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Gene expression in mycorrhizal orchid protocorms suggests a friendly plant–fungus relationship

Silvia Perotto, Marco Rodda, Alex Benetti, Fabiano Sillo, Enrico Ercole, Michele Rodda, Mariangela Girlanda, Claude Murat, Raffaella Balestrini

Abstract

Main conclusion

Orchid mycorrhiza has been often interpreted as an antagonistic relationship. Our data on mycorrhizal protocorms do not support this view as plant defence genes were not induced, whereas some nodulin-like genes were significantly up-regulated.

Orchids fully depend on symbiotic interactions with specific soil fungi for seed germination and early development. Germinated seeds give rise to a protocorm, a heterotrophic organ that acquires nutrients, including organic carbon, from the mycorrhizal partner. It has long been debated if this interaction is mutualistic or antagonistic. To investigate the molecular bases of the orchid response to mycorrhizal invasion, we developed a symbiotic in vitro system between *Serapias vomeracea*, a Mediterranean green meadow orchid, and the rhizoctonia-like fungus *Tulasnella calospora*. 454 pyrosequencing was used to generate an inventory of plant and fungal genes expressed in mycorrhizal protocorms, and plant genes could be reliably identified with a customized bioinformatic pipeline. A small panel of plant genes was selected and expression was assessed by real-time quantitative PCR in mycorrhizal and non-mycorrhizal protocorm tissues. Among these genes were some markers of mutualistic (e.g. nodulins) as well as antagonistic (e.g. pathogenesis-related and wound/stress-induced) genes. None of the pathogenesis or wound/stress-related genes were significantly up-regulated in mycorrhizal tissues, suggesting that fungal colonization does not trigger strong plant defence responses. In addition, the highest expression fold change in mycorrhizal tissues was found for a nodulin-like gene similar to the plastocyanin domain-containing ENOD55. Another nodulin-like gene significantly more expressed in the symbiotic tissues of mycorrhizal protocorms was similar to a sugar transporter of the SWEET family. Two genes coding for mannose-binding lectins were significantly up-regulated in the presence of the mycorrhizal fungus, but their role in the symbiosis is unclear.

Keywords

Orchid mycorrhiza Pyrosequencing *Serapias vomeracea* Transcriptome *Tulasnella calospora*

Abbreviations

EST Expressed sequence tag; GO Gene ontology

Electronic supplementary material

The online version of this article (doi:10.1007/s00425-014-2062-x) contains supplementary material, which is available to authorized users.

Introduction

In nature, orchids rely on interactions with other organisms to complete their life cycle. However, whereas interactions with pollinators are only required for sexual reproduction, mycorrhizal symbioses with soil fungi are required throughout the life history stages of orchids, from seed germination to adulthood. Orchid seeds are devoid of energy resources, and their germination is promoted by symbiotic fungi that provide the plant with signals and nutrients (see Smith and Read 2008, and references therein). After germination, all orchids form a protocorm, a structure intercalated between the embryo and the seedling (Peterson and Farquhar 1994) that lacks chlorophyll and is, therefore, fully heterotrophic. The protocorm entirely depends on a symbiotic fungus for carbon supply, a strategy termed mycoheterotrophy (Leake 2004). In the basal region of the orchid protocorm, most plant cells harbour viable, coiled intracellular fungal hyphae (the *pelotons*) that eventually collapse and undergo lysis. The cellular features of orchid mycorrhiza in the colonised protocorms, and the structure and components of the plant–fungus interface, are well described by Smith and Read (2008), who summarised earlier studies on this orchid life stage. Nutrient transfer in mycorrhizal orchid protocorms was mainly investigated with radioisotopes (Smith 1967), and carbon transfer was demonstrated to occur from the fungus, growing saprotrophically on complex polymeric substrates, to the mycorrhizal protocorm. Because the fungal pelotons are rhythmically digested within the plant cells, lysis of the fungus by the plant has been proposed as the main route to bring carbon and other nutrients to the orchid, a mechanism named tolypophagy (Rasmussen 1995). By contrast, more recent experiments strongly suggest that nutrient transfer mainly takes place across intact membranes (Cameron et al. 2008).

Protocorms develop into plantlets that become photosynthetic in most orchid species. The work by Cameron et al. (2006, 2008) suggests, in contrast to earlier studies (Hadley and Purves 1974; Alexander and Hadley 1985), that adult photosynthetic orchids can transfer carbon to their symbiotic fungus. However, some species remain fully achlorophyllous as adults, or with an inefficient photosynthesis due to adaptation to shaded environments (Gebauer and Meyer 2003; Selosse and Roy 2009). Thus, the nature of the symbiotic orchid–fungus relationship in the adult stage can be rather complex, depending on the trophic strategy of the host plant species and on the environmental conditions. By contrast, the protocorm is a more constant feature of all orchids and can also represent a more feasible experimental system to investigate molecular and cellular aspects of the early plant–fungus interactions in orchid mycorrhiza.

The plant–fungus interactions in mycorrhizal symbioses have been described as a continuum ranging from mutualism to parasitism (Johnson et al. 1997). Starting from early observations (Bernard 1909; Burges 1939), orchid mycorrhiza has often been portrayed as an example of parasitism because of the observation that the fungi occasionally attack and destroy the protocorm. This view was likely reinforced by the fact that the form-genus *Rhizoctonia*, comprising the most common symbionts of photosynthetic orchids (Dearnaley et al. 2012), also included *Rhizoctonia solani,* one of the most destructive plant pathogens (Parmeter 1970). *Rhizoctonia* has been later recognized as a polyphyletic group, with mycorrhizal and pathogenic isolates in distinct teleomorphic fungal genera (Taylor et al. 2002; Weiss et al. 2004), but orchid mycorrhiza is still sometimes referred to as a highly antagonistic interaction mainly because phytoalexins and other antifungal proteins can be accumulated in the plant tissues (Wang et al. 2001; Shimura et al. 2007). The orchid mycorrhizal relationship is also viewed as parasitic because the plant gains carbon from the fungus without an apparent reward, at least during the protocorm stage; for this reason, orchids have been described as mycophagous plants (Rasmussen and Rasmussen 2009; Selosse and Roy 2009). However, as orchids fully rely on their fungal partners, at least during the early developmental stages, mechanisms to attract and facilitate cellular accommodation of symbiotic fungi can be envisaged.

When compared to other mycorrhizal systems, such as arbuscular mycorrhiza (Balestrini and Lanfranco 2006; Hogekamp et al. 2011) or ectomycorrhiza (Plett and Martin 2011), the cellular and molecular bases of the plant–fungus interactions in orchid mycorrhiza have received very little attention (Watkinson and Welbaum 2003; Dearnaley 2007). Only recently Zhao et al. (2013) compared, by subtractive suppression hybridization (SSH), gene expression in symbiotically germinated and ungerminated seeds of the epiphytic orchid species *Dendrobium officinale* colonised by *Sebacina*.

Here, we describe the development and use of an in vitro system to investigate gene expression in mycorrhizal orchid protocorms, to understand the molecular bases of plant–fungus interactions. The plant host, the green terrestrial Mediterranean orchid *Serapias vomeracea*, and the fungal symbiont *Tulasnella calospora* (Basidiomycetes, Cantharellales) were chosen because of their common occurrence (Girlanda et al. 2011), their ability to grow in culture and their compatibility in germination assays. This in vitro system was used to generate a normalised cDNA library by 454 GS-FLX Titanium pyrosequencing, which represents an inventory of plant and fungal genes expressed in mycorrhizal *S. vomeracea* protocorms.

To investigate the contribution of each partner in a symbiotic relationship, a physical separation should ideally be carried out before RNA extraction, a task not possible for intracellular symbioses. As symbiotic cells contain transcripts produced by both partners, uncertainties in gene assignment to one or the other symbiont can arise, especially when both partners are eukaryotic, such as in mycorrhiza. The problem of gene assignment can be especially complex when non-model organisms are investigated, with little or no available genetic information. A bioinformatic pipeline was, therefore, applied to assign genes identified in the transcriptome of mycorrhizal *S. vomeracea* protocorms, using a combination of algorithms based on sequence alignment (BLAST) and on codon usage (EST3). A similar combination of approaches has been recently used by Fernandez et al. (2012) to define the plant or fungal origin of ESTs in a Roche 454 library of *Coffea arabica* infected by the obligate pathogenic fungus *Hemileia vastatrix*.

Among the genes identified in the mycorrhizal *S. vomeracea* transcriptome, we selected some plant genes that may provide insights into the orchid response to the mycorrhizal fungus. Molecular components of plant–microbe interactions are often conserved during plant evolution, the best example being the common features shared by mutualistic interactions as diverse as arbuscular mycorrhiza and nitrogen fixing root nodules (Parniske 2008; Bapaume and Reinhardt 2012). We, therefore, selected the orthologs of some genes described in either mutualistic or antagonistic symbioses and assessed their expression in mycorrhizal and non-mycorrhizal protocorm tissues.

