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

3

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26 **Summary**

- 27 • Orchids are highly dependent on their mycorrhizal fungal partners for nutrient supply, especially
28 during early developmental stages. In addition to organic carbon, nitrogen (N) is likely a major
29 nutrient transferred to the plant because orchid tissues are highly N-enriched. We know almost
30 nothing on the N form preferentially transferred to the plant and on the key molecular determinants
31 required for N uptake and transfer.
- 32 • We identified, in the genome of the orchid mycorrhizal fungus *Tulasnella calospora*, two
33 functional ammonium transporters and several amino acid transporters but no evidence of a nitrate
34 assimilation system, in agreement with N preference of the free living mycelium grown on different
35 N sources.
- 36 • Differential expression in symbiosis of a repertoire of fungal and plant genes involved in transport
37 and metabolism of N compounds suggests that organic N may be the main form transferred to the
38 orchid host and that ammonium is taken up by the intracellular fungus from the apoplatic symbiotic
39 interface.
- 40 • This is the first study addressing the genetic determinants of N uptake and transport in orchid
41 mycorrhiza, and provides a model for nutrient exchanges at the symbiotic interface, which may
42 guide future experiments.

43

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

45

46

47 **Introduction**

48 Like the majority of terrestrial plants, orchids form mycorrhizal associations with soil fungi that
49 provide them with essential nutrients. However, orchids are peculiar because seed germination and
50 early development in nature fully depend on the mycobionts (Rasmussen, 1995; Smith & Read,
51 2008), that provide the embryo with organic carbon (C) and other essential nutrients. Following
52 seed germination, orchids form the protocorm, a heterotrophic structure that precedes seedling
53 development and continue to rely on their mycobionts for organic C supply, a strategy known as
54 mycoheterotrophy (Leake, 2004). Some orchid species remain achlorophyllous or with inefficient

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

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

69 Nitrogen occurs in soil as inorganic forms, such as ammonium (NH₄⁺) and nitrate (NO₃⁻), or as
70 organic N compounds. Fungal uptake and transfer of soil-derived N to the host plant has been
71 extensively investigated in arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) fungi (see
72 references in Chalot *et al.*, 2006; Martin *et al.*, 2007; Müller *et al.*, 2007; Koegel *et al.*, 2015;
73 Bücking & Kafle, 2015). Nitrate transporters and genes responsible for nitrate utilization have been
74 identified mainly in AM fungi (Kaldorf *et al.*, 1998; Tian *et al.*, 2010; Koegel *et al.*, 2015), and
75 nitrophilous ECM fungi (Plassard *et al.*, 2000; Jargeat *et al.*, 2003; Montanini *et al.*, 2006). Genes
76 coding for high- and low-affinity ammonium transporters have been identified and characterized in
77 both AM (López-Pedrosa *et al.*, 2006; Perez-Tienda *et al.*, 2011; Calabrese *et al.*, 2016) and ECM
78 fungi (Montanini *et al.*, 2002; Javelle *et al.*, 2001; Willmann *et al.*, 2007). Amino acid permeases
79 and peptide transporters, that allow for organic N uptake, have been identified and characterized
80 mainly in ECM fungi (Nehls *et al.*, 1999; Wipf *et al.*, 2002a; Guidot *et al.*, 2005; Benjdia *et al.*,
81 2006; Shah *et al.*, 2013) but also in AM fungi (Cappellazzo *et al.*, 2008; Belmondo *et al.*, 2014),
82 although the ability of AM fungi to utilize organic N sources is considered to be relatively low.
83 Ammonium is thought to be the main N form transferred to the host plant in AM (Koegel *et al.*,
84 2015; Bücking & Kafle, 2015), whereas N transfer across the ECM interface is not fully clear and
85 may involve both organic (i.e. amino acids) and inorganic nitrogen forms (Chalot *et al.*, 2006;
86 Couturier *et al.*, 2007; Müller *et al.*, 2007).

87 Compared to ECM and AM fungi, very little is known on N metabolism in orchid mycorrhiza.
88 Some ORM fungi belonging to *Ceratobasidium*, *Tulasnella* and *Sebacina* can grow on organic N as
89 well as on ammonium (Hadley & Ong, 1978; Nurfadilah *et al.*, 2013). In addition, a
90 *Ceratobasidium* spp. isolate also grew on nitrate (Nurfadilah *et al.*, 2013). Uptake of N from
91 inorganic sources by a *Ceratobasidium* isolate and transfer to the host protocorm was demonstrated
92 by Kuga *et al.* (2014) after feeding the fungus with ¹⁵N-labelled NH₄NO₃. Cameron *et al.* (2006)
93 demonstrated uptake of double-labelled [¹³C-¹⁵N] glycine by *C. cornigerum* and transfer of labelled
94 ¹⁵N to the adult photosynthetic host *Goodyera repens*. However, the mechanisms underlying fungal
95 N uptake and transfer to the plant, as well as the form of N transferred to the orchid host, remain
96 unknown.

