Cell-specific expression of plant nutrient transporter genes in orchid mycorrhizae

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Abstract

Orchid mycorrhizal protocorms and roots are heterogeneous structures composed of different plant cell-types, where cells colonized by intracellular fungal coils (the *pelotons*) are close to non-colonized plant cells. Moreover, the fungal coils undergo rapid turnover inside the colonized cells, so that plant cells containing coils at different developmental stages can be observed in the same tissue section. Here, we have investigated by laser microdissection (LMD) the localization of specific plant gene transcripts in different cell-type populations collected from mycorrhizal protocorms and roots of the Mediterranean orchid *Serapias vomeracea* colonized by *Tulasnella calospora*. RNAs extracted from the different cell-type populations have been used to study plant gene expression, focusing on genes potentially involved in N uptake and transport and previously identified as up-regulated in symbiotic protocorms. Results clearly showed that some plant N transporters are differentially expressed in cells containing fungal coils at different developmental stages, as well as in non-colonized cells, and allowed the identification of new functional markers associated to coil-containing cells.

Keywords

Orchid symbiosis, *Serapias vomeracea*, *Tulasnella calospora*, laser microdissection, gene expression, nitrogen

1. Introduction

Orchids belong to one of the largest plant families [1] and an extraordinary floral diversification is correlated with the enormous number of species [2]. Their survival in nature is ensured by a complex symbiotic association with soil mycorrhizal fungi, which are particularly important during seed germination and early plant development. Orchid seeds are in fact very minute and without stored reserves, and germination and seedling development require therefore an external source of nutrients and organic C, provided by the mycorrhizal fungi [3,4]. Seed germination leads to the formation of a protocorm, a pre-seedling stage that generally lacks chlorophyll and is therefore heterotrophic and completely fungus-dependent for nutrients and organic C supply [5]. This peculiar ability of the plant to gain organic C from its mycorrhizal fungal partner is known as mycoheterotrophy [5,6]. Irrespective of the fact that orchid seedlings may then develop

photosynthetic leaves or remain fully or partially mycoheterotrophic, mature roots of adult orchids are generally found to be colonized by mycorrhizal fungi [4].

Orchid mycorrhizal (OM) protocorms and roots are heterogeneous structures composed of different plant cell-types, where cells colonized by intracellular fungal coils (the *pelotons*) are close to non-colonized plant cells. Moreover, the fungal pelotons undergo rapid turnover inside OM cells, so that plant cells containing pelotons at different developmental stages can be observed in the same tissue section [7]. Differential expression of orchid genes in mycorrhizal and non-mycorrhizal protocorm regions has been reported in the model system formed by the photosynthetic Mediterranean orchid *Serapias vomeracea* and the basidiomycete *Tulasnella calospora* [8]. In this study, protocorms were manually dissected to separate the basal part, which contained the mycorrhizal tissue, from the apical region, featuring a plant meristem that is never colonized by the fungus [7]. However, the basal protocorm region is made of a heterogeneous cell population, featuring colonized and non-colonized orchid cells. Thus, localized expression of plant genes in specific cell-types may be masked when RNA is extracted from the whole tissue, as already suggested for the arbuscular mycorrhizal (AM) symbiosis [9,10].

Laser microdissection (LMD) has been used over the last years to study cell-specificity in gene expression profiles in the AM interaction, where particular attention has been paid to the root cortical cells containing the arbuscule, the fungal structure typical of this symbiosis. The results indicate that LMD can be a powerful tool for the identification of plant genes involved in specific stages of fungal colonization [9,10,11,12,13,14,15,16,17), or to verify the expression of fungal genes during the symbiotic stage [9,14,18]. In AM, information derived from these studies has led to the identification of functional genetic markers associated with arbusculated cells. Among them, several plant and fungal transporter genes have been identified, suggesting that functions like nutrient exchange are cell-specific, and confirming the hypothesis that arbuscule-containing cells represent the core of a functional AM symbiosis [16, 10].

LMD has been successfully applied to study cell-type specific gene expression in orchid mycorrhiza for both plant [19] and fungal [20] genes. Here, we have used LMD to dissect the complexity of OM tissues and to investigate cell-type specific expression of plant genes related to nitrogen (N) transport. In fact, field (see [6]) and laboratory [21,22] studies provide evidence that orchids receive nitrogen (N) from their mycobionts, and the expression of fungal and plant genes potentially involved in N transport and assimilation has been recently investigated in *S. vomeracea* protocorms colonized by *T. calospora* [20]. The results suggest that organic N may be the main form

transferred by the fungus to the orchid host. They also suggest that the intracellular fungus takes up ammonium from the symbiotic interface, indicating for the first time a flow of nutrients back to the fungal partner from the non-photosynthetic orchid. Based on these and on previous data, Dearnaley & Cameron [23] have proposed a model for mycorrhizal nutrient exchange in non-photosynthetic orchid protocorms, where the plant receives P, N and C (the latter two nutrients as amino acids) from the fungal partner and exports NH_4^+. Nutrient transfer in OM cells is thought to occur across the symbiotic interface, an apoplastic matrix between the intracellular fungal hyphae and the orchid cell membrane. However, it is still debated whether fungal nutrients are transferred to the plant across an intact fungal membrane by viable intracellular hyphae [21,22,23,24], or whether nutrients are released to the plant by digestion of degenerating fungal pelotons [22,25], a strategy called tolypophagy [3].

