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Research

Fungal and plant gene expression in the *Tulasnella calospora–Serapias 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 apoplatic 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 & 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; Pérez-Tienda *et al.*, 2011; Calabrese *et al.*, 2016) and ECM fungi (Javelle *et al.*, 2001; Montanini *et al.*, 2002; Willmann *et al.*, 2007). Amino acid permeases and peptide transporters, which allow for organic N uptake, have been identified and characterized mainly in ECM fungi (Nehls *et al.*, 1999; Wipf *et al.*, 2002; 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 (Bücking & Kafle, 2015; Koegel *et al.*, 2015), whereas N transfer across the ECM interface is not completely clear and may involve both organic (i.e. amino acids) and inorganic N forms (Chalot *et al.*, 2006; Couturier *et al.*, 2007; Müller *et al.*, 2007).

Compared with ECM and AM symbiosis, very little is known about N metabolism in orchid mycorrhizas. Some orchid mycorrhizal fungi belonging to *Ceratobasidium*, *Tulasnella* and *Sebacina* can grow on organic N as well as on ammonium (Hadley & Ong, 1978; Nurfadilah *et al.*, 2013), and 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 *Ceratobasidium 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 largely unknown. In the colonized protocorm cells, orchid mycorrhizal 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 arbuscules, fungal pelotons in orchid mycorrhizas 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 mycorrhizas. 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.

Here, we used the orchid mycorrhizal fungus *Tulasnella calospora* isolate AL13/4D, either as free-living mycelium or in symbiosis with achlorophyllous protocorms of the photosynthetic orchid *Serapias vomeracea*, as a model system to explore the genetic bases of N uptake and transfer to the mycorrhizal orchid host. In particular, we investigated some fungal genes that may be involved in the uptake of inorganic and organic N forms and their transfer to the host plant. To this purpose, we used available genomic information as well as RNA-sequencing (RNA-Seq) experiments that compared fungal gene expression on two N sources (ammonium and glutamine) and/or in symbiosis. We show that *T. calospora* lacks a nitrate uptake system but has two functional ammonium transporters (*T. calospora* ammonium transporters 1 and 2, named TcAMT1 and TcAMT2) that 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 regarding the N pathway in orchid mycorrhizas.

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 flasks containing 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 *Serapias 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 *Serapias 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 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 ammonium permease (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*, characterized 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% 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 (OD_{600}) reached 0.6–0.8. Cells were harvested, washed twice, and resuspended in water to a final OD_{600} of 2, and 10-μl drops corresponding to a serial 10-fold dilution 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 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 BLASTX 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 quantified 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 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 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., Bensheim, 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 Balestrini *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 (*SvEF1α*) 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 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 (Pérez-Tienda *et al.*, 2011) showed that *TcAMT1* clustered with high-affinity transporters, whereas *TcAMT2* clustered with low-affinity transporters (Fig. 2).

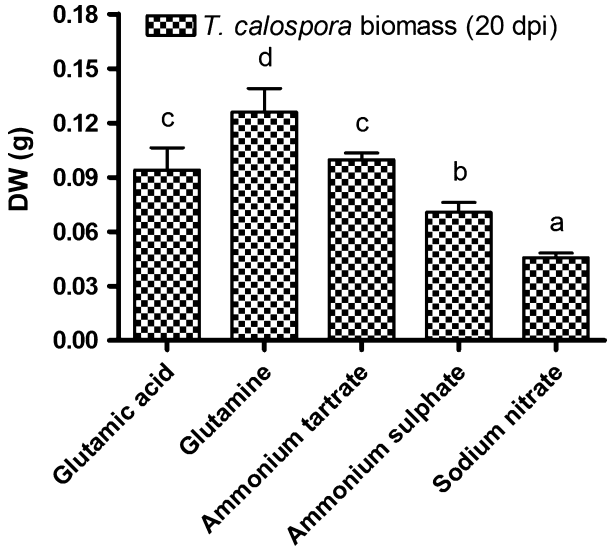

Fig. 1 Growth of *Tulasnella calospora* on different nitrogen (N) sources, as dry weight (DW). Biomass is expressed as mean values of three replicates ± SD. ANOVA was performed comparing all five media, and values with different letters above the bars differ significantly following Tukey's honest significant difference (HSD) test ($P < 0.05$). dpi, days post inoculum.

