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Fungal and plant gene expression in the *Tulasnella calospora*–*Serapis vomeracea* symbiosis provides clues about nitrogen pathways in orchid mycorrhizas

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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 probably a major nutrient transferred to the plant because orchid tissues are highly N-enriched. We know almost nothing about the N form preferentially transferred to the plant or about 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 found no evidence of a nitrate assimilation system, in agreement with the 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 the transport and metabolism of N compounds suggested 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 apoplastic symbiotic interface.
- This is the first study addressing the genetic determinants of N uptake and transport in orchid mycorrhizas, and provides a model for nutrient exchanges at the symbiotic interface, which may guide future experiments.

**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), which 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 the mycobiont 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, most experiments on nutrient transfer in orchid mycorrhizas have focussed on the acquisition and transfer of organic C, whereas little is known about other nutrients, such as nitrogen (N). 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 the soil in inorganic forms, such as ammonium (NH$_4^+$) and nitrate (NO$_3^-$), or in organic N compounds. Fungal uptake and transfer of soil-derived N to host plants 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 & Kaffe, 2015). Nitrate transporters and genes
Research

Several important points are discussed in this paper, which is focused on the uptake and transfer of nitrogen (N) forms in orchid mycorrhizas. The authors investigate the mechanisms underlying N uptake and transfer to the orchid host, Serapias vomeracea isolate AL13/4D. They emphasize the role of low-affinity ammonium transporters and the ability of AM fungi to utilize organic N. Several functional genes involved in N metabolism are identified, with a focus on the expression of additional fungal AMTs.

**Materials and Methods**

**Growth of the free-living mycelium**

Tulasnella calospora (Boud.) Juel AL13/4D was isolated from mycorrhizal roots of Anacamptis laxiflora (Lam.) R.M. Bateman, Pridgeon and M.W. Chase in northern Italy (Girlanda et al., 2011) and deposited in the mycological collection of the University of Turin (accession number MUT4182). Free-living mycelium was maintained on solid 2% malt extract agar at 25°C. To evaluate growth on different N sources, eight hyphal plugs (6 mm diameter) of 20-d-old T. calospora mycelia were transferred into 50 ml of modified synthetic Pachlewski P5 liquid medium adjusted to pH 5.5 (Kemppainen & Pardo, 2011) with L-glutamine, monosodium glutamate, ammonium tartrate, sodium nitrate or ammonium sulphate, each added as 0.49 g of N. Inoculated flasks were maintained at 25°C under constant shaking (120 rpm). After 20 d, the mycelium was recovered by filtration, washed with distilled water, weighed and dried to measure biomass. The pH of the culture medium was measured. 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 and 1% agar), were used to grow free-living mycelium 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 colonized (c. 20 d). The mycelium was then collected from the cellophane membrane, immediately frozen in liquid N and stored at −80°C.

**Symbiotic and asymbiotic germination of Serapis 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 Serapis vomeracea (Burm.f.) Briq. were surface-sterilized in 1% sodium hypochlorite and 0.1% Tween-20 for 20 min on a vortex, followed by three 5-min rinses in sterile distilled water. Seeds were resuspended in sterile water and dropped on strips of autoclaved filter paper (1.5 × 3 cm) positioned on solid oat medium (0.3% milled oats and 1% agar). Plates were inoculated with a plug of actively growing T. calospora mycelium and were incubated at 20°C in darkness.
Asymbiotic seed germination was obtained on modified BM1 culture medium (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 N 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 Joint Genome Institute (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 RNA-Seq database obtained in this study, as described in RNA-Seq experiments. Multiple protein alignments were performed with MUSCLE (Edgar, 2004). Phylogenetic trees were constructed with the maximum likelihood method using MEGA v.7.0 (Kumar et al., 2016); bootstrap analyses were conducted on the basis of 1000 resamplings 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 protocorm cDNA with the full-length primers reported in Supporting Information Table S1. PCR reactions were performed in a 50-μl final volume containing 10 μl of 5× Phusion HF Buffer, 1 μl of dNTPs (10 mM stock each), 1.5 μl of each primer (10 mM stock), 0.5 μl of Phusion High-Fidelity DNA polymerase (New England Biolabs, Hitchin, UK), 34.5 μl of water and 1 μl of cDNA. PCR amplifications were carried out in a thermal cycler (Biometra GmbH, Göttingen, Germany) using the following programme: 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 visualized on 1.2% agarose gels after electrophoresis in 0.5× Tris-acetate-EDTA (TAE) buffer, excised from the agarose gel and purified using the Wizard® SV Gel (Promega Corp., Madison, WI, USA) and PCR Clean-Up System (Promega) following the manufacturer’s instructions. 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 Escherichia coli chemically competent cells (Top10 cells; Invitrogen). Plasmids were then purified, from positive colonies, using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA). Sequencing was performed by the sequencing service at Munich University (Sequencing Service, 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 ammonium permease (mp) 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, characterized in the AM fungus Rhizobagus 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% yeast nitrogen base without 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 (Pérez-Tienda et al., 2011). For growth assays, yeast transformants were grown in liquid N-free medium plus arginine until the optical density at 600 nm (OD600) reached 0.6–0.8. Cells were harvested, washed twice, and resuspended in water to a final OD600 of 2, and 10-μl drops corresponding to a serial 10-fold dilution were spotted on solid N-free medium supplemented with different NH4Cl concentrations as the sole N source (0.1, 0.5, 1 and 5 mM). Yeast cells were also spotted on 1 mM NH4Cl-supplemented medium buffered at different pHs (4.5, 5.5, 6.5 and 7.5) with 50 mM Mes/Tris. Pictures of the plates were taken after 3 d of incubation at 30°C.