We have applied a LMD approach to investigate accumulation of specific plant gene transcripts in different cell-type populations collected from OM protocorms and roots of *S. vomeracea*. In particular, we addressed the following questions: i) can we identify, among the N transporters described as being up-regulated in mycorrhizal protocorms by Fochi et al. [20], some genetic markers of peloton-containing orchid cells, and ii) is the accumulation of transcripts coding for these N transporters modulated during the different stages of fungal peloton development?

2. Materials and methods

2.1 Biological materials

Symbiotic protocorms were obtained by co-inoculation, in 90 mm Petri dishes, of the mycorrhizal fungus *Tulasnella calospora* (isolate AL13/4D) and seeds of the Mediterranean orchid *Serapias vomeracea*, as described in [8]. Briefly, seeds of *S. vomeracea* were surface sterilized in 1% sodium hypochlorite and 0.1% Tween-20 for 20 minutes on a vortex, followed by three 5-minutes rinses in sterile distilled water. Seeds (re-suspended in sterile water) were dropped on strips of autoclaved filter paper (1.5 x 3 cm) previously positioned on solid oat medium (0.3% milled oats, 1% agar). A portion of actively growing mycelium of *T. calospora* was then placed in the centre of each Petri dish and the plates were incubated at 20°C in full darkness for 30 days. Mycorrhizal seedlings of *S. vomeracea* were obtained as described in Balestrini et al. [26]. Briefly, after symbiotic protocorms had developed the first leaf primordium, plates were moved to light for about 20 days in a growth chamber (23 °C for 16 h light/21 °C for 8 h dark) until the leaf primordia turned green. At this stage, protocorms were transferred to magenta jars containing fresh OA medium (7,5 g/L agar and 3 g/L oat flour) and were maintained in the growth chamber with the same parameters described

above until a radicle was formed at the protocorm base. At this point, they were transferred to new magenta jars containing a fresh OA medium (5 g/L agar and 3 g/L oat flour) supplemented with previously sterilized soil. Seedlings higher than 2 cm were finally transferred to new magenta jars containing twice-sterilized natural soil, which was watered with a sterile solution of water and oat flour (3 g/L), and maintained with the same photoperiodic parameters as above.

2.2 Tissue preparation for LMD

For paraffin embedding, *S. vomeracea* symbiotic protocorms and mycorrhizal root (cut into about 10 mm segments with a razor blade in fixative) were fixed in freshly prepared Farmer's fixative (absolute ethanol/glacial acetic acid 3:1) at 4°C overnight. Samples were subsequently dehydrated in a graded series of ethanol (70%, 90% in sterile water and 100% twice) followed by two incubations in Neoclear (Merck, Darmstadt, Germany), with each step on ice for 30 min, and embedded in paraffin as described in Balestrini et al. [9,26]. Neoclear was then gradually replaced with paraffin (Paraplast Plus) and samples were embedded in paraffin in Petri dishes as described in Balestrini et al. [26]. Sections of 12µm thickness were cut using a rotatory microtome, placed and stretched out on Leica RNase-free PEN foil slides (Leica Microsystems) with ddH_2O (filtered with a 0.2 µm filter). The sections were then dried on a 40°C warming plate, stored at 4 °C and used within 2 days.

2.3 LMD

A Leica LMD 6500 Laser microdissection system (Leica Microsystems, Inc., Germany) was used to isolate the different cell-types from the prepared tissue sections. Just before use, the slides with the sections were deparaffinized with Neoclear for 8-10 minutes, rinsed in 100% ethanol for one minute and then air-dried. The deparaffinized slides were placed face-down on the microscope and two different cell-types were selected from symbiotic protocorms and roots, microdissected and collected separately (Fig. 1): i) cells containing visible fungal coils (C), mainly selecting those in which the coil occupied the whole cell and ii) non-colonized cells (NM). In a second experiment, a third cell-type was collected from protocorm sections, i.e. cells with more condensed central coils (CCC). Approximately 1500 cells for each cell-type population were collected for each replicate, and the pools were brought to a final volume of 50 µl with Pico Pure extraction buffer and processed for RNA extraction. For protocorms, at least three independent biological replicates of each cell type were collected for downstream gene expression analyses, while two independent biological replicates were used for roots.

2.4 RNA extraction and RT-PCR

RNA was extracted using the Pico Pure kit (Life Technologies, Carlsbad, CA, USA), without DNase treatment in the kit column. The RNA was eluted in 20-25 µl of Elution buffer and treated with RNase-free DNAse (TURBO™ DNase, Ambion), according to the manufacturer's instructions. RNA quantification was determined using a NanoDrop 1000 spectrophotometer. A One-Step RT-PCR kit (Qiagen, Valencia, CA, USA) was used for the RT-PCR experiments on RNA extracted from the different LMD samples. All RNA samples were checked for DNA contamination through RT-PCR analyses with specific primers for plant and fungal elongation factor genes, i.e. *SvEF1a* and *TcEF1a*, respectively (Table 1), without a previous retrotranscription step (RT-).