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 NH_4^+ 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

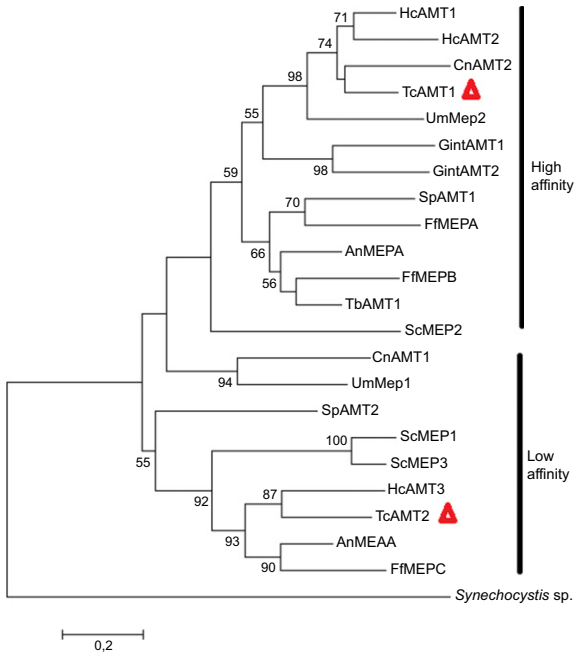

Fig. 2 Phylogenetic tree of fungal ammonium transporters, based on amino acid deduced sequences. The sequences were aligned using MUSCLE and a tree was constructed using the maximum likelihood method. Numbers indicate bootstrap values, and are given only for ≥50%. Bootstrap tests were performed using 1000 replicates. Sequences were obtained from the GenBank database with the following accession numbers: *Glomus intraradices* (GintAMT1, CAI54276; GintAMT2, CAX32490), *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* (SpAMT1, NP_588424; SpAMT2, NP_593462), *Aspergillus nidulans* (AnMEAA, AAL73117; AnMEPA, AAL73118), *Fusarium fujikuroi* (FfMEPA, CAJ44733; FfMEPB, CAJ44734; FbMEPC, CAK55531), *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.

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*

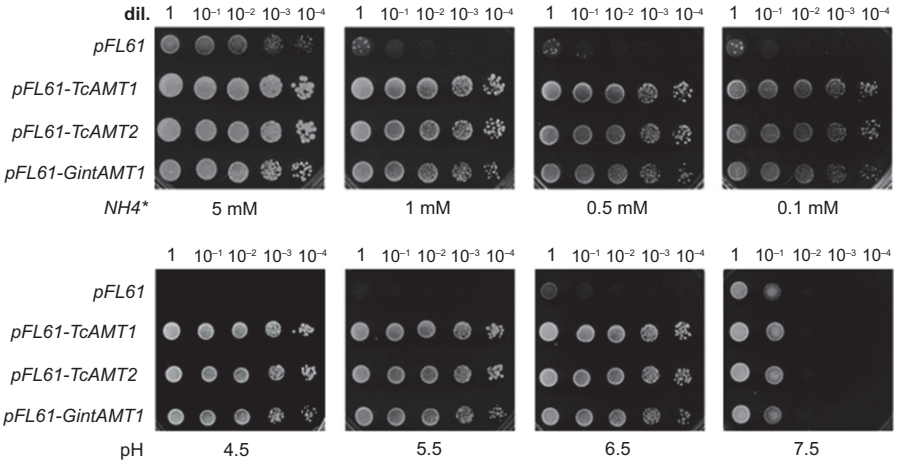

Fig. 3 Complementation of the ammonium permease (*mep*) yeast mutant with *Tulasnella calospora* ammonium transporters *TcAMT1* and *TcAMT2*. The ammonium uptake-deficient yeast 31019b (ΔΔΔmep1;2;3) was transformed with *TcAMT1*, *TcAMT2*, the positive control *Rhizophagus irregularis GintAMT* and the empty plasmid pFL61 as a negative control. Serial dilutions of the transformants were grown on ammonium as the sole nitrogen (N) source at different concentrations and pH or on arginine as a positive growth control.