RNA-Seq experiment

Two different RNA-Seq experiments were carried out to investigate expression of T. calospora and S. vomeracea genes, and the experimental set-up is illustrated in Fig. S1. In the first experiment (RNA-Seq-A), T. calospora transcripts were investigated in free-living mycelium and in mycorrhizal S. vomeracea protocorms grown on solid oat medium (0.3% milled oats and 1% agar). Preparation of libraries and 2 × 100 bp Illumina HiSeq2000 (Illumina Inc., San Diego, CA, USA) mRNA sequencing (RNA-Seq) was performed at the IGA Technology Services facilities (Udine, Italy). The complete series has been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE63869. For the second experiment (RNA-Seq-B), T. calospora free-living mycelium was grown on modified Melin–Norkrans medium with two different N sources, ammonium and glutamine, each added as 0.49 g of N. 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 in the ‘Symbiotic and asymbiotic germination of Serapias vomeracea seeds’ section. Illumina HiSeq2500 sequencing (2 × 150 bp) was performed at the JGI (Walnut Creek, USA). The complete series of fungal and plant transcripts was submitted to GEO (GSE86968 and GSE87120, respectively). For both RNA-Seq experiments, three separate libraries were prepared from three biological replicates for each condition.

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). Contigs were annotated following BLAST searches against the Arabidopsis thaliana (TAIR) and the Phalaenopsis equestris (Cai et al., 2015) proteome.

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 (Qiagen Bioinformatics; http://www.qiagenbioinformatics.com/products/clc-genomics-workbench/). For mapping, the minimum length fraction was 0.9, the minimum similarity fraction was 0.8 and the maximum number of hits for a read was set to 10. The unique and total mapped read numbers for each transcript were determined, and then normalized to reads per kilobase of exon model per million mapped reads (RPKM). The Baggerly test (Baggerly et al., 2003) implemented in the CLC Genomic workbench compares the proportions of counts in a group of samples against those of another group of samples and 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 the fold-change (FC) was ≥ 2.5 and the FDR was < 0.05, and down-regulated when the FC was ≤ 0.5 and the FDR was < 0.05.

Real-time quantitative PCR analyses

Total RNA for quantitative real-time polymerase chain reaction (qPCR) was extracted from symbiotic and asymbiotic S. vomeracea protocorms and from T. calospora free-living mycelia following the method of Chang et al. (1993). Genomic DNA was removed using the Turbo DNA-free™ reagent (Ambion, Austin, TX, USA), according to the manufacturer’s instructions. RNA was then quantitated using spectrophotometry (NanoDrop 1000; BioRad) and subjected to a reverse transcription–PCR reaction (RT-PCR) to exclude DNA contamination, using the One Step RT-PCR kit (QiaGen), before cDNA synthesis. SuperScriptII Reverse Transcriptase (Invitrogen) was used to synthesise 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 by 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, Thermo Fisher Scientific Inc., Waltham, MA, USA), 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 normalization to the geometric mean of the endogenous control genes, the elongation factor (EF) genes TcEF-1α and SvEF-1α. Gene expression data were calculated as expression ratios (quantity relative to that of control). All reactions were performed with three biological and three technical replicates.