Specific primers for several plant genes involved in N metabolism (Table S1) were designed using *PRIMER3PLUS* (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and tested for their specificity with *PRIMER BLAST* (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The specificity of the primers was tested through PCR experiments on DNA from *S. vomeracea* seeds and *T. calospora* mycelium (data not shown).

RT-PCR reactions were carried out in a final volume of 10 µl containing 2 µl of 5x buffer, 0.5 µL of 10mM dNTPs, 0.25 µl of each primer (10 mM), 0.25 µl of One-Step RT-PCR enzyme mix and 0.5 µl of total RNA (generally diluted 1:3). Samples were incubated for 30 min at 50°C, followed by 15 min at 95°C. Amplification reactions were run for 40 cycles at 94°C for 30s, 60°C for 30s, and 72°C for 40s using *T. calospora* and *S. vomeracea* specific primers. The PCR products were separated by agarose gel electrophoresis (1.5% in TAE 0.5x buffer).

Semi-quantitative RT-PCR experiments were carried out following the same protocol. Amplification reactions with specific primers for the selected genes and for the housekeeping gene (HKG) *SvEF-1α* (annealing temperature 60°C) were run for different numbers of cycles (29, 32 and 35 for the specific genes and 26, 27, 29 for the *SvEF-1α* gene) to determine the exponential amplification phase. To obtain a better resolution of the amplified bands, the PCR products were separated by agarose gel electrophoresis (2.5% in TBE 1x buffer). Images of the RT-PCR ethidium bromide-stained agarose gels were acquired with a Gel Doc™ EZ Imaging System (BioRad) and quantification of the bands was performed by Image Lab™ software. The relative quantity is the ratio of the band volume divided by the reference band volume, i.e. the band of the C sample, at 29 cycles for the HKG and at 32 cycles for specific genes except for *SvAMT1* where, due the low intensity of the band at 32 cycles, sample at 35 cycles have been used.

2.5 Quantitative real-time analyses

One-Step RT-qPCR was performed with the Rotor-Gene Q (Qiagen) apparatus. The reactions were carried out in a final volume of 15 µl with 7.5 µl of Rotor-GeneTM SYBR® Green Master Mix, 5.5 µl of a mix of forward and reverse primers (prepared by adding 16 µl of each primer at 10 µM stock concentration to 168 µl of water), 0,15 µl of RT-mix and 2 µl of cDNA (diluted 1:10). The RT-qPCR cycling program consisted of a 10 min/55°C RT step, 10 min/95 °C holding step followed by 40 cycles of two steps (15 s/95 °C and 1 min/60 °C).

3. Results and Discussion

In this study, LMD has been used to investigate whether cell-type specific expression of plant genes potentially involved in N uptake and transport occurs in orchid mycorrhiza. Fochi et al. [20] recently highlighted the differential expression of a repertoire of fungal and plant genes involved in the transport and metabolism of N compounds in symbiotic protocorms, as compared to the asymbiotic condition. Because the genome of *S. vomeracea* has not yet been sequenced, a *de novo* assembly of *S. vomeracea* transcripts was used by these authors to identify plant genetic functions that were then validated by RT-qPCR [20]. Putative *S. vomeracea* transporters for amino acids and oligopeptides were strongly up-regulated in mycorrhizal protocorms, suggesting transfer of organic N forms from the intracellular fungus to the host plant. To verify this hypothesis, we used LMD to localize expression of these *S. vomeracea* genes (Table 1) in mycorrhizal and non-mycorrhizal cells of both protocorms and mature roots (Fig. 1).

3.1 Detection of plant transcripts in colonized and non-colonized microdissected cell-type populations from OM protocorms and roots

In a first experiment, LMD was applied to collect colonized (C) and non-colonized (NM) from OM protocorms and roots. RNA extracted from these microdissected cell-type populations was used in One-Step RT-PCR reactions to investigate accumulation of specific plant transcripts. In particular, we focused on some *S. vomeracea* transcripts potentially involved in N uptake and transport, and shown by Fochi et al. [20] to be induced in mycorrhizal protocorms. These putative genes, listed in Table 1, were: *SvAAP1*, *SvAAP2* and *SvLHT*, coding for different putative amino acid transporters, *SvOPT1*, *SvOPT2*, *SvPTR1* and *SvPTR2* coding for different putative (oligo)peptide transporters, and *SvAMT1*, coding for a putative ammonium transporter.

Table 1. The table lists the *S. vomeracea* contigs analyzed in the current work, previously identified in a *de novo* assembly by Fochi et al. (2017).