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

106

107 **Materials and methods**

108 ***Growth of the free-living mycelium (FLM)***

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

119 Modified P5 solid medium with the same N sources, as well as oat agar medium (0.3% milled oats,
120 1% agar), were used to grow FLM for RNA extraction and expression studies. In this case, *T.*
121 *calospora* was inoculated onto a sterilized cellophane membrane placed on top of the agar medium
122 (Schumann *et al.*, 2013) and kept until the plate was fully colonised (about 20 days). The mycelium
123 was then collected from the cellophane membrane, immediately frozen in liquid nitrogen and stored
124 at -80°C.

125

126 ***Symbiotic and asymbiotic germination of S. vomeracea seeds***

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

138

139 ***Gene identification and phylogenetic analysis***

140 Fungal genes coding for proteins possibly involved in N uptake and transfer were identified in the
141 *T. calospora* genome database on the JGI fungal genome portal MycoCosm
142 (<http://genome.jgi.doe.gov/Tulca1/Tulca1.home.htm>). Plant transcripts coding for proteins
143 potentially involved in N uptake and transfer were selected from the RNASeq database obtained in
144 this study as described below. Multiple protein alignments were done with MUSCLE (Edgar,
145 2004). Phylogenetic trees were constructed with the maximum likelihood method using the MEGA
146 software, Version 7.0 (Kumar *et al.*, 2015); bootstrap analyses were conducted on the basis of 1,000
147 re-samplings of the sequence alignment.

148

149 ***Full-length TcAMT1 and TcAMT2 isolation***

150 *TcAMT1* (1467 bp) and *TcAMT2* (1611 bp) full length cDNA isolation was carried out through PCR
151 amplification of *T. calospora* or mycorrhizal *S. vomeracea* protocorms cDNA with the full-length
152 primers reported in Table S1. PCR reactions were performed in 50 µl final volume containing 10 µl
153 of 5X Phusion HF Buffer, 1 µl of dNTPs (10mM stock each), 1.5 µl of each primer (10 mM stock),
154 0.5 µl of Phusion High-Fidelity DNA polymerase (NEB, USA), 34.5 µl of water and 1 µl of cDNA.
155 PCR amplifications were carried out in a thermal cycler (Biometra) using the following program: 98
156 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
157 were visualised on 1.2% agarose gel after electrophoresis in 0.5X TAE buffer, excised from the
158 agarose gel and purified using Wizard® SV Gel and PCR Clean-Up System following the
159 manufacturer's instruction. The purified DNA was eluted in 30 µl of Nuclease-Free Water and
160 inserted into the p-GEM T plasmid (Promega) using a T4 DNA Ligase (Promega). Plasmids were
161 transformed into *E. coli* chemically competent cells (Top10 cells, Invitrogen). Plasmids were then
162 purified, from positive colonies, using QIAprep Spin Miniprep Kit (QIAGEN). Sequencing was
163 performed at the sequencing service at Munich University (Sequencing Server, Biocenter of the
164 LMU Munich, Germany).

165

166 ***Heterologous expression of TcAMT1 and TcAMT2 in yeast***

167 Full-length *TcAMT1* and *TcAMT2* cDNAs were cloned into the yeast expression vector pFL61
168 under the control of the constitutive yeast phosphoglycerate kinase (PGK) promoter (Minet *et al.*,
169 1992). The *Saccharomyces cerevisiae* triple mep mutant 31019b ($\Delta\Delta\Delta$ mep1;2;3; Marini *et al.*,
170 1997), kindly provided by Nuria Ferrol (CSIC, Granada, Spain), was transformed with the empty
171 pFL61 vector (negative control) or with the pFL61-*TcAMT1* and pFL61-*AMT2* constructs according
172 to Gietz & Schiestl (2007). The coding sequence of *GintAMT1*, characterised in the AM fungus
173 *Rhizophagus irregularis* (previously *Glomus intraradices*; López-Pedrosa *et al.*, 2006), was used as
174 a positive control. Transformed yeasts were selected on solid N-free medium (2% Agar, 0.17%
175 YNB w/o amino acids and ammonium sulphate) supplemented with 3% glucose and 0.1% arginine
176 as the sole N source, buffered to pH 6.1 with 50 mM MES/Tris. For growth assays, yeast
177 transformants were grown in liquid N-free medium plus arginine until OD600 reached 0.6–0.8.
178 Cells were harvested, washed twice, resuspended in water to a final OD600 of 2, and 10 µl drops
179 corresponding to a serial 10-fold dilutions were spotted on solid N-free medium supplemented with
180 different NH₄Cl concentrations as the sole N source (0.1, 0.5, 1 and 5 mM). Yeast cells were also