Laser microdissection 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 Pérez-Tienda et al. (2011). Sections (12 μm) were cut with a rotary microtome and transferred onto Leica RNase-free PEN foil slides (Leica Microsystems Inc., Ben-heim, Germany), dried at 40°C in a warming plate, stored at 4°C and used within 1 d. Specific cell types were collected from paraffin sections of S. vomeracea symbiotic protocorms with a Leica LMD 6500 system (Leica Microsystem), as described by Balestrieri et al. (2007). Approximately 1000–1500 cells for each type were collected, with three independent biological replicates. RNA was extracted from laser microdissected cells following the Pico Pure kit (Life Technologies, Carlsbad, CA, USA) 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) 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 (StEF1α) and the fungal (TcEF1α) EF 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 analyses

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

Growth of Tulasnella calospora on different N sources

After 20 d of culture on modified Pachlewski P5 medium containing some defined organic and inorganic N sources, the highest fungal biomass (as dry weight) was on glutamine and the lowest 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). At the end of the growth experiment, the pH of the culture media ranged from 4.3 to 4.9 for most N sources, with the exception of ammonium sulphate (pH = 3.6) and glutamate (pH = 5.7).

Identification of Tulasnella 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 genome. By contrast, three gene models coding for ammonium 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 ID 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 cylindrosporum (76% and 69% identity with TcAMT1 and TcAMT2, respectively) or Laccaria bicolor (74% and 65% identity with TcAMT1 and TcAMT2, respectively). Phylogenetic comparison with functionally characterized transporters from other fungi (Pérez-Tienda et al., 2011) showed that TcAMT1 clustered with high-affinity transporters, whereas TcAMT2 clustered with low-affinity transporters (Fig. 2).

Heterologous expression of T. calospora ammonium transporters 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 NH₄⁺ as the sole N source because it is defective in all three endogenous Mep ammonium transporters. Both TcAMT1 and TcAMT2 were able to complement the growth defect of the

Fig. 2 Phylogenetic tree of fungal ammonium transporters, based on amino acid deduced sequences. The sequences were aligned using MUSCLE and a tree was constructed using the maximum likelihood method. Numbers indicate bootstrap values, and are given only for ≥50%. Bootstrap tests were performed using 1000 replicates. Sequences were obtained from the GenBank database with the following accession numbers: Glomus intraradices (GintAMT1, CAI45276; GintAMT2, CA32490), Hebeloma cylindrosporum (HcAMT1, AAM21926; HcAMT2, AAK82416; HcAMT3, AAK82417), Tuber borchii (TbAMT1, AAL11032), Ustilago maydis (UmMep1, AAL08424; UmMep2, AAO42611), Saccharomyces cerevisiae (ScMEP1, P40260; ScMEP2, P41948; ScMEP3, P53390), Schizosaccharomyces pombe (SpMEP1, NP_588244; SpMEP2, NP_593462), Aspergillus nidulans (AnMEAA, AAL73117; AnMEPA, AAL73118), Fusarium fujikuroi (FfMEPA, CAJ44733; FfMEPB, CAJ44734; FfMEPC, CAX32490), Cryptococcus neoformans (CnAMT1, XP_566614; CnAMT2, XP_567361), and Synechocystis sp. (NP_442561). Red arrowheads point to the Tulasnella calospora ammonium transporter sequences TcAMT1 and TcAMT2.

Fig. 1 Growth of Tulasnella calospora on different nitrogen (N) sources, as dry weight (DW). Biomass is expressed as mean values of three replicates ± SD. ANOVA was performed comparing all five media, and values with different letters above the bars differ significantly following Tukey’s honest significant difference (HSD) test \((P < 0.05)\). dpi, days post inoculum.
mutant yeast strain in the presence of NH$_4^+$ (from 0.1 to 1 mM) 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 dependence, 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

RNA-Seq and RT-qPCR experiments were used to investigate expression of TcAMT1 and TcAMT2, as well as the expression of other T. calospora genes potentially involved in N uptake and metabolism, both in the free-living mycelium and in symbiosis. RNA-Seq expression data were derived from two separate experiments (illustrated in Fig. S1): RNA-Seq-A was run to identify genes differentially expressed by T. calospora in free-living mycelium and inside symbiotic protocorms, both obtained on oat meal, whereas RNA-Seq-B was run to investigate fungal gene expression patterns in free-living mycelia grown on two defined N sources, glutamine and ammonium. Gene expression in symbiosis was also measured in this second RNA-Seq experiment in order to gain some insights into N regulation in the protocorm environment. In the free-living mycelium grown on glutamine as the sole N source, expression of both TcAMT genes was reduced as compared with ammonium, but down-regulation was significant (FC < 0.5; P=0.44) only for TcAMT2 (RNA-Seq-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 up-regulated in symbiosis (FC = 3.6; P<0.05), whereas expression of TcAMT1 was not significantly different from expression in the free-living mycelium grown on oat medium, the same medium used for symbiotic seed germination (RNA-Seq-A in Table 1; Fig. 4).