Table 1 Expression of *Tulasnella calospora* genes potentially involved in nitrogen (N) metabolism in free-living mycelium (FLM) and in symbiotic mycelium (SYM)

				RNA-Seq-A				RNA-Seq-B								
				Mean raw read counts		Fold change		Mean raw read counts			Fold change		Fold change		Fold change	
Functional groups	Protein ID	Protein length	Code	FLM Oat	SYM Oat	SYM vs FLM Oat	*P*-value	FLM Am	FLM Gln	SYM Oat	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**	0.0713	380.84	195.35	329.02	**0.51**	0.0440	0.86	0.6541	1.68	0.0008	Ammonium transporter
	186135	537	*TcAMT2*	200.13	724.54	**3.62**	0.0172	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**	0.0001	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**	0.0015	24.10	24.45	87.63	**1.01**	1.0000	3.64	0.0749	3.58	0.0594	Amino acid transporter/permease
	74421	238	*TcAAT3*	366.77	581.75	**1.59**	0.0128	1000.38	548.83	374.69	**0.55**	0.1120	0.37	0.0331	0.68	0.0373	Amino acid transporter/permease
	74154	782	*TcAAT4*	55.72	80.85	**1.45**	0.0016	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**	1E-06	715.87	379.32	209.47	**0.53**	0.0010	0.29	4E-09	0.55	0.0047	Amino acid transporter/permease
	24746	512	*TcAAT6*	4.96	16.15	**3.25**	0.0074	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**	9E-08	75.45	74.87	89.02	**0.99**	0.8505	1.18	0.9024	1.19	0.7850	Amino acid transporter/permease
	33378	416	*TcAAT8*	23.66	40.98	**1.73**	0.0047	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**	0.0051	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**	0.0319	15.82	9.22	17.94	**0.58**	0.0624	1.13	1.0000	1.95	0.6214	Amino acid transporter/permease
	228655	449	*TcAAT11*	131.11	75.07	**0.57**	4E-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**	0.0021	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**	2E-08	30.31	19.83	13.40	**0.65**	0.1078	0.44	0.1210	0.68	0.6195	Amino acid transporter/permease
	23211	586	*TcAAT14*	27.09	9.38	**0.35**	7E-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**	0.0120	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**	0.0073	1.51	2.12	2.17	**1.40**	0.8669	1.44	0.9484	1.03	1.0000	Oligopeptide transporter OPT superfamily
	6542	1664		3.66	10.93	**2.98**	0.0062	5.06	2.68	8.84	**0.53**	0.2972	1.75	0.3380	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**	1.0000	2.64	0.0063	2.48	0.0061	Oligopeptide transporter OPT superfamily
	21934	989		29.03	17.51	**0.60**	0.0183	7.28	11.60	12.37	**1.59**	0.2914	1.70	0.6000	1.07	1.0000	Oligopeptide transporter OPT superfamily
	22904	1063		34.96	18.02	**0.52**	0.0005	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**	0.0231	10.66	7.40	5.43	**0.69**	0.3437	0.51	0.3178	0.73	0.7900	Oligopeptide transporter OPT superfamily
	229195	805		12.25	4.18	**0.34**	0.0037	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**	0.0008	7.18	6.93	7.02	**0.96**	0.9750	0.98	1.0000	1.01	1.0000	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**	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**	4E-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**	0.0280	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**	0.9539	52.68	75.84	83.63	**1.44**	0.0410	1.59	0.3923	1.10	0.8607	Glutamine synthetase
	242592	2163	*TcGOGAT*	16.49	104.95	**6.36**	0.0126	20.47	27.70	95.05	**1.35**	0.4985	4.64	0.0000	3.43	2E-12	Glutamate synthase
Urea cycle	245827	466	*TcASL*	118.15	337.12	**2.85**	0.0000	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**	0.0558	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	0.0720	Urease
Lysine biosynthesis	241089	388		51.86	225.57	**4.35**	1E-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**	0.0051	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**	0.0000	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**	0.0000	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**	4E-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

In RNA-Seq-A, *T. calospora* gene expression was compared in free-living mycelium and in mycorrhizal protocorms grown on the same oat medium. In RNA-Seq-B, *T. calospora* gene expression was compared in free-living mycelium grown on two different N sources (Am, ammonium; In = glutamine) and in symbiosis. The experimental set-up is illustrated in Supporting Information Fig. S1.
P-value, false discovery rate (FDR) *P*-value correction. NB, only transcripts with FDR ≤0.05 in at least one sample comparison are listed in the table.
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).