Materials and methods

Symbiotic and asymbiotic germination of *Serapias vomeracea* seeds

Mature yellowing capsules from naturally pollinated plants of *S. vomeracea* (Burm.f.) Briq. 1910 were obtained and dried at room temperature for 2–3 weeks. After capsule dehiscence, seeds were cleaned from capsule debris and stored in glass vials on silica gel at 4 °C. Co-inoculation of mycorrhizal fungi and orchid seeds was performed in 9 cm Petri. Briefly, seeds of *S. vomeracea* were sterilized in 1 % sodium hypochlorite and 0.1 % Tween-20 for 20 min on vortex, followed by three 5-min rinses in sterile distilled water. Seeds were suspended in sterile water and single drops of the seeds suspension were dropped on a square of autoclaved filter paper (5 × 5 cm) previously positioned on solid oat medium (0.3 % milled oats, 1 % agar). An inoculum of *T. calospora* isolate AL13, obtained from *Anacamptis laxiflora* in Northern Italy (Girlanda et al. 2011) and consisting of a 3 mm × 3 mm portion of actively growing mycelium, was then placed in the center of each Petri dish. Plates were incubated at 20 °C in full darkness. Protocorms were

collected 20 and 30 days post inoculation and were either frozen immediately in liquid nitrogen and stored at −80 °C before RNA extraction, or fixed and embedded in LR White resin, as described below. For gene expression studies, the basal (mycorrhizal) and the apical (non-mycorrhizal) tissues of symbiotically germinated protocorms were separated with a sterile blade before freezing. Asymbiotic seed germination was obtained on modified BM culture media (Van Waes and Deberg 1986) at 20 °C for 90 days in darkness.

RNA extraction

RNA for the construction of the transcriptomic library and for the expression studies was extracted from protocorms following the protocol developed by Chang et al. (1993). For 454 GS-FLX pyrosequencing, total RNA was extracted from ten different samples of pooled protocorms at stage 2 (swollen seed with rhizoids but without a well-defined apex) and 3 (fully developed protocorm with first scale), according to Otero et al. (2004). Each sample was made of 100–200 mg fresh weight of plant material. For gene expression studies, total RNA was extracted from a pool of about 10 asymbiotic *S. vomeracea* protocorms, or apical (A) and basal (B) portions of mycorrhizal protocorms. Samples were ground in liquid nitrogen in 2 ml Eppendorf tubes using a Retsch Tissue Lyser (2 × 2 min at 20 Hz). The extraction buffer (2 % CTAB, 2 % PVP, 100 mM Tris–HCl pH 8.0, 25 mM EDTA, 2.0 M NaCl, 2 % beta-mercaptoethanol, 2 % PVPP) was immediately added and incubated for 5 min at 65 °C. An equal volume of chloroform: isoamyl alcohol (24:1, v/v) was then added and, after 10 min of centrifugation at 2,300*g* at room temperature, the upper phase was transferred to a new tube and extracted again with chloroform: isoamyl alcohol (24:1, v/v). The supernatant was recovered and mixed with 1:4 (v/v) 10 M LiCl for overnight RNA precipitation at 4 °C. The RNA pellet was centrifuged for 20 min at 18,300*g* at 4 °C and resuspended in sodium-SDS-Tris–EDTA buffer (SSTE) (1.0 M NaCl, 0.5 % SDS, 10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0), followed by three extractions with acid phenol and chloroform: isoamyl alcohol (24:1, v/v). The aqueous phase was transferred to a clean tube and the RNA was ethanol precipitated at −20 °C for at least 2 h, followed by centrifugation at 10,000*g* for 10 min at 4 °C and an 80 % ethanol wash. After a final centrifugation at 10,000*g* for 10 min at 4 °C, the air-dried RNA pellet was resuspended in 25 µl RNase-free water.

To eliminate contaminating DNA, total RNA was digested using a Turbo DNA-free™ reagent (Ambion, Austin, TX, USA), according to the manufacturer's instructions. The treated RNA was quantified using spectrophotometry (NanoDrop 1000, BioRad), and quality was assessed as A260 nm/A280 nm ratio. The quality of RNA used for the cDNA library construction was also verified on 1.2 % agarose gel.

For the construction and sequencing of the normalised cDNA library, the RNA was sent to DNAVision (Belgium). We opted for a normalization step in the library construction to avoid over-representation of abundant transcripts and to increase the probability of sequencing more rare mRNAs.

Sequence processing and bioinformatic analyses

The reads were extracted from the sff files using the python script sff_extract_0_2_8.py (http://bioinf.comav.upv.es/_downloads/sff_extract_0_2_8) with default parameters except of the option—min_left_clip = 45 that was used to clip at least 45 bp at left of the reads. The reads were cleaned using Seqclean (http://compbio.dfci.harvard.edu/tgi/software/) that removed vector contaminant using UniVec (http://www.ncbi.nlm.nih.gov/tools/vecscreen/univec/), reads shorter than 50 bp, removed terminal N's and polyA/T as well as low complexity reads. The reads were then assembled using MIRA assembler (http://sourceforge.net/projects/mira-assembler/) with the following parameters: job = denovo,est,normal,454; 454_SETTINGS -LR:mxti = no -CL:qc = no.

Unigenes were either compared with the NCBI non-redundant protein (Nr) database at http://www.ncbi.nlm.nih.gov using the BLASTX algorithm on Blast2Go program (Conesa et al. 2005), or analysed with the binary SVM classifier EST3, developed by Emmersen et al. (2007) to determine the origin of sequences in mixed sequence sets by codon frequencies. The EST3 classifier was trained with 500 fungal/plant sequences previously identified in the cDNA library by BlastX (cutoff value of $E < 1.0^{-10}$). EST3 assignment was reiterated 50 times, every time trained with 500 randomly chosen plant/fungal sequences. We obtained the GO (Gene Ontology) terms of each *S. vomeracea* sequence by the Blast2GO (Conesa et al. 2005) software using the default parameters.

Further analyses were performed to better characterize 11 selected genes assigned to the plant partner and chosen for the high similarities with proteins involved in mutualistic and/or pathogenic plant–microbe interactions. The corresponding sequences were deposited in GenBank (Accession numbers HG970048-HG970060, including housekeeping genes). The corresponding amino acid sequences were analyzed using the Pfam database to assess the presence of conserved functional domains, and SignalP (Bendtsen et al. 2004) to verify whether they also contained a signal peptide. Multiple protein alignment was constructed in MUSCLE (Edgar 2004). Phylogenetic trees were constructed with the maximum likelihood method, using the MEGA software, Version 5.0 (Tamura et al. 2011); bootstrap analyses were conducted on the basis of 1,000 re-samplings of the sequence alignment.

Fixation and resin embedding for light microscopy

Mycorrhizal protocorms *of S. vomeracea* were fixed in 2.5 % (v/v) glutaraldehyde in 10 mM Na-phosphate buffer (pH 7.2) overnight at 4 °C. After washing in the same buffer, the samples were post-fixed in 1 % (w/v) osmium tetroxide in water for 1 h, washed three times with water, and dehydrated in an ethanol series [30, 50, 70, 90, and 100 % (v/v); 15 min each step] at room temperature. Samples were infiltrated in 2:1 (v/v) ethanol/LR White (Polysciences, Warrington, PA, USA) for 1 h, 1:2 (v/v) ethanol/LR White resin for 2 h, 100 % LR White overnight at 4 °C, and embedded in LR White resin, according to Balestrini et al. (1996). Semi-thin sections (1 µm) were stained with 1 % (w/v) toluidine blue for morphological observations.

One-Step RT-PCR and quantitative RT-PCR

Primers for RT-PCR and qRT-PCR (Table S2) were designed to investigate gene expression by using PerlPrimer (http://perlprimer.sourceforge.net/) and tested for their specificity with Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). To design primers specific for non-conserved regions, sequences were first aligned using Mega5 (Tamura et al. 2011). Prior to use in qRT-PCR, primers were tested on genomic DNA extracted from the *T. calospora* mycelium (as negative control) and *S. vomeracea* seeds (as positive control) with the CTAB method (Doyle and Doyle 1990).

The RT-PCR reactions (OneStep RT-PCR, Qiagen) were performed in 20 µl final volume containing 4 µl of 5× buffer, 1 µl of dNTPs (10 mM), 0.5 µl of each primer (10 mM), 0.5 µl of Enzyme Mix, 12.5 µl of water and 1 µl of RNA (about 10 ng). RT-PCR reactions were carried out in thermal cycler T3000 Biometra® (http://www.biometra.de), using the following amplification program: retrotranscription at 50 °C for 30 min, initial denaturation at 95 °C for 15 min; 35 cycles, each cycle consisting of one step of denaturation at 94 °C for 30 s, annealing for 30 s and extension at 72 °C for 1 min; a final extension at 72 °C for 10 min. Amplicons were separated on 1.8 % agarose gels by electrophoresis in 0.5 × TAE buffer (Tris base, acetic acid, EDTA) and visualized with ethidium bromide.

