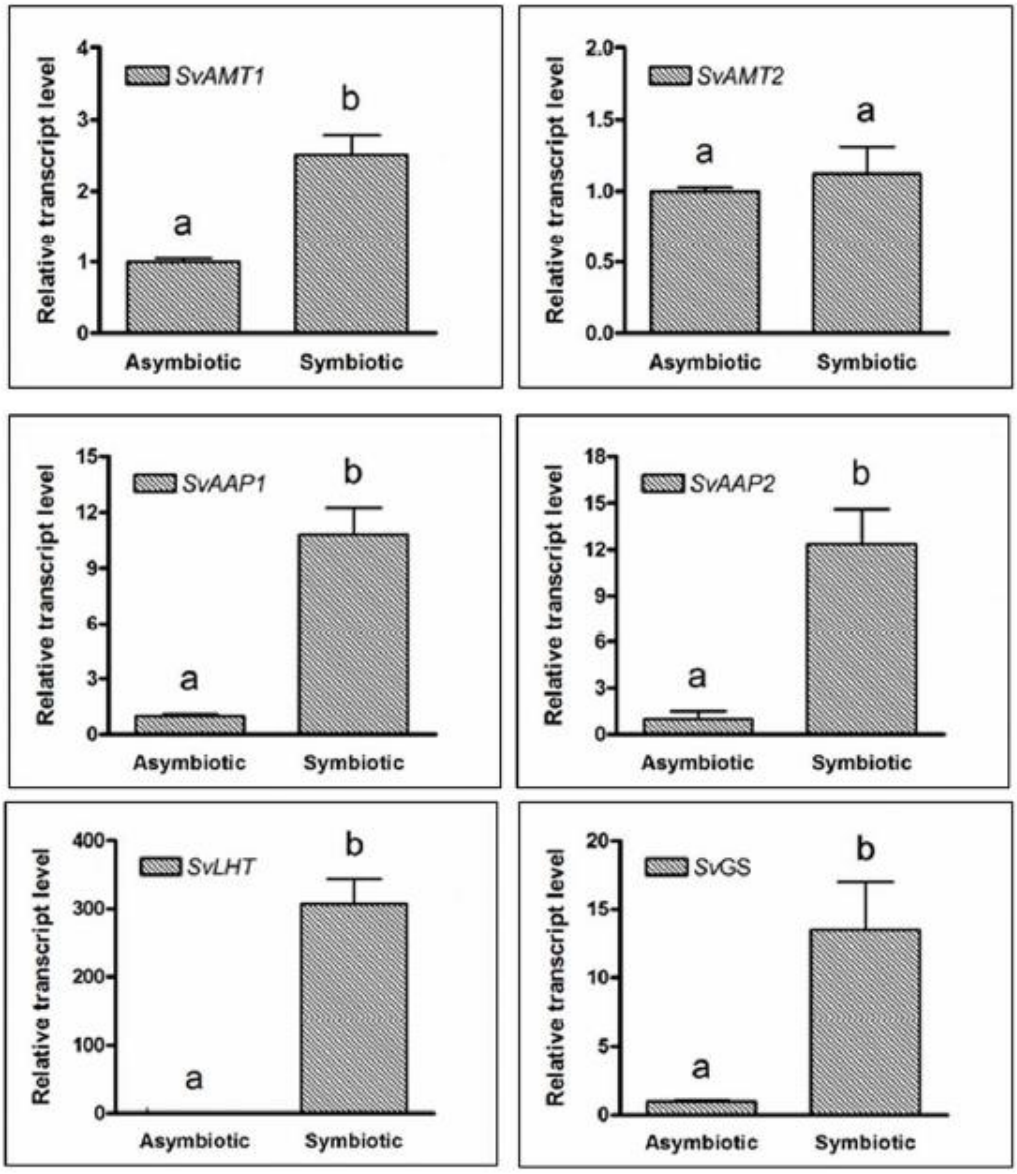
Relative transcript level
SvAMT1
a
b
Asymbiotic
Symbiotic
SvAMT2
SvAAP1
SvAAP2
SvLHT
SvGS

Figure 6

945