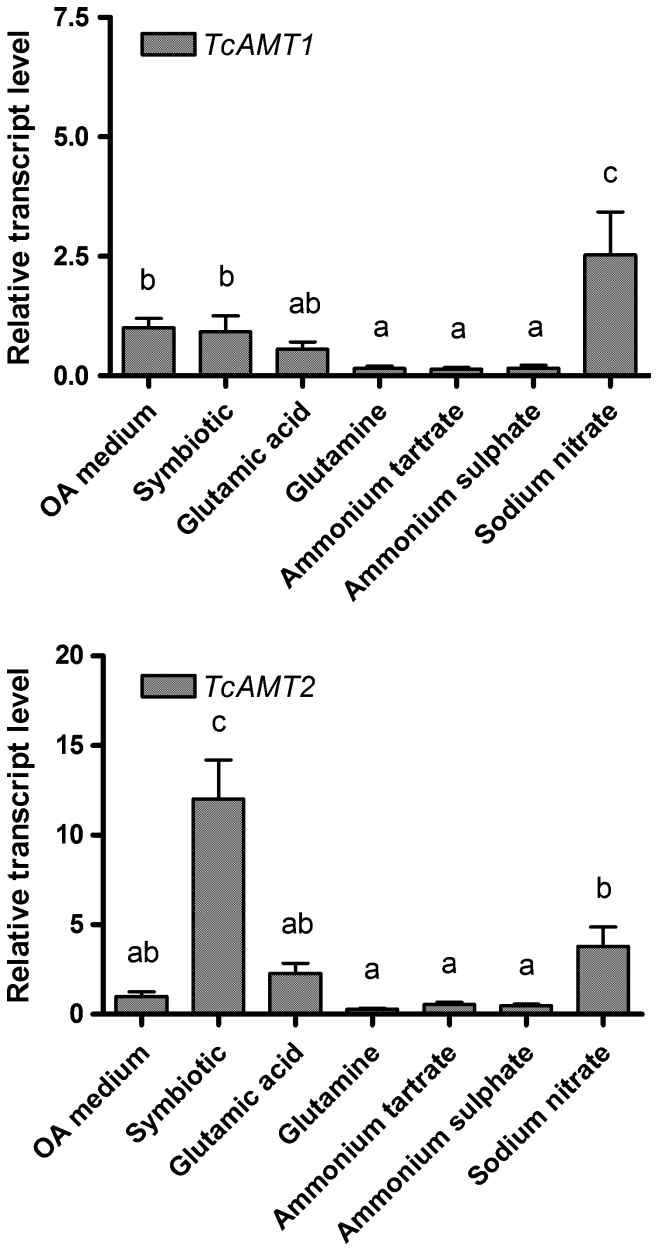

Fig. 4 Quantification by quantitative real-time polymerase chain reaction (qPCR) of the expression of the *T. calospora* ammonium transporter genes *TcAMT1 and TcAMT2* in free-living mycelium grown on different organic and inorganic nitrogen (N) sources and in symbiosis. Gene expression was calculated 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.

and *TcGS2*) were identified in the *T. calospora* genome. Their expression in free-living mycelium did not change on ammonium- or glutamine-containing media (Table 1), although *TcGS1* was more strongly expressed (as a raw read number) and up-regulated in symbiosis (FC = 2.7; $P < 0.05$). Both *TcGS1* and *TcGS2* transcripts could be amplified from laser microdissected protocorm cells containing fungal pelotons (Fig. S3). The *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).