For qRT-PCR, SuperScriptII Reverse Transcriptase (Invitrogen) was used to synthesize cDNA starting from five hundred nanograms of total RNA for each sample, following the manufacturer's instructions. RT-qPCR reactions were carried out in a StepOne apparatus (Applied Biosystem), following the protocol described in Zampieri et al. (2011). The baseline range and CT values were automatically calculated using the StepOne software. The expression of candidate genes was normalized to that of *S. vomeracea* elongation factor gene (*SvEF-1α*; Table S2) by subtracting the CT value of *SvEF-1α* from the CT value of the candidate gene resulting from the ΔCT. The expression ratios were calculated without the PCR efficiency correction from equation $2^{-\Delta\Delta CT}$, where ΔΔCT represents the ΔCTsample − ΔCTcontrol. All reactions were performed on three biological and three technical replicates. Statistical analyses were carried out using Rest2009, version 2.0.13, considering data with a *P* value <0.05 as being significantly different.

Results

454 GS-FLX Titanium pyrosequencing and assignment of transcripts to the symbiotic partners

Pyrosequencing (454 GS-FLX Titanium) of the cDNA library constructed from *S. vomeracea* mycorrhizal protocorms produced 260,200 raw reads that were reduced to 256,543 after cleaning (average and median reads length of 498 and 501 bp, respectively, with SD = 145 bp). MIRA assembled reads into 23,486 contigs with an average length of 508 bases (SD = 210 bp, range = 40–3,624). These contigs (unigenes) were assigned following the pipeline shown in Fig. 1. The assembled unique transcripts from mycorrhizal protocorms were compared with the sequences in the NCBI non-redundant protein (Nr) database using the BLASTX algorithm. A cutoff value of $E < 1.0e^{-10}$ was used and transcripts that found significant sequence similarity to known plant or fungal genes in the NCBI database were assigned to *S. vomeracea* or *T. calospora*; 2,549 sequences (21.2 % of assigned sequences) could be assigned to the fungal partner, whereas 9,333 (77.8 % of assigned sequences) could be attributed to the plant partner. In addition, 70 sequences corresponded to plant transposable elements, 27 were of viral origin, and 11 of bacterial origin.

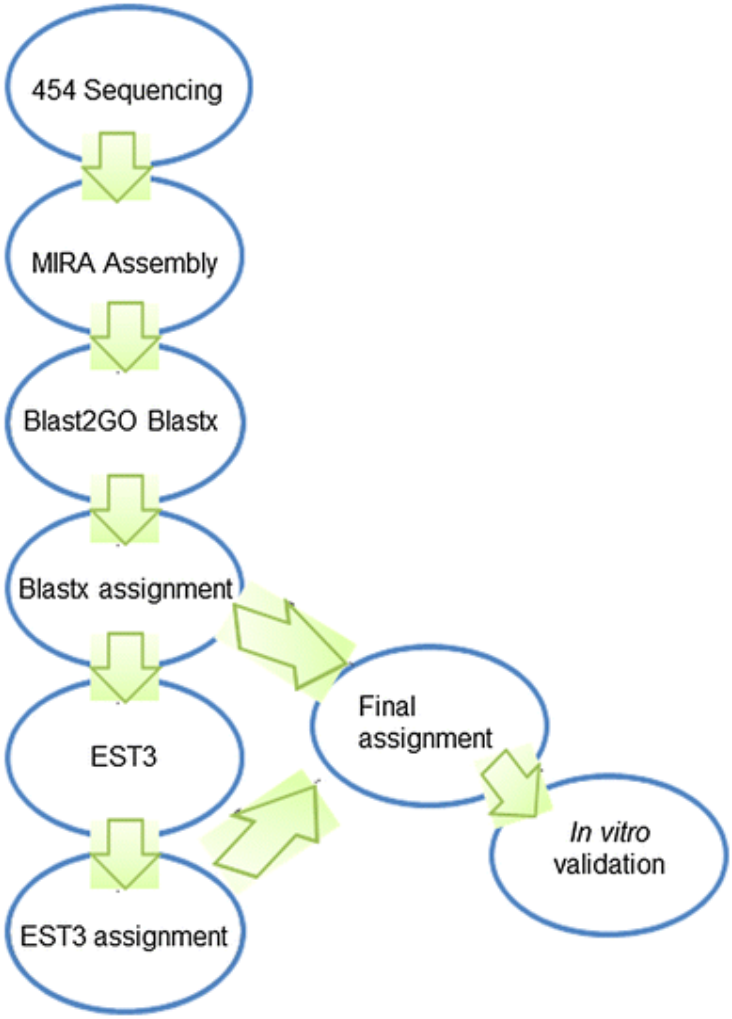

Fig. 1

Pipeline of the bioinformatic analysis. Sequences derived from 454 pyrosequencing of mRNA extracted from mycorrhizal *S. vomeracea* protocorms were assembled by MIRA, and contigs were subject to BlastX searches in the Blast2Go program. Sequences that found matches with a value of *E* <1.0e^{-10} with either plant of fungal sequences in the NCBI non-redundant protein (Nr) database were directly assigned to *S. vomeracea* or *T. calospora*. Sequences unassigned after BlastX search were further analysed with the binary Support Vector Machine classifier EST3. Some sequences were randomly validated by PCR amplification of genomic DNA extracted from *S. vomeracea* seeds or from *T. calospora* free living mycelium

With this high *E* value cutoff, 11,496 sequences (48.9 % of total sequences) could not find a significant match after BlastX search and were not assigned to any partner. These unassigned sequences were then analysed with the binary Support Vector Machine classifier EST3 (Emmersen et al. 2007), an algorithm developed to determine the origin of sequences in mixed sequence sets by codon frequencies, trained as described in Materials and methods. The EST3 analysis allowed the assignment of further 8,492 sequences to the plant (73.8 % of unassigned sequences), and 1,922 sequences (16.7 % of unassigned sequences) to the fungus. At the end of the pipeline, 1,082 sequences remained unassigned (9.4 % of unassigned sequences, corresponding to 4.6 % of the total sequences). The database of plant-assigned transcripts that could be identified (cutoff value of *E* <1.0e^{-6}) with the BLASTX algorithm is provided in Table S1

(Supplementary material). We also used the GO (Gene Ontology) annotations for sequences matching to known genes in other species, to provide a description of gene products associated with biological processes, cellular components and molecular functions. The results are reported in Fig. S1 (Supplementary material). According to the GO annotation, the terms related to metabolic (24 %) and cellular (25 %) processes, binding (43 %) and catalytic activity (40 %), membrane-bounded organelle (29 %) and cell parts (36 %) were dominant, respectively, in biological process, molecular function and cellular components (Fig. S1, Supplementary material). By contrast, 8 % of transcripts were functionally classified as being involved in response to biotic and abiotic stimuli, and only 2 % were specifically involved in stress response.

Plant gene expression in symbiotic and asymbiotic protocorm tissues

Among the sequences assigned to the plant partner, a small panel of genes was chosen because the corresponding proteins, or conserved protein functional domains, showed high similarities with proteins involved in plant–microbe interactions and induced in mutualistic and/or pathogenic interactions. The putative functions assigned to these *S. vomeracea* genes are reported in Table 1. In particular, from the EST contigs database we selected sequences encoding four different nodulin-like proteins (out of 20 contigs encoding for nodulin-like proteins, named *SvNod1*, *SvNod6*, *SvNod9*, *SvNod10*), a class III acidic chitinase (out of 4 contigs, named *SvChit3*), a pathogenesis-related protein (out of 7 contigs, *SvPR10*), a putative remorin (the only contig found, *SvRemor3*), two mannose-binding lectins (out of 48 contigs, named *SvLect3* and *SvLect5*) and two wound-induced proteins (out of 11 contigs, named *SvWound2* and *SvWound3*) with different putative functions. In particular, *SvWound2* codes for a protein containing a lipoxygenase, LH2 (IPR001024) conserved domain, while *SvWound3* encodes a protein with similarity with a serine carboxypeptidase (containing the conserved domains IPR001563 and IPR018202 corresponding to the PF00450 peptidase_S20 and to PS00560 CARBOXYPEPT_SER_HIS). A signal peptide was recognized only in the gene products of *SvLect5* and *SvWound2*, although its absence in other sequences could derive from the fact that they were not complete at the N-terminal.