Fungal pelotons are thought to be key structures for nutrient exchange 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, and transcripts corresponding to TcAMT1 and TcAMT2 were identified in laser microdissected protocorm cells containing both younger (i.e. occupying the whole plant cell) and older (i.e. more condensed) fungal pelotons (Figs 5, S3).

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

In addition to inorganic N, 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 up-regulation was recorded for only one amino acid transporter/permease gene (named TcAAT9) in free-living mycelium grown on glutamine (FC = 3.8; P < 0.05), as compared with ammonium (RNA-Seq-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 free-living mycelia 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 up-regulated in symbiosis (RNA-Seq-A in Table 1). For example, TcAAT1, TcAAT2 and TcAAT6 were significantly up-regulated in mycorrhizal protocorms (FC > 2.5; P < 0.05), as compared with free-living mycelia (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).

N assimilation and pathways in T. calospora

Glutamine synthetase is an essential enzyme in N assimilation, and two genes coding for glutamine synthetase (named TcGS1
In RNA-Seq A, *T. calospora* gene expression was compared in free-living mycelium and in symbiosis. The experimental set-up is illustrated in Supporting Information Fig. S1. In RNA-Seq B, *T. calospora* gene expression was assayed in symbiotic and asymbiotic conditions on media containing two different N forms, glutamine and ammonium.)
transcripts could be amplified from laser microdissected protocorm cells containing fungal pelotons (Fig. S3). The difference (HSD) test (P<0.05). Results are mean values of three biological and three technical replicates ± SD. OA, oat agar.

Fig. 4 Quantification by quantitative real-time polymerase chain reaction (qPCR) of the expression of the *T. calospora* ammonium transporter genes *TcAMT1* and *TcAMT2* in free-living mycelium grown on different organic and inorganic nitrogen (N) sources and in symbiosis. Gene expression was calculated as the expression ratio relative to the free-living mycelium grown on oat medium (relative transcript level). Different lowercase letters above the bars denote significant differences by Tukey’s honest significant difference (HSD) test (P<0.05). Results are mean values of three biological and three technical replicates ± SD. OA, oat agar.

T. calospora gene coding for glutamate synthase, the other enzyme taking part in the N assimilation pathway, was also up-regulated (FC = 6.4; P < 0.05) in mycorrhizal protocorms (Table 1).

As no information is currently available on the N pathways inside orchid mycorrhizas, 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 is involved in arginine biosynthesis and is a key enzyme of the anabolic arm of the urea cycle; in the *T. calospora* free-living mycelium, the corresponding gene was found to be slightly induced by glutamine (FC = 2.5; P < 0.05), as compared with 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 the N pathway in AM, they are thought to release ammonium from arginine in the intraradical hyphae of AM fungi (Tian *et al*., 2010). Arginase (*CAR* gene expression was investigated in *T. calospora* by RNA-Seq (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).

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

As the genome of *S. vomeracea* has not yet been 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* (*P. equestris* Cai *et al*., 2015) proteomes. 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 up-regulation in symbiotic protocorms, but their induction was not statistically significant in the RNA-Seq experiment (Table 2). RT-qPCR demonstrated that *SvAMT1* was slightly but significantly up-regulated (FC = 2.5; P < 0.05) in symbiotic protocorms, whereas *SvAMT2* was not up-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
Some of them were validated by RT-qPCR, which confirmed the RNA-Seq results (Fig. 6). Two contigs coding for amino acid permeases (SvAAP1 and SvAAP2) and a contig with very high identity with lysine histidine transporter 1 (LHT1) in BLASTP searches (SvLHT) were the most up-regulated in symbiotic protocorms (Table 2). Interestingly, the strong up-regulation of SvLHT expression in symbiosis was accompanied by the up-regulation of the biosynthetic pathways for lysine and histidine in the symbiotic fungus (RNA-Seq-A in Table 1). Histidinol dehydrogenase, the gene coding for the last steps in histidine biosynthesis, was significantly up-regulated 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 up-regulated (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). Although the expression level was not very high in terms of raw read numbers, one contig (SvGS) was significantly up-regulated (FC = 245; P < 0.05) in mycorrhizal protocorms (Table 2), a situation confirmed by RT-qPCR (Fig. 6).