Putative function	Trinity Contig	Putative Gene Name	Mean raw read count *		Fold Change	*P*-value	BlastX annotation against *A. thaliana***	E-value
			ASYM	SYM				
Ammonium transporters	DN68801_c0_g1_i1	*SvAMT1*	7.69	21.75	**2.83**	0.8961	AT1G64780.1 ammonium transporter 1;2	0
Amino acid transporters/ permeases	DN71918_c0_g1_i1	*SvAAP1*	0.35	43.97	**125.27**	4E-05	AT1G77380.1 amino acid permease 3	0
	DN71918_c0_g2_i1	*SvAAP2*	0.93	49.83	**53.32**	2E-12	AT5G63850.1 amino acid permease 4	1.00E-60
	DN63460_c0_g1_i1	*SvLHT*	1.79	167.52	**93.43**	4E-05	AT5G40780.2 lysine histidine transporter 1	9.00E-133
Oligopeptide transporters	DN78718_c1_g1_i2	*SvOPT1*	3.69	36.53	**9.90**	2E-11	AT4G26590.1 oligopeptide transporter 5	0
	DN1177_c0_g1_i1	*SvOPT2*	0.85	381.43	**451.23**	0.0108	AT5G55930.1 oligopeptide transporter 1	1.00E-43
	DN69777_c0_g1_i2	*SvPTR1*	0.58	11.01	**19.02**	3E-05	AT3G54140.1 Peptide transporter 1	0
	DN75842_c0_g4_i1	*SvPTR2*	9.36	41.38	**4.42**	0.0005	AT5G46050.1 Peptide transporter 3	4.00E-35
EXOCYST complex component (EXO70)	DN73752_c2_g2_i1	*SvEXO*	5.77	42.61	**7.38**	0.0087	AT2G39380.1 exocyst subunit exo70 family protein H2	1.00E-98

*Expression values derived from a previous RNAseq experiment (Fochi et al. 2017). The expression of *S. vomeracea* contigs in mycorrhizal protocorms (SYM) was compared with expression in non-mycorrhizal protocorms at a similar developmental stage (ASYM). P-value: FDR p-value correction.
**Annotation from Fochi et al. (2017).

In addition, a transcript coding for a putative protein annotated as a subunit of the EXOCYST complex (*SvEXO70*) was investigated. Interestingly, an EXO70I protein was described in AM as a marker of arbusculated cells, being involved in the biogenesis of the perifungal membrane that surrounds the arbuscule [27].

An amplified fragment of the expected size was observed in all RNA samples after RT-PCR using specific primers for a *S. vomeracea* HKG, the elongation factor *SvEF-1α* (Fig. 2). Absence of an amplified product in the RT minus reactions excluded genomic DNA contamination. RNA samples were also amplified by RT-PCR using primers specific for the *T. calospora* elongation factor *TcEF-1α*, in order to verify the presence of the fungus in the collected cells (Fig. 2).