Table 1

Serapias vomeracea and *Tulasnella calospora* genes investigated by RT-PCR and real-time quantitative PCR

Sequence code	**Sequence name**	**Blastx (best identified match)**	**Plant species**	**Query coverage (%)**	***E* value**	**Conserved domain**
Serapias vomeracea genes						
R2_rep_c11088	SvNod1	Early nodulin 55-2, putative	*Ricinus communis*	50	2,00E-18	Cu_bind_like (PF02298), Plastocyanin-like domain
R2_rep_c10018	SvNod6	Auxin-induced protein 5NG4-like (nodulin MtN21-like protein)	*Brachypodium distachyon*	88	5,00E-79	–
R2_c5820	SvNod9	MtN3-like protein	*Arabidopsis*	64	3,00E-	MtN3_slv (PF03083),

Sequence code	Sequence name	Blastx (best identified match)	Plant species	Query coverage (%)	*E* value	Conserved domain
		(bidirectional sugar transporter SWEET, putative)	*thaliana*		16	Sugar efflux transporter for intercellular exchange
R2_rep_c1368	SvNod10	Nodulin-related protein	*Oryza sativa*	68	9,00E-50	–
R2_rep_c6084	SvLect3	Lectin	*Bulbophyllum morphologorum*	54	2,00E-19	B_lectin (PF01453), d-mannose binding lectin
R2_rep_c46	SvLect5	Mannose-binding lectin	*Crocus vernus*	65	1,00E-47	B_lectin (PF01453), d-mannose binding lectin
R2_rep_c9327	SvChit3	Class III acidic chitinase	*Medicago truncatula*	77	3,00E-44	Glyco_hydro_18 (PF00704)
R2_rep_c1768	SvPR10	Pathogenesis-related protein	*Hyacinthus orientalis*	53	5,00E-34	Bet_v_1 (PF00407), Pathogenesis-related protein Bet v I family
R2_rep_c745	SvWound2	Wound/stress protein precursor	*Zea mays*	48	4,00E-64	PLAT/LH2 domain (PF01477)
R2_c8258	SvWound3	Serine carboxypeptidase	*Vitis vinifera*	85	8,00E-155	Peptidase_S10 (PF00450), Serine carboxypeptidase
R2_c12546	SvRemor3	Remorin family protein	*Arabidopsis lyrata*	63	2,00E-32	Remorin_C (PF03763), Remorin, C-terminal region
Tulasnella calospora genes						
R2_c9021	TcMprot1	Extracellular elastinolytic metalloproteinase	*Coprinopsis cinerea*	66	7,00E-47	Peptidase_M36 (PF02128), Fungalysin metallopeptidase (M36)
R2_c4090	TcMprot2	Metalloprotease	*Piriformospora indica*	84	1,00E-17	Peptidase M16 domain (IPR011237)

As confirmed by phylogenetic analysis (Fig. S2), SvWound3 belongs to the serine carboxypeptidase-like (SCPL) proteins and it clustered in a well-supported group (bootstrap value = 99 %) together with other sequences from monocots and dicots. The SCPL group including *S. vomeracea* SvWound3 was distinct from SCPLs with known acyltransferase activity (Fig. S2). Phylogenetic analyses of *S. vomeracea* mannose-binding lectins SvLect3 and SvLect5 showed that, whereas SvLect3 did not cluster close to other lectins (probably due to its short sequence), SvLect5 clustered in a well-supported group (bootstrap value = 98 %) together with other mannose-binding lectins from monocots (Fig. 2). Interestingly, all other lectins from orchids clustered in two different groups, separated from the SvLect5 cluster (Fig. 2).

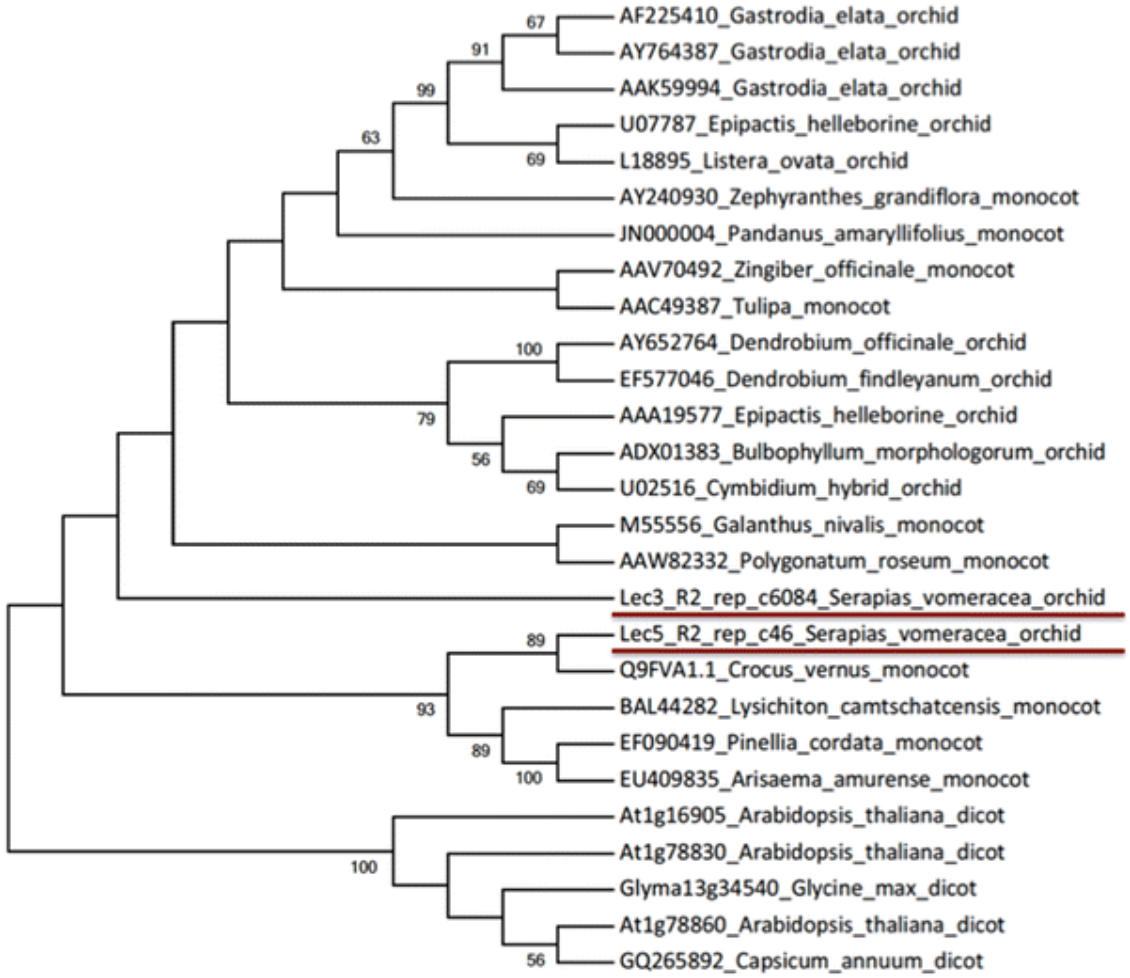

Fig. 2

Phylogenetic relationships between mannose-binding lectins from monocots and dicots, based on amino acid deduced sequences. The sequences were aligned using Muscle and the unrooted tree was constructed using maximum likelihood. Sequences from other orchid lectins were also included in the tree and grouped in two different cluster, both distinct from the *S. vomeracea* lectins. *Numbers* indicate bootstrap values, and are indicated only when ≥50 %. *S. vomeracea* sequences are *underlined*

Expression of the plant genes was assessed in mycorrhizal *S. vomeracea* protocorms collected at stage 3 (Otero et al. 2004). At this stage, symbiotic protocorms displayed the typical features illustrated in Fig. 3, with *T. calospora* hyphae forming intracellular pelotons in plant cells mostly in the basal region of the protocorm. The apical protocorm region featured a meristem that was never colonized by the fungus.

Protocorms obtained through asymbiotic seed germination were used as reference for evaluation of gene expression in non-mycorrhizal tissues. However, asymbiotic protocorms required a different, very rich growth medium for germination and showed a much slower growth rate when compared to symbiotically germinated protocorms, only reaching stage 2. To avoid misinterpretation of the results due to these differences, an internal control was obtained by physically separating the basal (mycorrhizal) and the apical (non-mycorrhizal) tissues with a sterile blade (see Fig. 3).