Serapias 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* (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

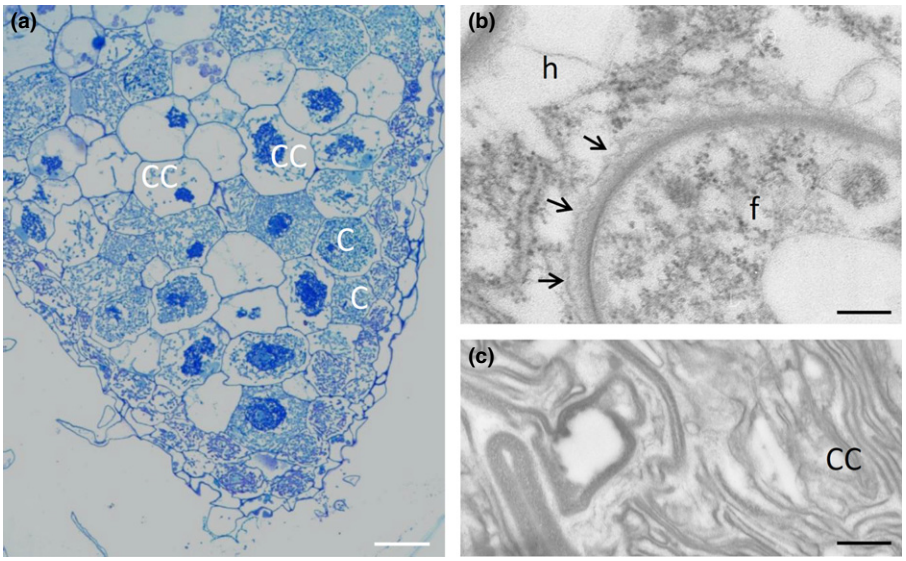

Fig. 5 Mycorrhizal protocorms of *Serapias vomeracea* 30 d after sowing with *Tulasnella calospora*. (a) Semi-thin section of a resin-embedded protocorm, showing the basal mycorrhizal region. Colonized cells containing hyphal pelotons (coils) at different stages of development correspond to the laser microdissected cell types: cells containing younger, well-developed pelotons occupying the whole plant cell (C), and cells containing older, more condensed or collapsing fungal pelotons (CC). (b) Under the electron microscope, an interfacial matrix surrounded by plant membrane proliferation (arrows) can be seen around an intracellular hypha (f) inside the host plant cell (h). (c) A colonized protocorm cell containing a collapsed coil (CC). Bars: (a) 80 μm; (b) 0.25 μm; (c) 0.35 μm.

verified by BLASTX and with FDR ≤ 0.05 are reported in Table 2. 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).

New Phytologist (2017) 213: 365–379
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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

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
			ASYM	SYM											
Ammonia permeases	DN68801_c0_g1_i1	*SvAMT1*	7.69	21.75	**2.83**	0.8961	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**	0.9877	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	nd			
	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	nd			
	DN63460_c0_g1_i1	*SvLHT*	1.79	167.52	**93.43**	4E-05	AT5G40780.2	Lysine histidine transporter 1	987	9.00E-133	87.0	nd			
	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**	0.0198	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**	0.0018	AT5G55930.1	Oligopeptide transporter 1	224	3.00E-22	57.3				
	DN1177_c0_g1_i1		0.85	381.43	**451.23**	0.0108	AT5G55930.1	Oligopeptide transporter 1	391	1.00E-43	60.5	PEQU_41210	525	9.00E-68	72.0
	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	56.0	PEQU_40777	432	3.00E-55	89.0
	DN48315_c0_g1_i1		0.37	279.21	**750.97**	2E-10	AT4G26590.1	Oligopeptide transporter 5	807	8.00E-101	58.0	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	nd			
	DN69777_c0_g1_i2		0.58	11.01	**19.02**	3E-05	AT3G54140.1	Peptide transporter 1	2148	0	71.2	nd			
	DN75842_c0_g4_i1		9.36	41.38	**4.42**	0.0005	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**	0.0318	AT5G35630.3	Glutamine synthetase 2	925	3.00E-121	60.4	nd			

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).