181 spotted on 1 mM NH₄Cl-supplemented medium buffered at different pH (4.5, 5.5, 6.5, 7.5) with 50
182 mM Mes/Tris. Pictures of the plates were taken 3 days incubation at 30°C.

183

184 **RNA-Seq experiment**

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

192 For the second experiment (RNASeq-B), *T. calospora* FLM was grown on Modified Melin-
193 Norkrans medium with two different N sources, ammonium and glutamine, each added as 0.49g of
194 N. For each condition, three separate libraries were prepared from three biological replicates. To
195 investigate fungal and plant gene expression in symbiosis, RNA was also extracted in a parallel
196 experiment from symbiotic and asymbiotic *S. vomeracea* protocorms obtained as described above.
197 Independent libraries were prepared from three replicates for each condition. Illumina HiSeq2500
198 sequencing (2x150bp) was performed at the Joint Genome Institute (JGI, Walnut Creek, USA). The
199 complete series was submitted to GEO (XXXX).

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

209 Reads Alignments: Reads were aligned either to the *T. calospora* reference transcripts
210 (<http://genome.jgi-psf.org>) or to the *S. vomeracea de novo* assembly using CLC Genomics
211 Workbench. For mapping, the minimum length fraction was 0.9, the minimum similarity fraction
212 0.8 and the maximum number of hits for a read was set to 10. The unique and total mapped reads
213 number for each transcript were determined, and then normalized to RPKM (Reads Per Kilobase of

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

219

220 **Real Time quantitative PCR (RT-qPCR) analyses**

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

240

241 **Laser Microdissection (LMD) analysis**

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

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

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

261

262 **Microscopy**

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

269

270 **Statistical analysis**

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

276

277

278

279 **Results**

280 ***Growth of *Tulasnella calospora* on different nitrogen sources***

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

285

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

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

301

302 ***Heterologous expression of *TcAMT1* and *TcAMT2* in a yeast *mep* mutant***

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

310 4.5 to 7.5 on minimal medium containing 1 mM NH₄⁺ as the sole N source. Both transporters
311 showed pH dependency, and growth promotion was best at acidic pH and strongly decreased at pH
312 7.5 (Fig. 3).

313

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

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

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

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

336

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

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

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

352

353 ***Nitrogen assimilation and pathways in T. calospora***

354 Glutamine synthetase (GS) is an essential enzyme in N assimilation, and two genes coding for
355 glutamine synthetase (named *TcGS1* and *TcGS2*) were identified in the *T. calospora* genome. Their
356 expression in FLM did not change on ammonium or on glutamine containing media (Table 1),
357 although *TcGS1* was more expressed (as raw reads number) and upregulated in symbiosis ($FC = 2.7$;
358 $P < 0.05$). Both *TcGS1* and *TcGS2* transcripts could be amplified from LMD protocorm cells
359 containing fungal pelotons (Fig. S3). The *T. calospora* gene coding for glutamate synthase, the
360 other enzymes taking part to the GS/GOGAT nitrogen assimilation pathway, was also upregulated
361 ($FC = 6.4$, $P < 0.05$) in mycorrhizal protocorms (Table 1).

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

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

377

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

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

393 Several contigs in the *S. vomeracea* transcriptome matched genes annotated as amino acid
394 transporters/permeases in the *A. thaliana* and *P. equestris* genomes. Only contigs manually verified
395 by BlastX and with $FDR \leq 0.05$ are reported in Table 2. Some of them were validated by RT-qPCR,
396 that confirmed the RNASeq results (Fig. 6). Two contigs coding for amino acid permeases (*SvAAP1*
397 and *SvAAP2*) and a contig identified as a putative lysine histidine transporter (*SvLHT*) with very
398 high identity with LHT1 transporters in Blastp searches, were the most upregulated in symbiotic
399 protocorms (Table 2). Interestingly, the strong upregulation of *SvLHT* expression in symbiosis was
400 mirrored by the upregulation of the biosynthetic pathways for lysine and histidine in the symbiotic
401 fungus (RNASeq-A in Table 1). Histidinol dehydrogenase, the gene coding for the last steps in
402 histidine biosynthesis, was significantly upregulated inside mycorrhizal protocorms (FC=5.7,
403 $P<0.05$). One sequence corresponding to saccharopine dehydrogenase, the final enzyme responsible
404 of lysine biosynthesis (protein ID 241089), was also upregulated (FC=4.4, $P<0.05$) in symbiosis
405 (Table 1).