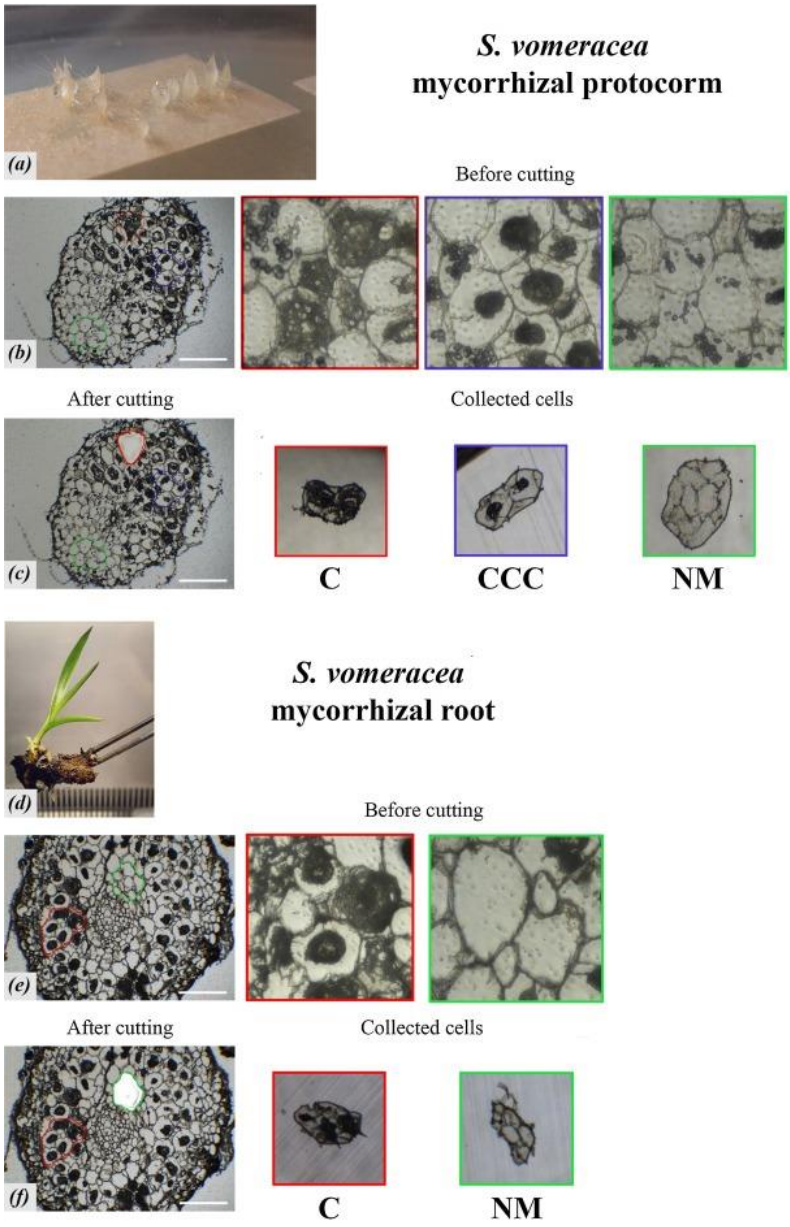

Figure 1. Morphological features of *S. vomeracea* mycorrhizal protocorms and roots. (a) Mycorrhizal protocorms of *Serapias vomeracea* 30 days after sowing (stage 3) with the mycobiont *Tulasnella calospora*. (b)-(c) Paraffin section of a *S. vomeracea* mycorrhizal orchid protocorm before (b) and after (c) cutting cells containing large coils (C, circled by a red line). On the right, magnification of cells fully colonized by large coils (C, circled by a red line), cells containing central condensed coils (CCC, circled by a blue line) and cells without the fungus (NM, circled by a green line), before (above) and after microdissection (below). (d) Magenta jar containing a 4 months old plantlet. (e)-(f) Paraffin section of a *S. vomeracea* mycorrhizal root before (e) and after (f) cutting cells containing the fungal coils (C, circled by a red line). On the right, a magnification

of root cells containing fungal coils (C, circled by a red line) and cells without coils (NM, circled by a green line). Bars: 350 μm in (b) and (c), 300 μm in (e) and (f).

In general, fungal RNA was not detected in NM cells microdissected from mycorrhizal protocorms (Fig. 2), despite some variability among replicates. Only NM protocorm samples devoid of fungal RNA, or showing only a very faint amplification product after RT-PCR of the fungal housekeeping gene, were used for further analyses. By contrast, due to the low amount of starting material and, likely, to the low percentage of NM cells in the cortical tissue of mycorrhizal roots, it was not possible for this tissue to obtain a NM sample completely devoid of fungal RNA.

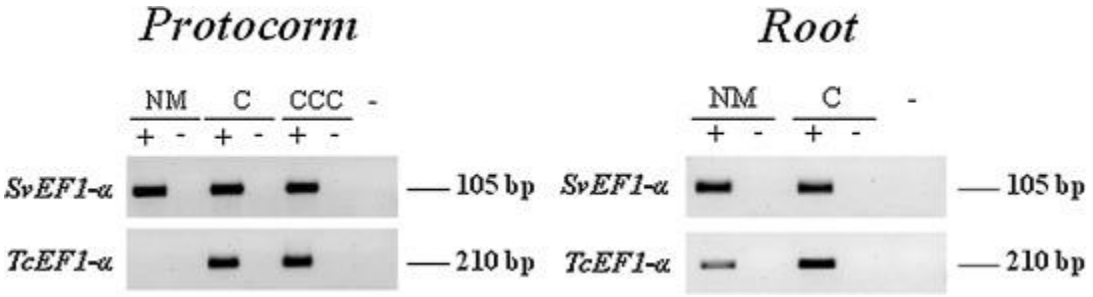

Figure 2. One-step RT-PCR analysis of microdissected cells from mycorrhizal *S. vomeracea* protocorms and roots colonized by *T. calospora*, using primers specific for the plant (*SvEF1-α*) and the fungal (*TcEF1-α*) elongation factors as housekeeping genes. Each RNA sample was tested for potential DNA contamination by omitting the RT step. The presence of an amplified product in the RT sample (+) and its absence in the RT-negative reaction (-) excluded DNA contamination. C, protocorm cells containing large coils; CCC, protocorm cells containing central condensed coils; NM, non-colonized cells from colonized protocorms.

Whereas transcripts coding for the plant elongation factor (*SvEF-1α*) were found in both mycorrhizal (C) and non-mycorrhizal (NM) cells collected from symbiotic protocorms (Fig. 2), transcripts corresponding to *SvEXO70* and to the amino acids transporters (*SvAAP1*, *SvAAP2*, *SvLHT*) could be amplified exclusively from mycorrhizal protocorm cells (Fig. 3). Although we cannot exclude that these transcripts may be also found in NM cells, below the threshold of detectability, our results suggest that these genes may be considered as markers of coil-containing cells in mycorrhizal protocorms. It is worth noting that real-time RT-qPCR experiments (see below) confirmed that the absence of an amplified band fragment in NM cell-types was not due to a lower

RNA yield with respect to cells containing the fungus (Table S2). Another *S. vomeracea* putative gene (*SvNod1*), coding for a nodulin-like protein containing a plastocyanin-like domain, was also found to be expressed only in protocorm cells containing intracellular fungal coils [18].