Discussion

N preference in orchid mycorrhizal fungi

Orchids are peculiar mycorrhizal partners because they acquire 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 probably derive from organic compounds available in soil because these fungi are known saprotrophs that utilize soil nutrient sources for growth and survival (Smith & Read, 2008; Waterman et al., 2011). Nurfadilah et al. (2013) reported different N source preference in orchid mycorrhizal 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 a preference for ammonium and organic N sources, like other cultivable orchid mycorrhizal fungi (Hadley & Ong, 1978; Nurfadilah et al., 2013). The different abilities of Tulasnella and Ceratobasidium to use inorganic N forms is probably explained by the fact that the T. calospora genome lacks genes involved in nitrate uptake and reduction, whereas these genes could be found in the recently sequenced genome of a Ceratobasidium sp. isolate, available on the Mycocosm portal (http://genome.jgi.doe.gov/CerAGI/CerAGI.home.html). The ability of orchid mycorrhizal fungi to use different N sources has important ecological implications because most photoautotrophic orchids host a diverse community of orchid mycorrhizal fungi in their roots and protocorms, often including both Tulasnella and Ceratobasidium species (see e.g. Jacquemyn et al., 2010; Girlanda et al., 2011; Ercole et al., 2015). 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).
Table 2: Expression of *Serapias vomeracea* contigs potentially involved in nitrogen (N) metabolism, identified in a de novo assembly annotated by BLASTX against the *Arabidopsis thaliana* and *Phalaenopsis equestris* proteome

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<th>Functional groups</th>
<th>Trunity contig name</th>
<th>Code</th>
<th>Mean raw read count</th>
<th>Fold change</th>
<th>A. thaliana protein ID</th>
<th>Putative function in A. thaliana</th>
<th>Score</th>
<th>e-value</th>
<th>Percent identity</th>
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Expression of *S. vomeracea* contigs in mycorrhizal protocorms (SYM) was compared with expression in nonmycorrhizal protocorms at a similar developmental stage (ASYM). nd, no match found. 
P-value, false discovery rate (FDR) P-value correction.

In bold, fold changes of the most significative comparisons (i.e. T. calospora gene expression in symbiotic and asymbiotic conditions on oat medium, and T. calospora gene expression in asymbiotic conditions on media containing two different N forms, glutamine and ammonium).
N pathways in the fungal hyphae

In the current model proposed for AM fungi, the best studied among mycorrhizal fungi, 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 it is broken down via the catabolic arm of the urea cycle (see Bücking & Kafle, 2015). This inorganic N form is then assimilated in the plant cytoplasm through the up-regulation of the plant GS/GOGAT pathway (Bücking & Kafle, 2015). The urea cycle also seems to be 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 because the gene coding for urease, the main enzyme involved in arginine breakdown and ammonium release, is up-regulated in the intraradical AM fungal mycelium (Koegel et al., 2015) but strongly down-regulated in the symbiotic T. calospora. Moreover, argininosuccinate lyase, a marker gene of arginine biosynthesis up-regulated in the extraradical AM fungal mycelium (Koegel et al., 2015), was instead up-regulated in symbiosis in T. calospora. It should, however, be noted that, also as a consequence of the obligate symbiotic nature of AM fungi, gene expression and enzymatic activities in AM fungi were assessed in two different but connected compartments, that is, 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 free-living mycelium and symbiotic conditions. The metabolic pathway and the form of N transferred inside the T. calospora hyphae that connect the substrate to the protocorm remain therefore to be understood.
N transfer inside the mycorrhizal orchid protocorm

One way to elucidate the form of N delivered by the mycorrhizal fungus in symbiosis is to investigate the plant import system. For example, ammonium transfer in AM symbiosis is suggested by the high and localized up-regulation of plant AMTs in arbuscle-containing cells (Gomez et al., 2009; Guether et al., 2009; Kobae et al., 2010; Koegel et al., 2013). In Lotus japonicus, LjAMT2;2 was the most up-regulated gene in mycorrhizal roots (Guether et al., 2009). In our orchid mycorrhizal system, by contrast, the importance of ammonium transfer to the plant remains unclear because the two S. vomeracea SvAMT1 and SvAMT2 genes were weakly expressed (as raw read numbers) and not strongly up-regulated in mycorrhizal protocorms.