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

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

410

411 **Discussion**

412

413 *Nitrogen preference in orchid mycorrhizal fungi*

414 Orchids are peculiar mycorrhizal partners because they acquires all nutrients through the fungal
415 symbiont, including organic C, at least during the mycoheterotrophic life stages (Smith & Read,
416 2008; Selosse & Martos, 2014). For terrestrial orchids associated with *Tulasnella*, *Ceratobasidium*
417 and *Sebacinales*, nutrients likely derive from C, N and P forms available in soil because these fungi
418 are known saprotrophs that utilize soil nutrient sources for growth and survival (Waterman *et al.*,
419 2011; Smith & Read, 2008). Nurfadilah *et al.* (2013) reported different N source preference in
420 ORM fungi because isolates in the genus *Tulasnella* could use ammonium but not nitrate as
421 inorganic N forms, whereas isolates in the genus *Ceratobasidium* could use both ammonium and
422 nitrate. Growth of *T. calospora* isolate AL13/4D on different N sources confirmed, like other
423 cultivable ORM fungi (Hadley & Ong, 1978; Nurfadilah *et al.*, 2013), a preference for ammonium
424 and organic N sources. The different ability of *Tulasnella* and *Ceratobasidium* to use inorganic N
425 forms is likely explained by the fact that the *T. calospora* genome lacks genes involved in nitrate
426 uptake and reduction, whereas these genes can be found in the recently sequenced genome of
427 *Ceratobasidium*, available on the Mycosm portal
428 (<http://genome.jgi.doe.gov/CerAGI/CerAGI.home.html>). The ability of ORM fungi to use different
429 N sources has important ecological implications because most photoautotrophic orchids host a
430 diverse community of ORM fungi in their roots and protocorms, usually including both *Tulasnella*
431 and *Ceratobasidium* species (see e.g. Girlanda *et al.*, 2011; Ercole *et al.*, 2015; Jacquemyn *et al.*,
432 2010). Co-occurrence of fungal symbionts able to utilize a wide variety of nutrient sources and to
433 exploit different soil N forms would represent an advantage for the host plant because it could
434 broaden the habitat range as well as the ability of the orchid to grow in a wide range of soil types
435 (Nurfadilah *et al.*, 2013).

436

437 *Nitrogen pathways in the fungal hyphae*

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

441 arginine *via* the biosynthetic arm of the urea cycle (Bago *et al.*, 2001). Arginine is then transported
442 to the intraradical mycelium, where is broken down *via* the catabolic arm of the urea cycle to
443 release ammonium (see Bücking & Kafle, 2015). The free ammonium is released into the plant-
444 fungus interface, where it is taken up by the host plant thanks to local induction of plant ammonium
445 transporters (Guether *et al.*, 2009; Gomez *et al.*, 2009; Kobae *et al.*, 2010; Koegel *et al.*, 2013). This
446 inorganic N form is then assimilated in the plant cytoplasm thanks to the upregulation of the plant
447 GS/GOGAT pathway (Bücking & Kafle, 2015). The urea cycle seems to be also involved in the N
448 pathway of some ECM fungi, as urea was found to accumulate in the extraradical mycelium
449 together with gene transcripts related to the urea cycle (Morel *et al.*, 2005; Wright *et al.*, 2005).
450 Although solely based on transcriptional evidence, it seems unlikely that *T. calospora* uses this N
451 pathway to transfer ammonium to the orchid protocorm. Argininosuccinate lyase, a marker gene of
452 arginine biosynthesis upregulated in the extraradical AM fungal mycelium (Koegel *et al.*, 2015), is
453 upregulated in symbiosis in *T. calospora*. Moreover, the gene coding for urease, the main enzyme
454 involved in arginine breakdown and ammonium release, is upregulated in the intraradical AM
455 fungal mycelium (Koegel *et al.*, 2015) but was strongly down-regulated in *T. calospora* when inside
456 ORM protocorms. It should be however noted that, also due to the obligate symbiotic nature of AM
457 fungi, gene expression and enzymatic activities in AM fungi were assessed in two different but
458 connected compartments, i.e. the extraradical and intraradical AM fungal mycelium (Gomez *et al.*,
459 2009; Tian *et al.*, 2010; Koegel *et al.*, 2015), whereas gene expression in *T. calospora* was
460 measured separately in FLM and symbiotic conditions. The metabolic pathway and the form of N
461 transferred inside the *T. calospora* hyphae that connect the substrate to the protocorm remains
462 therefore to be understood.