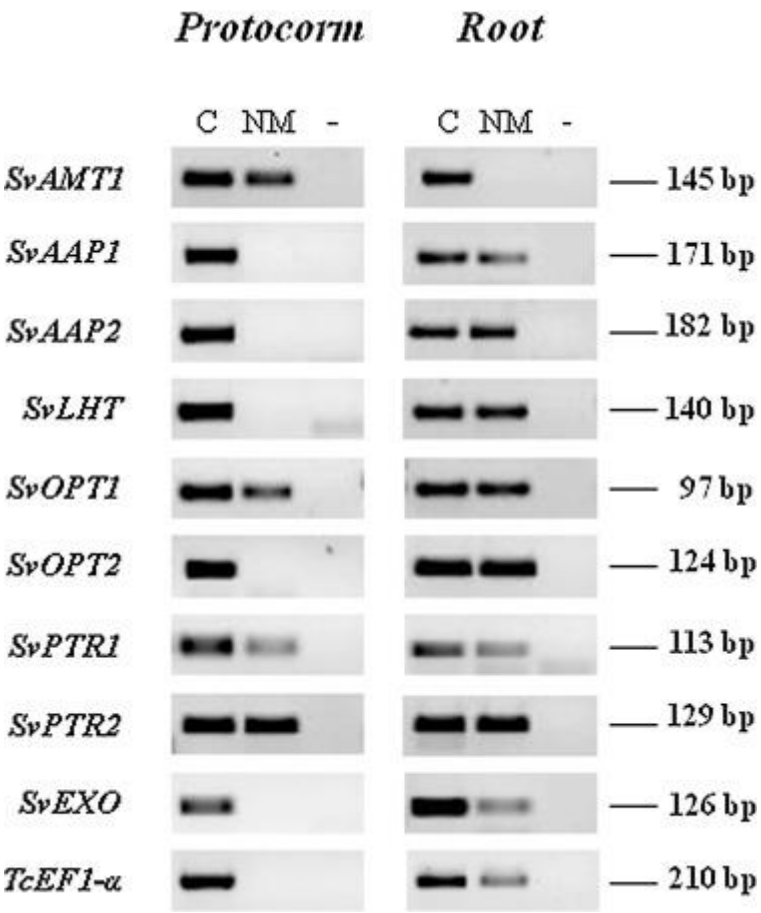

Figure 3. One-step RT-PCR analysis of *S. vomeracea* genes in two microdissected cell-type populations: C, protocorm cells containing large coils; CCC, protocorm cells containing central condensed coils. *TcEF-1α* was used to test the fungal presence in the RNA from the different cell-type populations.

After RT-PCR amplification with primers for the putative (oligo)peptide transporters *SvOPT1* and *SvPTR1,* an amplicon was always observed in both C and NM cell-type populations collected from mycorrhizal protocorms. By contrast, transcripts corresponding to *SvOPT2* and *SvPTR2* were found in colonized cells (C), whereas they were not always detected in collected NM cells from several independent biological replicates (not shown). Most plant transcripts were detected in both C and NM cell-type populations microdissected from OM roots (Fig. 3).

The amplification of some transcripts in both mycorrhizal (C) and non-mycorrhizal (NM) cells may be due the fact that small pelotons, either very young or already collapsed, may go undetected in some cortical cells during microdissection. The expression, in some replicates, of the fungal housekeeping gene *TcEF-1α* in plant cells that should not contain the fungus (NM) suggests a difficulty in the collection of specific cell-types, mainly from OM roots. Similarly, a greater variability in gene expression has been reported for the non-colonised cell-type population, as compared to other cell types in the AM symbiosis [15]. Thus, the variable identification of some plant transcripts in NM cells could be due to the sporadic presence of intracellular fungal hyphae, not visible under microscope inspection. However, some transcripts (e.g. *SvOPT1* and *SvPTR2*) were consistently amplified from NM cell samples, also when fungal RNA could not be detected, suggesting that the non-colonized cell-type population may comprise cortical cells where these genes may be induced prior to (or independently from) fungal colonization [16].

The presence of transcripts coding for transporters in coil-containing orchid cells is similar to the AM symbiosis, where the application of LMD has allowed the identification of several plant nutrient transporter genes mainly, or exclusively, expressed in arbusculated cells [10,14,16]. In particular, members of the gene categories involved in N uptake considered in this study have been already reported in AM roots as being mycorrhiza-specific and, in some cases, arbuscule-specific. Among them, genes coding for a PTR [16] and for two proton-dependent oligopeptide transporters [10] were found to be expressed in arbusculated cells but not in non-colonized cells of mycorrhizal roots. The specific localization of these transcripts in the arbusculated cells suggests that the corresponding transporters may be involved in N transfer from the fungus. In AM, transcripts of the *LjLHT1.2* gene, which encodes a lysine-histidine-transporter (LHT)-type amino acid transporter, have been located mainly in arbusculated cells, but also in the non-colonized cells of the root cortex, suggesting that *LjLHT1.2* is consistently expressed in cortical cells from AM roots [29]. A similar expression pattern has been observed for an ammonium transporter gene (*LjAMT2;2*) shown to be exclusively expressed in mycorrhizal roots. Transcripts of this ammonium transporter in AM were preferentially (but not exclusively) located in the arbusculated cells [17], providing evidence of its involvement in N uptake during AM symbiosis. The expression of an AMT gene in arbusculated cells was also confirmed in *M. truncatula* mycorrhizal roots [14].