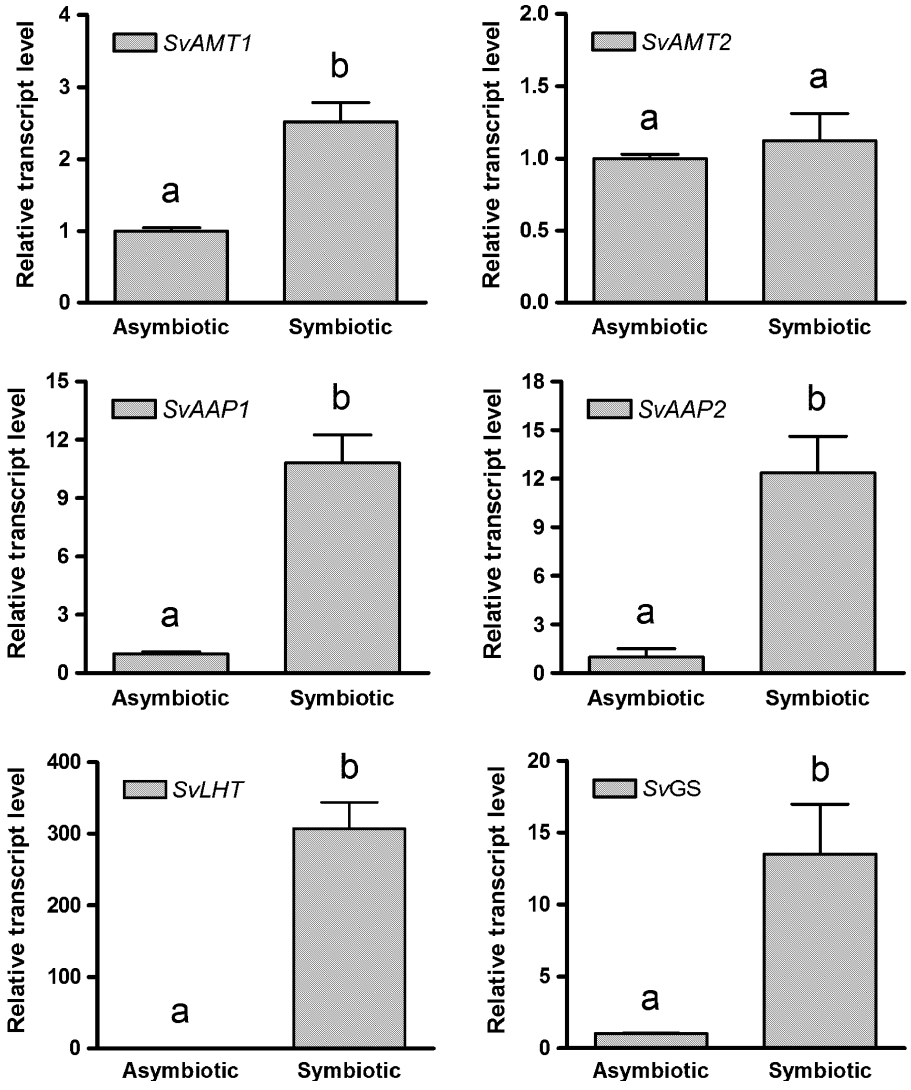

Fig. 6 Quantitative real-time polymerase chain reaction (qPCR) assessing the expression of several *Serapias vomeracea* contigs in symbiotic and asymbiotic protocorms. Gene expression was calculated as the expression ratio relative to asymbiotic protocorms (relative transcript level). Different lowercase letters above the bars denote significant differences by two-tailed Student's *t*-test ($P < 0.05$). Results are mean values of three biological and three technical replicates ± SD.

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 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 through local induction of plant ammonium transporters (Gomez *et al.*, 2009; Guether *et al.*, 2009; Kobae *et al.*, 2010; Koegel *et al.*, 2013). 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 arbuscule-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 LHTs 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* LHTs 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 (TcAMT2) 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 mycoheterotrophic 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 (Selosse & 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 biological materials and extracted the RNA for the cDNA libraries. A.K., V.R.S., E.L., K.B., I.V.G. and F.M. supervised and/or conducted the transcriptome sequencing and the bioinformatic analyses. S.P. and R.B. wrote the manuscript. All authors read and approved the manuscript.

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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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