463

464 ***Nitrogen transfer inside the mycorrhizal orchid protocorm***

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

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

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

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

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

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

509

510 ***Nitrogen uptake by T. calospora inside mycorrhizal protocorm cells***

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

522

523 ***Conclusions***

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

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

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

543

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551

552 **Author Contribution Statement**

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

558

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

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

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

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

P-value: FDR *p*-value correction

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

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

n.d: No match found

P-value: FDR p-value correction

875 **Figures legends**

876

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

880

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

894

895 **Figure 3.** Complementation of *mep* yeast mutant with *TcAMT1* and *TcAMT2*. The ammonium
896 uptake-deficient yeast 31019b ($\Delta\Delta\Delta mep1;2;3$) was transformed with *TcAMT1*, *TcAMT2*, the
897 positive control *GintAMT* and the empty plasmid pFL61 as negative control. Serial dilutions of the
898 transformants were grown on ammonium as sole N source at different concentrations and pH or on
899 arginine as positive growth control.

900

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

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

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

910 **Figure 6.** RT-qPCR assessing expression of several *Serapias vomeracea* contigs in symbiotic and
911 asymbiotic protocorms. Different lowercase letters above the bars denote significant differences by
912 two-tailed Student's *t* test ($P < 0.05$).

913

914

915 **List of supplementary material**

916

917 **Table S1.** List of primers used in this study

918

919 **Figure S1.** Diagram showing the experimental setup of the two RNASeq experiments that were
920 used to identify fungal and plant genes involved in N uptake and metabolism

921

922 **Figure S2.** Control experiment to verify the absence of DNA in the RNA extracted from LMD cells
923 and amplified by RT-PCR. C, cells containing coils occupying the whole plant cell; CC, cells with
924 older more condensed coils; NM, non-colonized cells; -, water. The signs + and - indicate presence
925 or absence of the RT step before PCR amplification with primers for the housekeeping genes
926 coding for the plant (*SvEF1 α*) and the fungal (*TcEF1 α*) elongation factor.

927

928 **Figure S3.** One-step RT-PCR analysis of *T. calospora* genes in three populations of laser
929 microdissected (LMD) cells: C, cells containing coils occupying the whole plant cell; CC, cells with
930 older and more condensed coils; NM, non-colonized cells; -, water. Fungal transcripts coding for
931 transporters and enzymes involved in N uptake and metabolism were amplified with the primers
932 listed in Table S1 and separated by agarose gel electrophoresis.

933

934 **Figure S4.** Phylogenetic tree of plant ammonium transporters, based on amino acid deduced
935 sequences. The sequences were aligned using Muscle and the unrooted tree was constructed using
936 the maximum likelihood method. Bootstrap tests were performed using 1,000 replicates. Numbers
937 indicate bootstrap values, and are given only for ≥ 50 %. Arrows point to the *S. vomeracea*
938 sequences SvAMT1 and SvAMT2.