SvAMT1, a *S. vomeracea* contig annotated as an ammonium transporter, was shown to be slightly but significantly up-regulated in symbiotic protocorms by RT-qPCR [20]. Our LMD data indicate that, similar to AM, transcripts corresponding to *SvAMT1* are expressed in cells containing fungal

coils as well as in non-colonized cells collected from mycorrhizal protocorms, whereas expression was restricted to mycorrhizal cells in orchid roots. These results suggest that localized expression of some transporters may change in different organs or at different stages during the orchid's life cycle, but whether this reflects changes in N metabolism is currently unknown.

3.2 Detection of plant transcripts in protocorm cell-type populations containing fungal coils at different developmental stages

An intriguing but unresolved question in OM is whether nutrient transfer from the fungus to the host plant occurs while the fungus is alive inside the colonized plant cell, or whether nutrients are released to the plant after the peloton collapses, through plant digestion of the fungal structures (see in [7]).

As described before, cells containing two different stages of fungal coil development, i.e. well-developed coils that filled the whole cell (C) and more condensed central coils (CCC) that may correspond to collapsed pelotons, could be recognised in sections of paraffin embedded protocorms (Fig. 1) and were separately collected by LMD. RNA extracted from these two cell-type populations was then tested in order to verify possible differential accumulation of plant transcripts coding for N transporters. Before performing the experiments on the specific genes, RNA extracted from the three different cell-type populations (C, CCC, NM) was tested by real-time RT-qPCR to obtain quantitative data, and expression of two plant housekeeping genes, *SvEF-1α* and ubiquitin (*SvUBI*) confirmed a similar amount of RNA in the three samples (Table S2). However, due to technical difficulties in the quantification of specific transcripts by real-time RT-qPCR, especially in RNA samples with low transcripts level, further experiments on RNA samples from microdissected cell-type populations were performed by semi-quantitative RT-PCR. In these experiments, RNA samples from NM microdissected cells were included only for those transcripts that were also detected in these protocorm cells (see Fig. 3).

Primers for the plant housekeeping gene *SvEF-1α* yielded a similar pattern of amplification for the three RNA samples (Fig. 4), in agreement with the real-time RT-qPCR results (Table S2). Primers for the fungal housekeeping gene *TcEF-1α* have been used in order to verify the presence of the fungus inside the several collected cell-types (Fig. S1). After 29, 32 and 35 cycles of amplification using specific primers for the transcripts coding for the three amino acid transporters (*SvAAP1*, *SvAAP2* and *SvLHT*), the amount of amplified products was considerably higher in cells containing large fungal coils (C, Fig. 4) than in the CCC cell-type population, suggesting that C cells are the

preferential site for the expression of these genes in a symbiotic protocorm. These results were confirmed by measurement of band intensity in the gel image (Table S3).