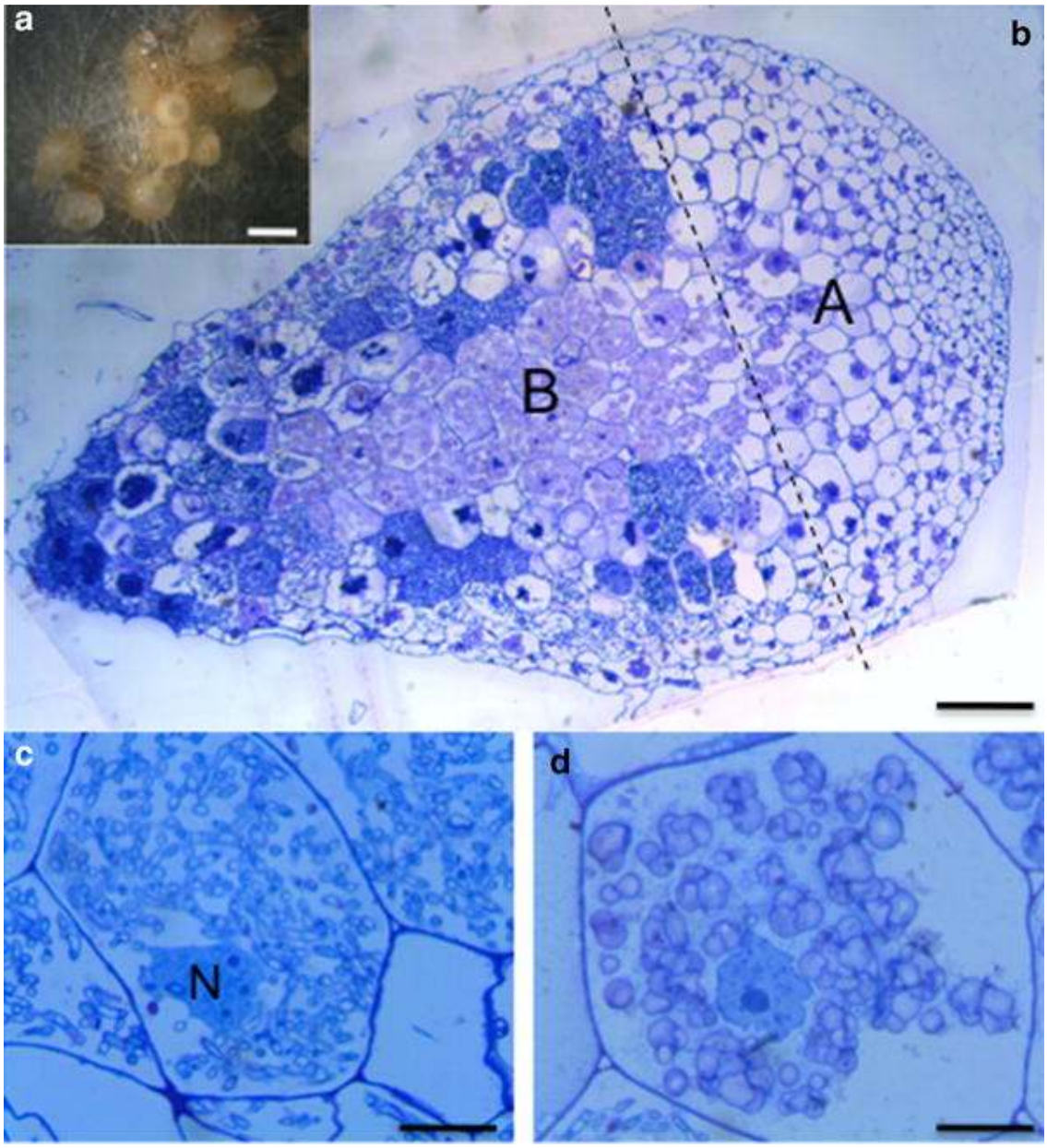

Fig. 3 Mycorrhizal protocorms of *Serapias vomeracea* 30 days after sowing (stage 3) with *Tulasnella calospora*. **a** Stereomicroscopic image of protocorms, showing well-developed rhizoids. *Bar* 1 mm. **b** Longitudinal section of a resin-embedded protocorm, showing the basal, mycorrhizal region (*B*) and the apical, uninfected region (*A*). These two regions were manually dissected. Starch containing cells can be observed in the central part of the basal region. *Bar* 120 µm. **c** A magnification of a mycorrhizal cell containing a well-developed fungal peloton. The plant nucleus is visible (N). *Bar* 21 µm. **d** A *S. vomeracea* cell from the basal protocorm region, showing starch granules around the nucleus. *Bar* 21 µm

Primers designed to the plant and fungal elongation factor (*SvEF-1α, TcEF-1α*) and ubiquitin (*SvUBI, TcUBI*), as house-keeping genes (Table S2), were used by one-step RT-PCR to assess RNA quality and possible contaminations of the protocorm RNA samples with genomic DNA. The absence of an amplified product in the RT-minus reactions excluded genomic DNA contamination (data not shown). The *SvEF-1α* and *SvUBI* primers amplified *S. vomeracea* transcripts in mycorrhizal and non-mycorrhizal tissues of symbiotic protocorms. However, only primers specific for *SvEF-1α* also amplified a DNA fragment from asymbiotic protocorms, whereas amplicons were not obtained for this sample with the *SvUBI* specific primers. For this reason, we only used *SvEF-1α* primers in the quantitative PCR experiments. As expected, primers for the two *T. calospora* house-keeping genes mainly amplified transcripts in the basal mycorrhizal samples (Fig. 4), but a faint band was also amplified from the apical protocorm sample, indicating that manual dissection could not fully separate mycorrhizal and non-mycorrhizal tissues. However, this fungal contamination was not observed after amplification of two *T. calospora* metalloprotease genes (*TcMprot1* and *TcMprot2*; Fig. 4), likely due to a lower level of expression.

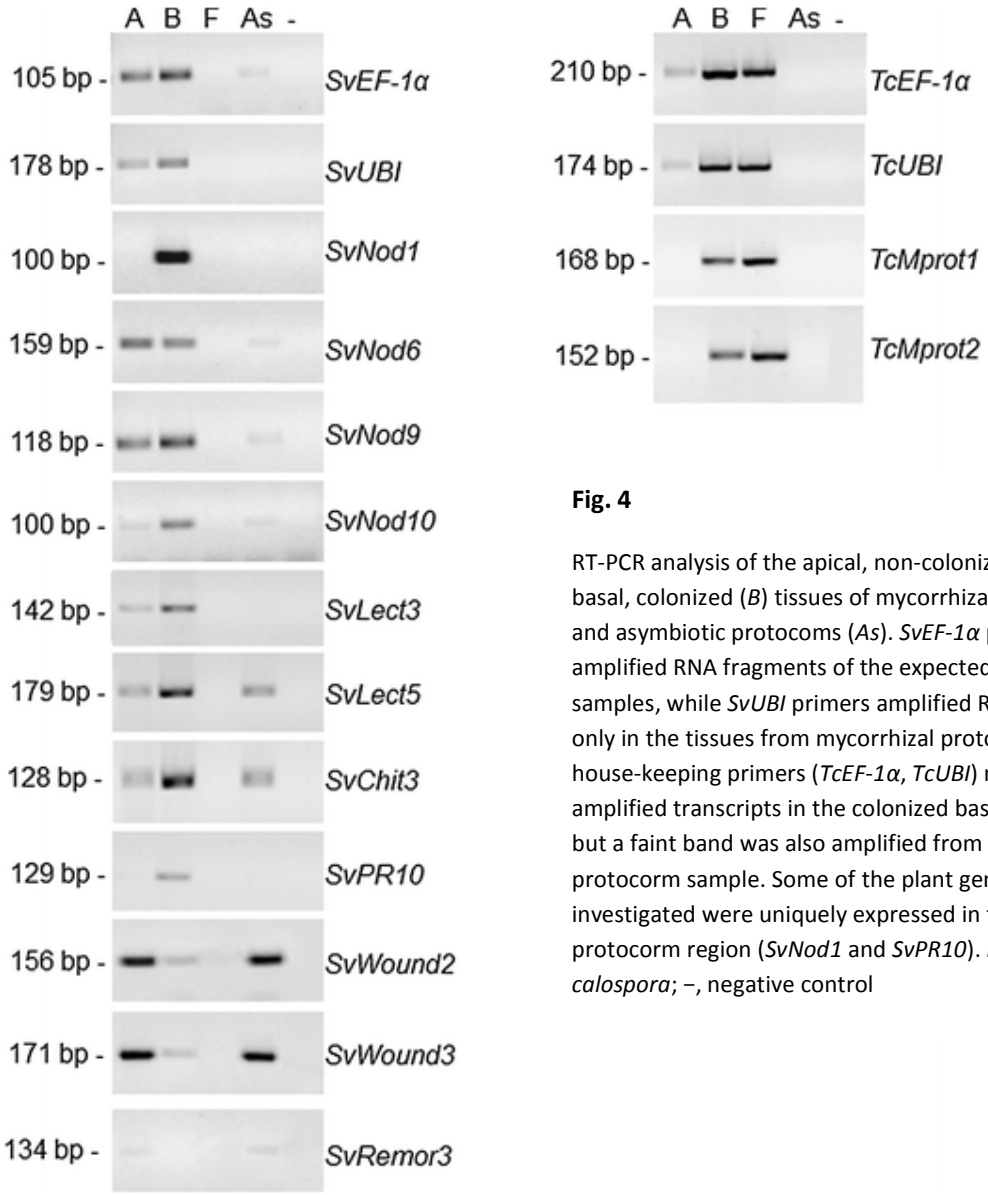

Fig. 4

RT-PCR analysis of the apical, non-colonized (*A*) and basal, colonized (*B*) tissues of mycorrhizal protocorms and asymbiotic protocoms (*As*). *SvEF-1α* primers amplified RNA fragments of the expected size in all the samples, while *SvUBI* primers amplified RNA fragments only in the tissues from mycorrhizal protocorms. Fungal house-keeping primers (*TcEF-1α*, *TcUBI*) mainly amplified transcripts in the colonized basal samples, but a faint band was also amplified from the apical protocorm sample. Some of the plant genes investigated were uniquely expressed in the colonized protocorm region (*SvNod1* and *SvPR10*). *F*, *Tulasnella calospora*; –, negative control

One-step RT-PCR experiments with the plant gene-specific primers listed in Table S2 showed that some of the genes investigated were uniquely expressed in the basal, mycorrhizal region of the protocorms. This was the case for the *SvNod1* gene, coding for a protein containing a plastocyanin-like domain (PF02298.12), and for a putative *SvPR10* gene (Fig. 4). The other genes were also expressed to different degrees in the apical, non-mycorrhizal tissue of symbiotic protocorms, as well as in the asymbiotic protocorms (Fig. 4).