939

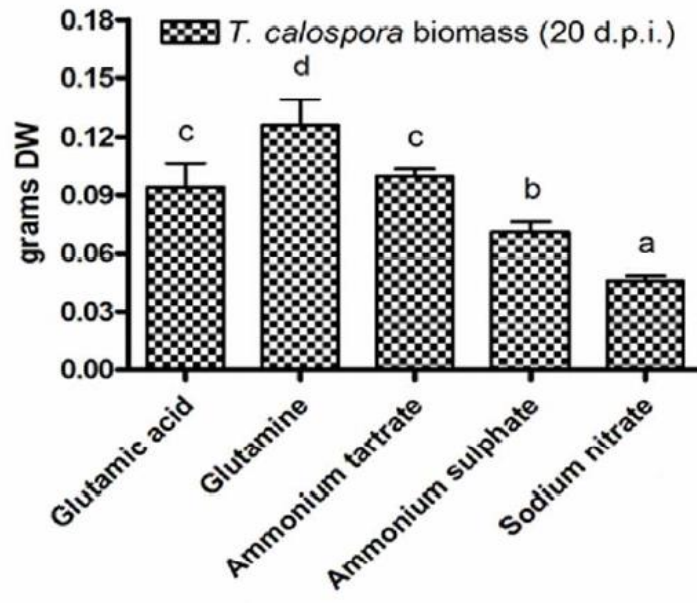


Figure 1

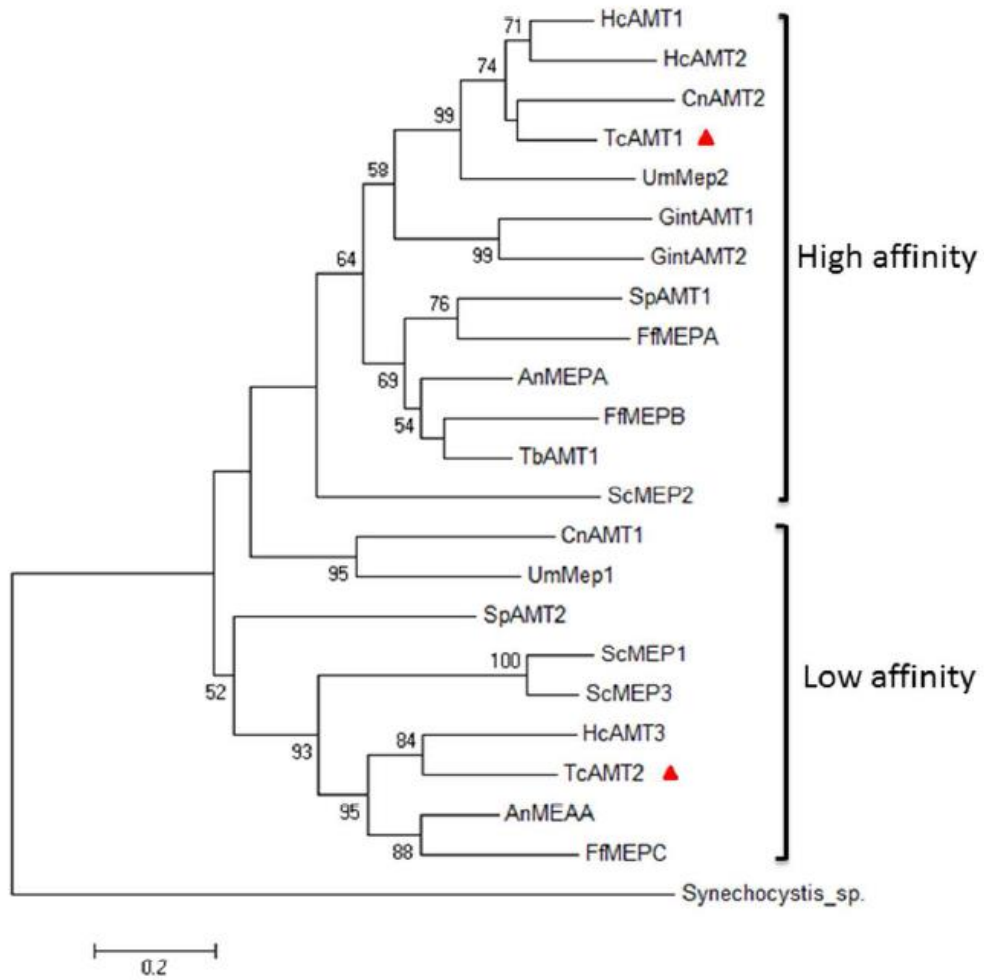


Figure 2