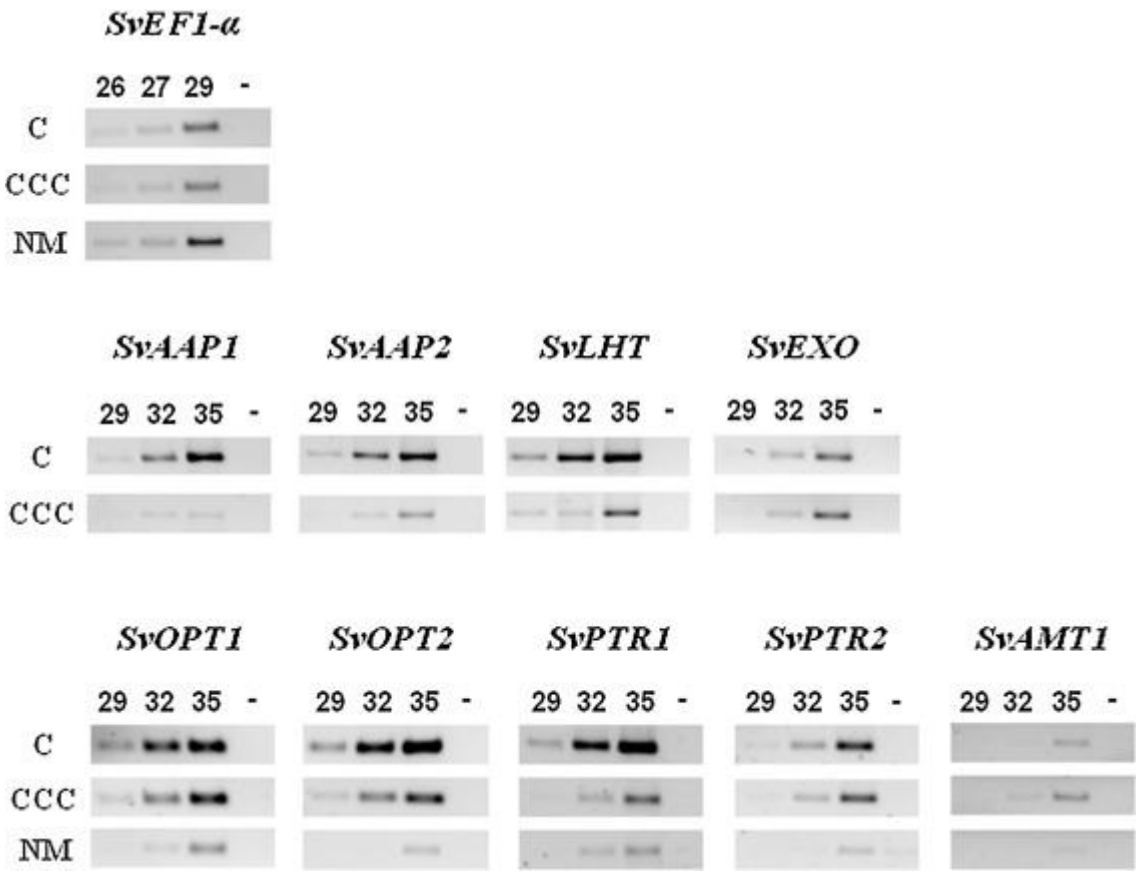

Figure 4. Semi-quantitative one-step RT-PCR analyses on microdissected protocorm cells. The housekeeping elongation factor gene (*SvEF1-α*) was used as endogenous control. C, protocorm cells containing large coils; CCC, protocorm cells containing central condensed coils; NM, non-colonized cells from colonized protocorms. Numbers correspond to the RT-PCR cycles.

Primers for the two oligopeptide transporters *SvOPT1* and *SvOPT2* amplified transcripts from C and CCC cell-type populations with a similar pattern, although the CCC/C intensity ratio suggested a slightly lower transcript level in CCC with respect to C cell type population, and a weak amplification product was also visible in the NM sample at the higher numbers of PCR cycles (Fig. 4; Table S3). A similar amplification pattern was also observed for one peptide transporter (*SvPTR1*) and for the ammonium transporter (*SvAMT1*), while no differences in the transcript level for the other PTR gene (*SvPTR2*) considered were observed in CCC and C cell-type populations (Fig. 4; Table S3).

These data clearly showed that some plant N transporters are differentially expressed in cells containing fungal coils at different developmental stages. In detail, transcripts coding for the three amino acid transporters investigated were much more abundant in protocorm cells containing large and viable pelotons. This finding provides support to the hypothesis that fungal-derived N is transferred as organic (i.e. amino acid) forms to the host plant in orchid mycorrhiza [20,21], and also suggests that amino acids may be transferred to the plant mainly when the fungus is alive inside the colonized cells, across an intact fungal membrane.

The identification of transcripts coding for putative plant (oligo)peptide transporters in all protocorm cell-type populations, with a more similar expression level in C and CCC cell-type populations with respect to amino acid transporter genes, may have different explanations. Kuga et al. [22] suggested that protocorm cells may acquire fungal-derived nutrients from both viable and collapsed intracellular hyphae. Thus, OPTs and PTRs may have a general role in the recovery of peptides from the symbiotic interface, especially during peloton digestion. However, OPT transporters seem to have different biological functions [30] and, in addition to peptide transport, they can transport glutathione derivatives and metal complexes, and may be involved in stress resistance [31]. PTR genes could also have a different role in symbiosis, as suggested by studies in *Arabidopsis*, where a PTR gene (*AtPTR3*) was reported to protect the plant against biotic and abiotic stresses [32].

4. Conclusions

In conclusion, we have identified new functional markers associated to coil-containing cells, suggesting that some functions, related to nutrient exchanges and to polarized secretion mediated by exocytotic fusion, are cell-type specific. From a technical point of view, the results confirm that LMD is a suitable technology to investigate the cellular complexity of OM tissues, although some difficulties in the collection of homogenous cell-type populations, mainly from mycorrhizal root tissues, were highlighted. As already described in the AM symbiosis for arbusculated cells [9,18,33], LMD allowed us to detect transcripts corresponding to fungal [20] and plant (this work) nutrient transporters with similar functions (e.g. amino acid and ammonium transporters) in peloton containing cells. The up-regulation of three putative plant amino acid transporters (*SvAAP1*, *SvAAP2* and *SvLHT*) in cells containing well developed, viable pelotons further suggests that the plant acquires this organic N form during the biotrophic stage of the OM interaction, but the nature of these amino acids remains to be established. Our data ([20] and this work) are fully consistent

with the model of nutrient exchange proposed for the OM interaction by Dearnaley and Cameron [23], where transfer of amino acids to the protocorm cells would support both C and N requirements of the mycoheterotrophic stages, whereas ammonium would be exported back to the symbiotic fungus and may entice colonization [23].

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