Some putative S. vomeracea transporters strongly induced in mycorrhizal protocorms suggest transfer of organic N forms to the host plant in orchid mycorrhizas. 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 up-regulated in mycorrhizal protocorms. Up-regulation of genes coding for LHT1 in mycorrhizal roots has also been demonstrated by deep sequencing in the tropical orchid Cymbidium hybridum (Zhao et al., 2014) and in L. japonicus AM roots (Guether et al., 2011), suggesting a role during symbiosis. LHT1 was first identified in A. thaliana and AtLHT1 was originally reported as a lysine and histidine selective transporter (Chen & Bush, 1997), but later studies showed that LHT1s can transport quite a broad range of amino acids (Hirner et al., 2006; Guether et al., 2011). Further experiments are therefore needed to characterize the putative LHT1 identified in this work.

Cameron et al. (2006) suggested amino acid transfer in orchid mycorrhizas, 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 in orchid mycorrhizal roots was significantly lower than the ratio in the source glycine. As discussed by these authors, if co-transport 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 the $^{13}$C : $^{15}$N ratio (Cameron et al., 2006). Although substrate specificity of the S. vomeracea LHT’s remains to be established, we can speculate that the amino acids preferentially transferred may be N-enriched amino acids such as arginine, lysine or histidine, as suggested by the RNA-Seq data showing that the biosynthetic pathways of these amino acids are up-regulated in T. calospora when inside the mycorrhizal protocorm.

In addition to transporters/permeases for single amino acids, several S. vomeracea contigs identified as putative oligopeptide transporters were very strongly up-regulated in symbiotic protocorms (Table 2), similarly to what has been observed in mycorrhizal roots of Cymbidium hybridum (Zhao et al., 2014). However, the role of oligopeptide transporters in N transport is unclear, as these transporters seem to be also involved in the transport of metals and glutathione (Lubkowitz, 2011).

N 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 induction of a low-affinity ammonium transporter (TeAMT2) and of the ammonium scavenging enzyme glutamine synthetase (TcGS1) suggests that ammonium is at high concentrations in the plant–fungus interface and is rapidly assimilated once taken up by the fungal peloton. Fungal transcripts corresponding to high- and low-affinity AMTs in root colonized cells have been reported in AM fungi (Pérez-Tienda et al., 2011; Calabrese et al., 2016), where a role in ammonium retrieval from the apoplastic interface surrounding the arbuscule has been suggested. As hypothesized for AM fungi (Guether et al., 2009; Calabrese et al., 2016), the presence of both fungal and plant AMTs in the same colonized orchid 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 transfer in orchid mycorrhizas. The T. calospora genome contains two genes coding for functional ammonium transporters and several amino acid transporters/permeases that allow this fungus to exploit organic and inorganic N sources (but not nitrate). Based on transcriptional evidence, we suggest that organic N is mainly transferred to the host plant, possibly in the form of N-rich amino acids. The transfer of amino acids with a high N : C ratio would explain the unusually high N content in orchid tissues (Hynson et al., 2013).

Up-regulation in symbiosis of plant and fungal genes coding for membrane transporters suggests active transport processes in orchid mycorrhiza. However, our findings do not exclude a role of pelotons lysis in nutrient transfer, and the strong induction of plant amino acid and oligopeptide transporters may reflect recovery of organic N forms from collapsing hyphae. Further studies on the expression of these plant genes in laser microdissected protocorm cells containing pelotons at different stages of development may help to elucidate this point.

Recent studies in AM interactions have demonstrated that reciprocal reward strategies guarantee a ‘fair trade’ between the two mycorrhizal partners, where phosphorus and N 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 about the mechanisms controlling the nutrient flux, at least during the mycoherotrophic stages (Selosse & Rousset, 2011). In fact, orchid mycorrhizal 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 orchid mycorrhizas (Sélosse & Rousset, 2011). The identification of the molecular components involved in this nutrient flow may provide some tools with which to start addressing this question.

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Author contributions

S.P., R.B. and M.G. conceived and designed the research. V.F. and W.C. conducted all wet lab experiments. S.V. prepared the manuscript. All authors read the manuscript. All authors read and approved the manuscript.

References


Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Experimental set-up of the two RNA-Seq experiments.

Fig. S2 RT-PCR experiments on LMD samples using primers for housekeeping genes.

Fig. S3 RT-PCR analysis of Tulasnella calospora genes in LMD cell-type populations.

Fig. S4 Phylogenetic tree of plant ammonium transporters.

Table S1 List of primers used in this study

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