941

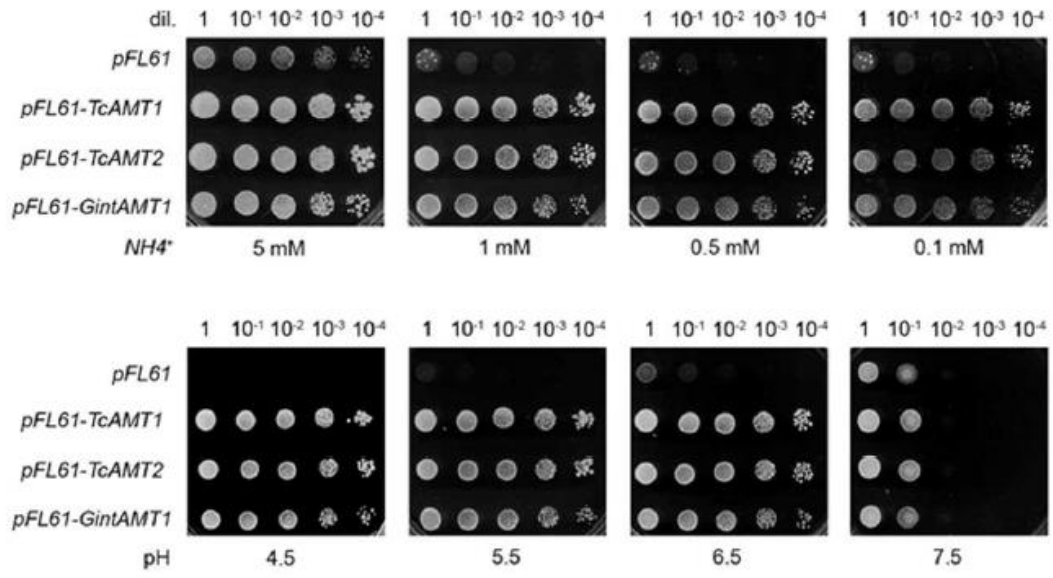


Figure 3

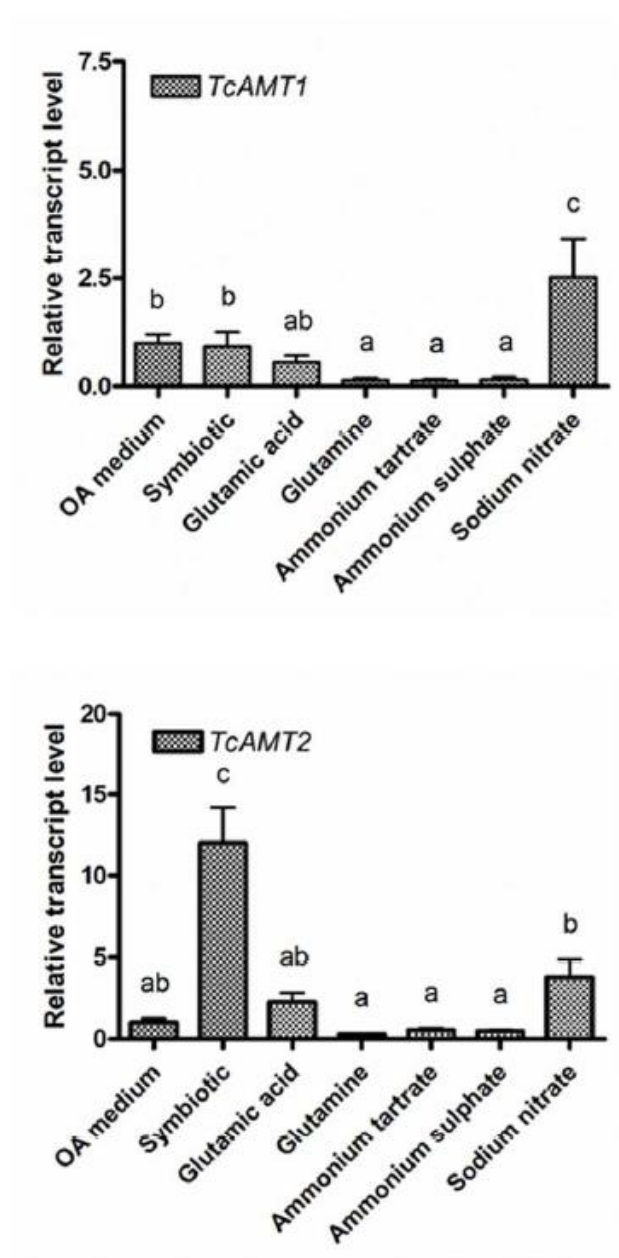


Figure 4