As one-step RT-PCR is not a quantitative technique, real-time quantitative PCR (qRT-PCR) experiments were performed to confirm the results for *SvNod1* and *SvPR10* expression and to better understand regulation of the other genes. qRT-PCR confirmed (Table 2) a very strong up-regulation in the expression of the *SvNod1* gene in the basal mycorrhizal protocorm tissues as compared to both the apical non-mycorrhizal tissues of the same protocorms and to the asymbiotic protocorms. By contrast, up-regulation of *SvPR10* was not confirmed by qRT-PCR (Table 2). Significant up-regulation of the two lectin genes (*SvLect3* and *SvLect5*) and for a putative bidirectional sugar transporter (*SvNod9*) was found in the basal mycorrhizal protocorm tissues, but it was statistically significant for *SvNod9* only when compared with the apical non-mycorrhizal tissues of the same symbiotic protocorms (Table 2). Expression in the mycorrhizal tissue appeared to be up-regulated also for the gene encoding the class III chitinase (*SvChit3*), but it was not statistically significant.

Table 2

Comparison of gene expression in mycorrhizal and non-mycorrhizal protocorm tissues as measured by qRT-PCR

Sequence code	Fold change qRT-PCR B/A	Fold change qRT-PCR B/As
SvNod1	1,451.5 ± 883.3*	256.9 ± 198.9*
SvNod6	4.5 ± 7.3	0.2 ± 1.6
SvNod9	54.2 ± 52.4*	4.3 ± 3.2
SvNod10	4.0 ± 1.5	0.4 ± 0.5
SvLect3	1,097.5 ± 297.4*	630.4 ± 264.3
SvLect5	388.9 ± 120.1*	10.8 ± 14.4
SvChit3	169.3 ± 233.7	42.5 ± 164.1
SvPR10	3.6 ± 1.9	1.6 ± 0.6
SvWound2	1.9 ± 0.2	0.6 ± 0.1
SvWound3	1.3 ± 0.04	0.1 ± 0.01
SvRemor3	0.9 ± 0.207	0.8 ± 0.1

Expression in basal mycorrhizal protocorm regions (B) was compared with apical non-mycorrhizal regions (A) of the same protocorms (B/A ratio), or with asymbiotically germinated protocorms (B/As ratio)

* *P* value <0.05

Discussion

Identification of the genes expressed by the individual partners of a symbiosis is a first step to understand the molecular bases of their interactions, either mutualistic or antagonistic. High-throughput sequencing technologies provide large databases, and the assignment of the expressed genes to the individual partners is commonly based, for non-model organisms with no genomic data available, on homology searches in GeneBank databases with the Blast algorithm. This method has limitations in the absence of genome data from phylogenetically close organisms. To identify plant genes expressed in mycorrhizal orchid protocorms, we have combined BlastX searches with the use of EST3, a predictive SMV program developed by Emmersen et al. (2007) to define the plant or fungal origin of genes expressed in plant–pathogen interactions, and based on GC percentage and codon usage.

Our results indicate that the majority of sequences in the normalised cDNA library were of plant origin, a result confirmed in our subsequent sequencing experiments. Similarly, only few fungal genes were identified by Zhao et al. (2013) in a SSH library enriched in transcript up-regulated in symbiotically germinated seeds. We currently do not know whether the low occurrence of fungal transcripts may mirror a lower metabolic activity or simply a dilution by the plant transcripts. Whereas no genetic information was available on the *S. vomeracea*–*T. calospora* system when our analyses were carried out, the *T. calospora* genome has been recently sequenced as part of a DOE JGI Community Sequencing program coordinated by F. Martin (INRA, Nancy, France). This has allowed us to evaluate the error of our bioinformatics pipeline by Blastn search of plant-assigned genes on the *T. calospora* genome. The results indicate that out of 9,403 sequences assigned by Blast2GO to *S. vomeracea*, only 79 sequences (0.84 %) matched *T. calospora* genes with an *E* value $<1.0e^{-10}$. Not surprisingly, most of these genes coded for highly conserved proteins (e.g. histone proteins, ribosomal proteins, tubulin and chaperonins). This is a further confirmation that a combination of bioinformatics approaches based on different algorithms (i.e. BlastX and EST3) can be helpful in gene assignment in mixed EST libraries from non-model organisms.

Normalized cDNA libraries are used to discover genes transcribed at relatively low levels because they decrease the prevalence of highly abundant transcripts. Therefore, they cannot be used to make estimates of relative gene expression. Despite this limitation, GO annotations suggest that *S. vomeracea* does not activate strong defences during fungal colonization of mycorrhizal protocorms. Most transcripts in the mycorrhizal protocorms were involved in normal metabolic and cellular processes, whereas only a small proportion was classified as being involved in stress response (3 %), or in the response to biotic and abiotic stimuli (8 %). Similar results were reported by Zhao et al. (2013), who compared gene expression in ungerminated and symbiotically germinated seeds of *D. officinale* by SSH. In this library, a low percentage of transcripts specifically expressed in symbiotically germinated *D. officinale* seeds were GO classified as being involved in response to stress (about 13 %) or to biotic and abiotic stimuli (14 %).

The small panel of orchid genes used to investigate the nature of plant–fungus interactions in mycorrhizal protocorms also suggests that the plant does not activate strong defences during fungal colonization. qRT-PCR indicated that a *S. vomeracea* gene coding for a putative pathogenesis-related (SVPR10) protein, known in other biological systems to be part of defence mechanisms and pathogenesis (Campos-Soriano and San Segundo 2011), was not significantly up-regulated in mycorrhizal orchid tissues.

SvChit3, coding for a class III acidic chitinase, was slightly activated in mycorrhizal tissues, but the data were not statistically significant. Although chitinases can be induced in pathogenic interactions (Salzer et al. 2000), specific class III chitinases have been found to be up-regulated in arbuscular mycorrhizal (AM) *Medicago truncatula* roots, but not in interactions with pathogens (Salzer et al. 2000). Predominant

expression of these chitinases was in the arbuscule-containing cells (Bonanomi et al. 2001; Hogekamp et al. 2011), where they may play a role in suppressing plant defence by the reduction of chitin-like elicitors during the formation of functional symbiotic interfaces (Salzer et al. 2000). A similar role may be envisaged in *S. vomeracea* mycorrhizal protocorms, but further studies are required to investigate regulation of Sv*Chit3* by other biotic and abiotic stimuli.

The two genes randomly selected in the EST library as markers of wound-induced response (*SvWound2* and *SvWound 3*) were not up-regulated in the mycorrhizal tissues. *SvWound2* codes for a putative lipoxygenase; these enzymes catalyse the hydroperoxidation of polyunsaturated fatty acids, the initial step in the biosynthesis of many signalling compounds in plants, including jasmonic acid (Feussner and Wasternack 2002). The second wound-related gene (*SvWound3*) encodes for a putative serine carboxypeptidase. Serine carboxypeptidases belong to a large family of hydrolyzing enzymes that play several roles in cellular processes, including defence responses against pathogen infection and oxidative stress (Liu et al. 2008). Lack of induction in mycorrhizal protocorm tissues would, therefore, indicate high plant–fungus compatibility. However, a *M. truncatula* putative serine carboxypeptidase gene (*MtSCP1*) is up-regulated in arbusculated and neighbouring cells and is considered a marker gene for AM symbiosis (Liu et al. 2003; Hogekamp et al. 2011). Currently, we cannot exclude a cell-type-specific induction of *SvWound3* in orchid mycorrhizal protocorms. The *S. vomeracea* RNA sample for qRT-PCR derived from a mixture of fungal infected and uninfected cell types, and apparent lack of induction may be the consequence of a dilution of the *SvWound3* transcripts. A higher resolution technique, such as laser microdissection, may help to clarify this point because it allows the selection of specific cell types (Balestrini et al. 2009).

The genes *SvLect3* and *SvLect5* code for two putative mannose-binding lectins, recently reclassified as B-lectins (Jiang et al. 2010), a large family of proteins originally identified in monocots. These proteins were selected because some mannose-binding lectins have been identified and characterized in orchids (Van Damme et al. 1994; Wang et al. 2001; Sattayasai et al. 2009). Antifungal activity against a range of phytopathogenic fungi has been demonstrated for some of these proteins both in vitro and in transgenic plants (Wang et al. 2001; Cox et al. 2006; Sattayasai et al. 2009), and they were thus proposed to be part of a defence mechanisms against the invading fungus. It is, therefore, possible that up-regulation of *SvLect3* and *SvLect5* may be part of a mechanism to limit fungal growth in the mycorrhizal protocorm. However, it should be noted that plant genomes feature several members of B-lectins (38 in *Arabidopsis*, 87 in *Oryza sativa* and 105 in *Glycine max*; Jiang et al. 2010), and about 48 contigs identified as mannose-binding lectins were found in the *S. vomeracea* 454 cDNA library. Expression studies by Jiang et al. (2010) showed that the different B-lectin isoforms responded differently to various biotic and abiotic stresses, and only some of them were up-regulated in the presence of fungal and/or bacterial pathogens. Moreover, the same authors showed a plant tissue-specific expression for 23.7 % of *Arabidopsis* and 8.0 % of rice B-lectin genes. Overall, these data suggest that, in addition to a role as antifungal proteins, B-lectins likely play biological functions in the developing plant. Interestingly, the sequence of the *S. vomeracea* SvLect5 protein did not cluster with the orchid lectins known to be antifungal. Thus, the role of these lectins in the orchid mycorrhizal protocorm remains to be established.