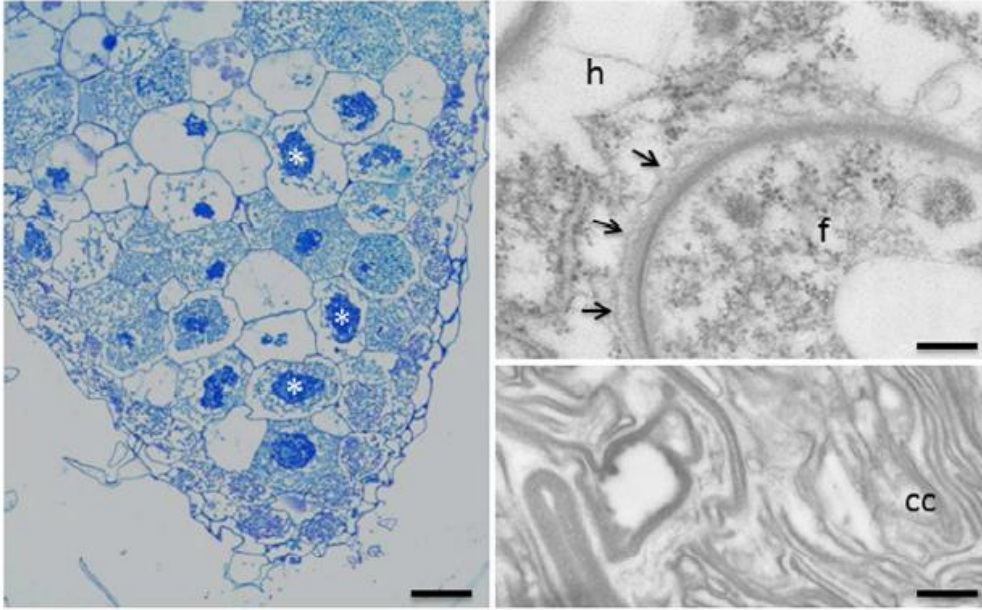


Figure 5

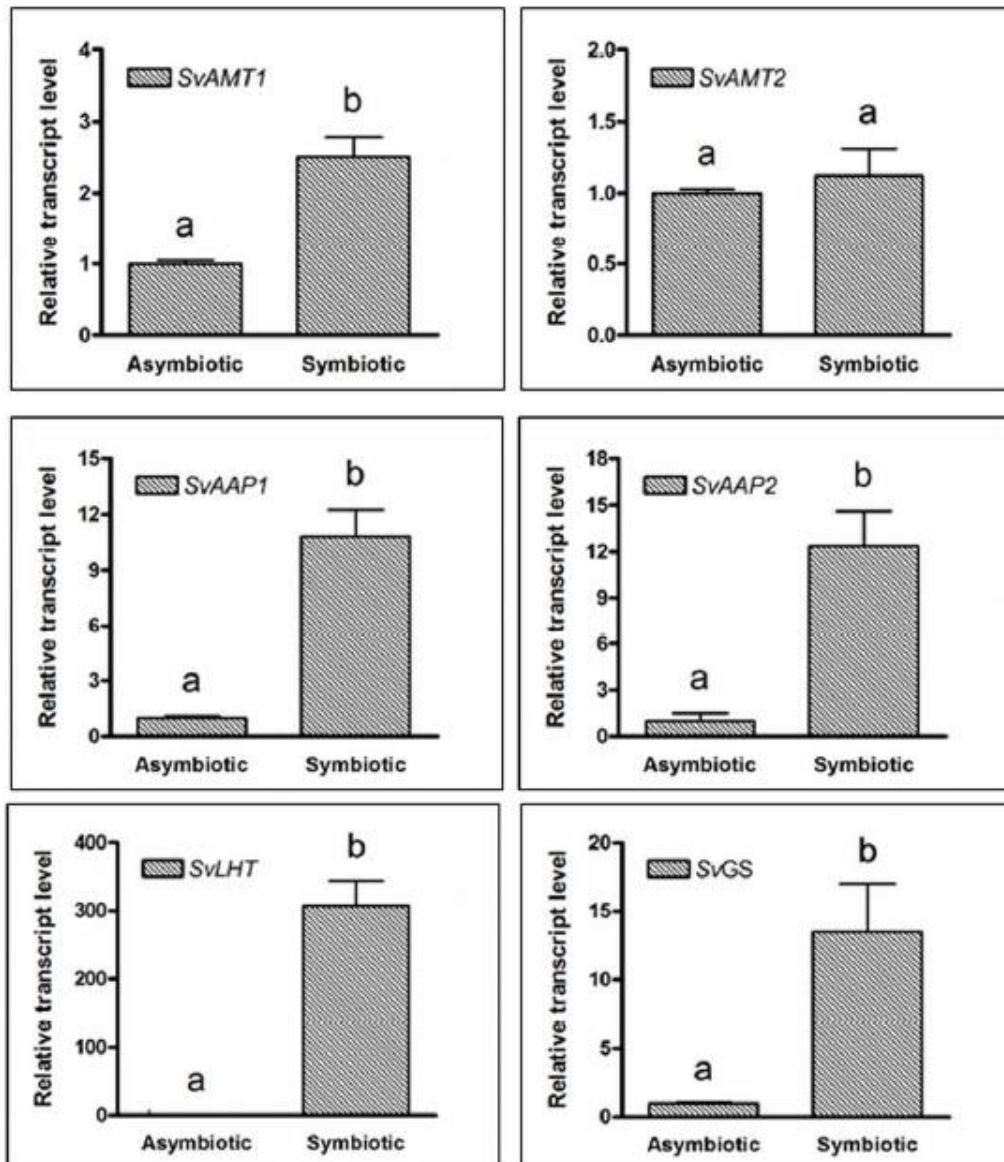


Figure 6