Some of the orchid genes tested in our experiments were selected because they shared domains with nodulin-like genes first identified in legume nodules. Several nodulin genes are also activated in response to inoculation with AM fungi (Harrison 1998). We have considered the expression of four *S. vomeracea* genes coding for proteins that found matches with putative nodulins by BlastX searches. *SvNod1*, which codes for an early nodulin-like protein containing a plastocyanin-like domain, resulted to be the most up-regulated gene in the mycorrhizal protocorm tissue. Structurally related early nodulins containing plastocyanin-like

domains (e.g. *Pisum sativum*/*Vicia sativa* ENOD5, *M. truncatula* MtENOD16 and MtENOD20, and *Glycine max* N315/ENOD55) are usually arabinogalactan-like proteins (Frühling et al. 2000; Mashiguchi et al. 2009), thought to be involved in cell surface interactions during the infection process (Scheres et al. 1990). Their common feature is the loss or substitution of key amino acids in the copper-binding site, so that these proteins are actually unable to bind copper (Greene et al. 1998; Khan et al. 2007). In non-legume species, orthologs of ENOD55 have been found in the plasma membrane of phloem cells (Khan et al. 2007) or involved in osmotic stress tolerance (Wu et al. 2011). Thus, the role for this group of proteins is not clear, but the strong up-regulation in the mycorrhizal protocorm suggests a role during symbiotic development. Preliminary experiments using laser microdissection to separate cells containing viable or collapsed pelotons, and non-colonized cells, suggest that *SvNod1* transcripts accumulate only in the colonized cells and could be considered a marker of orchid symbiosis (unpublished results).

The other nodulin gene that was up-regulated significantly in the basal part of mycorrhizal protocorms containing the fungal pelotons was *SvNod9*, which codes for a putative bidirectional sugar transporter of the SWEET family. These are uniporters (Chen et al. 2010) that can transfer hexoses across the plasma membrane in both directions, depending on the sugar gradient. SWEETs can serve for sugar export from cells, and they can be exploited by bacterial and fungal pathogens to acquire carbohydrates for energy and carbon (Chen et al. 2010). The nodule-specific MtN3 is a member of the *M. truncatula* SWEET family, and an involvement in bacteroid nutrition inside the nodule has been suggested (Bapaume and Reinhardt 2012), even though previous studies indicated that dicarboxylic acids are likely the carbon source supplied to intracellular bacteroids (see in White et al. 2007). Whether proteins of the SWEET family play a functional role also in mycorrhizal symbiosis remains to be established (Casieri et al. 2013; Tarkka et al. 2013), although the peculiarities of orchid mycorrhiza with respect to carbon flow would envisage differences with other plant–microbe interactions, at least during the mycoheterotrophic protocorm stage.

Other nodulin genes (*SvNod6* and *SvNod10)* and a putative remorin (*SvRemor3*) have been found to be induced in some mutualistic plant–microbe interactions (Jarsch and Ott 2011; Heller et al. 2012), but they did not show any significant up-regulation in *T. calospora*-colonized tissues.

Conclusion

Despite the low number of plant genes validated by RT-qPCR, our study provides no evidence of strong defence activation in *S. vomeracea* mycorrhizal protocorms. On the contrary, some nodulin genes were up-regulated, thus suggesting a high level of plant–fungus compatibility. These findings are in agreement with ultrastructural observations of orchid cells harbouring viable fungal pelotons, where similarities have been found with mycorrhizal cells in the highly compatible AM, including cell reorganization, some membrane-associated enzyme activities and the build-up of a plant–fungus interface (Dexheimer and Serrigny 1983; Peterson et al. 1996; Uetake and Peterson 1998; Peterson and Massicotte 2004).

Thus, the emerging picture of the orchid–fungus relationship (Zhao et al. 2013; this study) is far from that of a “life-and-death struggle” still proposed, for example, by Shimura et al. (2007). This is somehow not unexpected, when considering that all orchid species depend entirely on their symbiotic fungi for seed germination and protocorm development. As these fungi do not seem to be rewarded from the plant at the protocorm stage, at least in the form of carbon, it seems more likely that, to maintain this symbiosis crucial for their survival and evolution, orchids should attract their fungal partners and provide a hospitable environment, rather than exclude them by building strong defence.

Finally, many of the genes we have investigated are part of large gene families. High-throughput and genome-sequencing techniques are providing, for each species, a more complete spectrum of the different isoforms. A future challenge will be to test these isoforms for their specific biological functions.

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest.

Electronic supplementary material

Fig. S1 Gene Ontology assignments for *S. vomeracea* transcripts. Level 3 annotations are shown for the biological process and cellular component graphs, and level 2 annotations for the molecular function graph

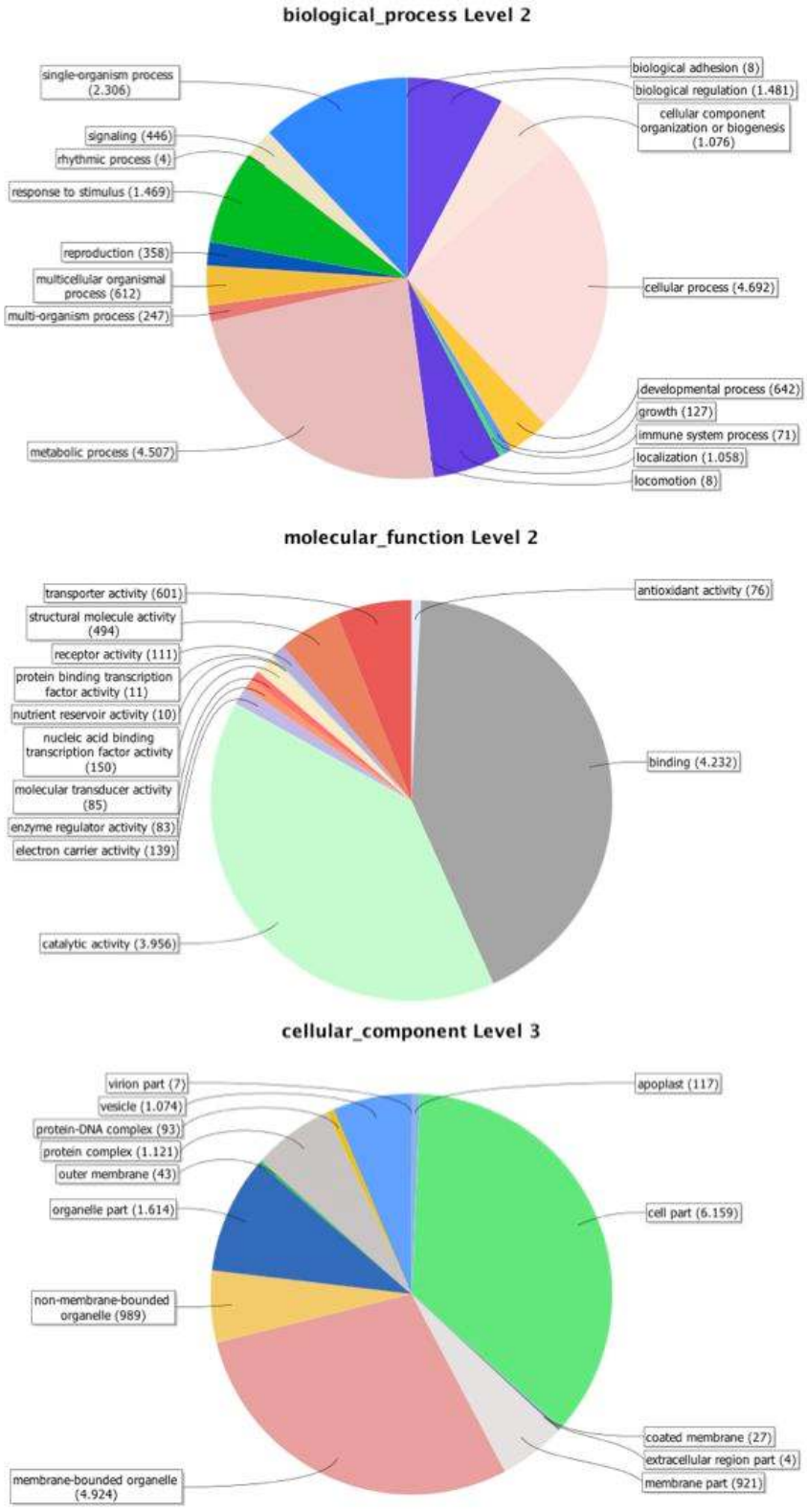

Fig. S2 Phylogenetic relationships of plant serine carboxipeptidases-like (SCPL) proteins based on amino acid deduced sequences. The sequences were aligned using Muscle and the unrooted tree was constructed using maximum likelihood. Characterized SCPL with acyltransferase activity from *Arabidopsis* and *Oryza sativa* are included in the tree and form a separate cluster. Characterized SCPL acyltransferase enzymes included in the tree are also from *Avena sativa* (ACT21078_SCPL1) and *Brassica napus* (AAQ91191_SCT). Characterized serine carboxypeptidase sequences from *Hordeum vulgare* were included (Hv, P07519_SCPI and P2159.2_SCPIII), in addition to sequences from *Vitis vinifera* (Vv, XP_002264454), *Ricinus communis* (Rc, XP_002527263) and *Medicago truncatula* MtSCP1 (Mt, XP_003592239). Numbers indicate bootstrap values and are indicated only when ≥ 50 %. Arrows point to the *S. vomeracea* sequence (SvWound3)

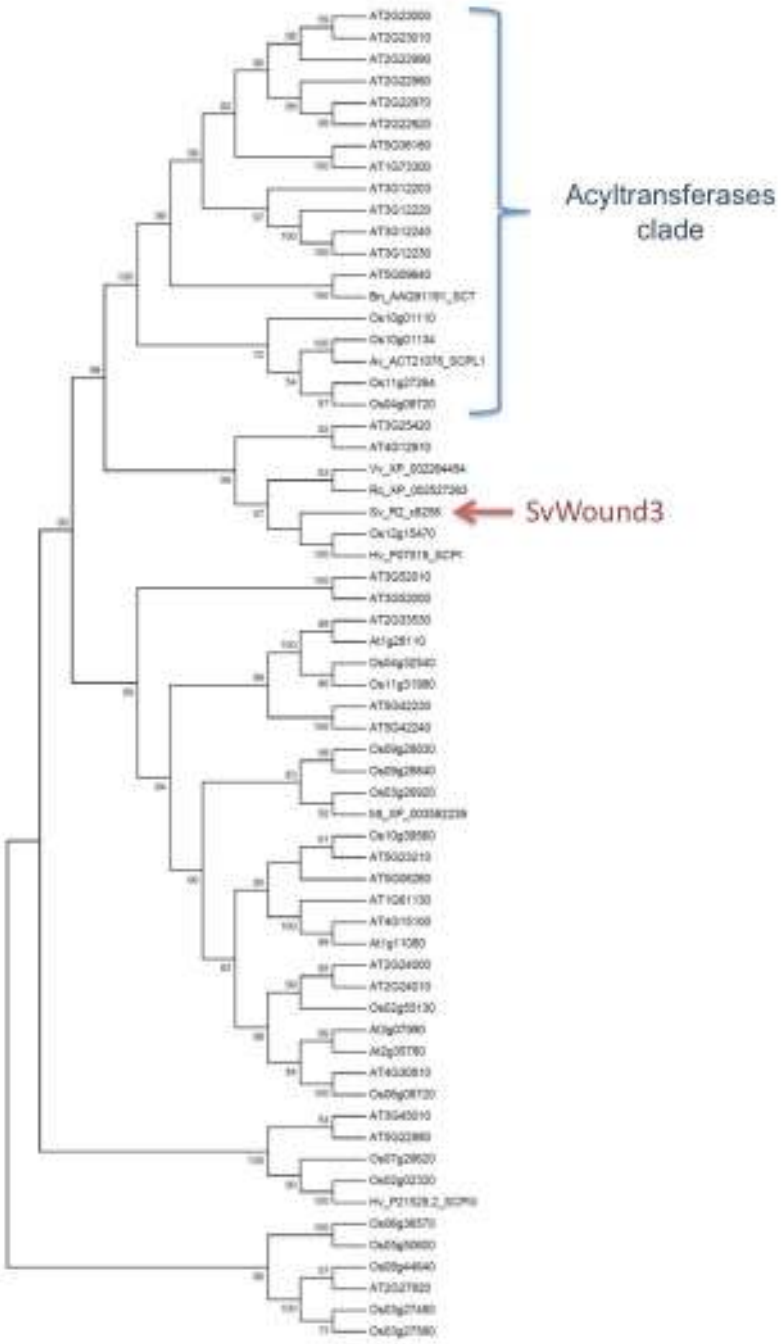

Table S1 Database of plant-assigned transcripts that could be identified (cutoff value of E < 1e-6) with the BLASTX algorithm. The nearest match is indicated for each sequence

blah425_2014_2062_MOESM3_ESM.xls (5142528) Supplementary material 3 (XLS 5022 kb)

Table S2. List of primers used in this study. Accession numbers of plant and fungal sequences are HG970048-HG970064. *Sv* and *Tc* in sequence names identify *Serapias vomeracea* and *Tulasnella calospora*, respectively.

Sequence code	Sequence name	Primer sequences	Annealing T°	Amplicon bp
R2_lrc124	*SvEF-1α*	*For:* CGATCTCGTAGTGATCTGAGCAAG	60°C	105
		Rev: GAGACAGAAATAACAACCAGCAACA		
R3_c218	*TcEF-1α*	*For:* CAAGCCTATGTGCGTTGAGA	60°C	210
		Rev: ACGAGAATGCGAAGGAAGAA		
R2_lrc444	*SvUBI*	*For:* TCTATCTATTCTTGCGGGGG	60°C	197
		Rev: CACATGGCGCAGTCAATAAA		
R3_lrc4	*TcUBI*	*For:* GGCATCATCGAACCTTCATT	60°C	174
		*Rev:*GTGTGGGCGAGTCGTTTACT		
R2_rep_c11088	*SvNod1*	*For:* TGACGGCATCCGCCGAGTTCT	64°C	100
		Rev: ACTTGGAATCTCATACGCTCCGCCC		
R2_rep_c10018	*SvNod6*	*For:* CGGCGGGAAACGCGGCGACA	66°C	159
		Rev: GCACCGGCGCCTGGAGAACCA		
R2_c5820	*SvNod9*	*For:* AGCTCGGCCTTTGCGAGCCCA	64°C	118
		Rev: TGACCGCCGCCGTTGCACCC		
R2_rep_c1368	*SvNod10*	*For:* GCTCACTGGGCTATCGTGCCTGCGG	66°C	100
		Rev: AGCCGGCGGGGTTCGCCACT		
R2_rep_c6084	*SvLect3*	*For:* CGTCCTCCTCCTCGCTCCCGCT	64°C	142
		Rev: GCGCCGCAGCACGAGGTTGC		
R2_rep_c46	*SvLect5*	*For:* CCTCCGCCAGCAGCACCAAATGC	64°C	179
		Rev: TGACCACACAGCGGGGCCGT		
R2_rep_c9327	*SvChit3*	*For:* CGACACGTGCGCCTCCGGCAA	66°C	128
		Rev: GGGCGGTGCAGCCGTCGGAG		
R2_c8258	*SvPR10*	*For:* GTGCTGCCGGCCTCGCCGTG	64°C	129
		Rev: CCCAAGCCCCGCCTGTTCCCC		
R2_rep_c46	*SvWound2*	*For:* GCGGCAACGACGGTGACTGCG	64°C	156
		Rev: CGCCCATCAGGCCGCCCCAA		
R2_rep_c1768	*SvWound3*	*For:* GGGTCGTGCTTGGCCTTTGAGGGCT	60°C	171
		Rev: GGGCCTGTCACGCTTTCCGGCT		
R2_c8258	*SvRemor3*	*For:* ACTGCGAAGGCAGAAGCACAATCG	60°C	134
		Rev: CGTGCTGCGGCTCTCCACTC		
R2_c9021	*TcMprot1*	*For*: CCCCAAGCACGGCAACGCCC	62°C	168
		Rev: CCACGACTGGCGAATCCAGCCCA		
R2_c9021	*TcMprot2*	*For*: CGACGGCGACGAACCGTTGCTCT	64°C	152

Table S3. Ct values derived from qRT-PCR experiments. Data are the average of Ct values obtained from three biological replicates (± SD).

	SvEF-1α	***SvNod1***	***SvNod6***	***SvNod9***	***SvNod10***	***SvLect3***	***SvLect5***	***SvChit3***	***SvPR10***	***SvWound2***	***SvWound3***	***SvRemor3***
CT A	32,95±1,84	42,91±0,01	35,74±1,39	38,41±3,04	32,23±2,21	34,95±3,51	34,17±1,37	34,48±2,21	32,38±0,56	29,64±2,51	31,15±1,79	31,80±0,85
CT B	33,26±0,83	32,75±3,11	34,58±2,07	33,00±1,73	31,23±4,14	25,20±4,28	25,92±2,12	28,07±6,69	30,87±2,13	29,04±3,06	31,81±3,88	33,01±2,28
CT As	31,73±2,47	39,39±3,10	32,42±2,76	33,71±4,16	29,84±3,44	33,13±1,81	27,97±4,85	33,48±0,66	30,15±2,92	26,87±3,39	28,92±4,10	29,49±3,04

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