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Raffaella Balestrini; Luca Nerva; Fabiano Sillo; Mariangela Girlanda; Silvia Perotto. Plant and fungal gene expression in mycorrhizal protocorms of the orchid Serapias vomeracea colonized by Tulasnella calospora. PLANT SIGNALING & BEHAVIOR. 9 (11) pp: e977707-e977707.
DOI: 10.4161/15592324.2014.977707

Plant and fungal gene expression in mycorrhizal protocorms of the orchid Serapias vomeracea colonized by Tulasnella calospora

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Abstract

Little is known on the molecular bases of plant–fungal interactions in orchid mycorrhiza. We developed a model system to investigate gene expression in mycorrhizal protocorms of *Serapias vomeracea* colonised by *Tulasnella calospora*. Our recent results with a small panel of genes as indicators of plant response to mycorrhizal colonization indicate that genes related with plant defense were not significantly up-regulated in mycorrhizal tissues. Here, we used laser microdissection to investigate whether expression of some orchid genes was restricted to specific cell types. Results showed that *SvNod1*, a *S. vomeracea* nodulin-like protein containing a plastocyanin-like domain, is expressed only in protocorm cells containing intracellular fungal hyphae. In addition, we investigated a family of fungal zinc metallopeptidases (M36). This gene family has expanded in the *T. calospora* genome and RNA-Seq experiments indicate that some members of the M36 metallopeptidases family are differentially regulated in orchid mycorrhizal protocorms.

Keywords

- fungalysins,
- gene expression,
- mannose-binding lectins,
- nodulin genes,
- orchid protocorm

Most land plants associate with mycorrhizal fungi that play a pivotal role in plant mineral nutrition.[1] In orchids, mycorrhizal fungi are also needed for seedling development,[2] because they provide the heterotrophic protocorm, an early orchid developmental stage, with organic carbon.[3] Although orchids strictly depend on mycorrhizal fungi, at least for early development, this interaction has been often described as a balanced parasitism, where the plant defends itself from an aggressive fungus thanks to the production of antifungal compounds. For a review see refs. [4-8]

To better understand the molecular bases of plant-fungal interactions in orchid mycorrhizal protocorms, we developed a model system with the Mediterranean orchid *Serapias vomeracea* colonised by *Tulasnella calospora* (Basidiomycetes, Cantharellales). We used this model system to generate a normalized cDNA library by 454 GS-FLX Titanium pyrosequencing, which represents an inventory of plant and fungal genes expressed in mycorrhizal protocorms.[9]

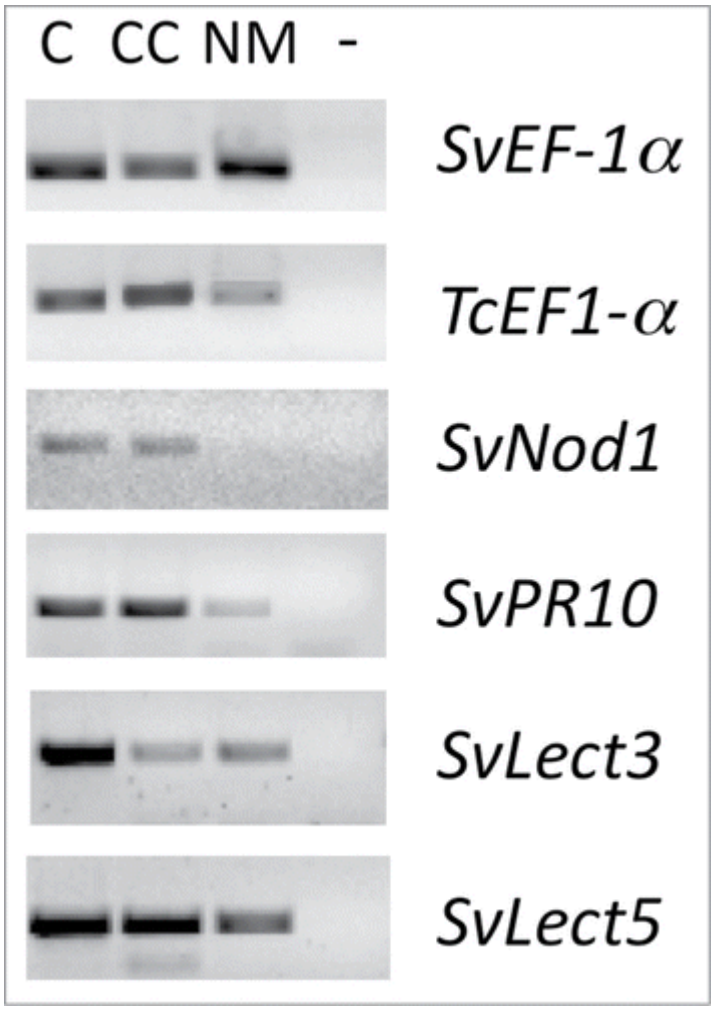
Gene expression in orchid mycorrhiza has received very little attention,[10,11] and only recently Zhao et al.[12] investigated the transcriptome of *Dendrobium officinale* colonised by *Sebacina*. Despite the different experimental approaches and model systems, a common finding in the transcriptomes of *D. officinale* and *S. vomeracea* mycorrhizal protocorms was the small proportion of plant transcripts related to stress response or response to biotic and abiotic stimuli.[9,12]

We previously assessed, in mycorrhizal and non-mycorrhizal protocorm tissues of *S. vomeracea*, expression of some plant genes that may provide insights into the orchid response to the mycorrhizal fungus.[9] Despite the small number of genes tested, none of the pathogenesis or wound/stress related genes were significantly upregulated in mycorrhizal tissues, suggesting that fungal colonization did not trigger strong plant defense responses.

In this work, we characterized further the expression of some of these plant and fungal genes. In particular, laser microdissection (LMD) was used to separate *S. vomeracea* protocorm cells containing fungal pelotons at different developmental stages (viable and collapsed), as well as non-colonized cells. Accumulation of specific transcripts in RNA extracted from these LMD cells was assessed by one-step RT-PCR for 4 orchid genes: a nodulin-like proteins (*SvNod1*), a putative pathogenesis-related protein (*SvPR10*) and 2 mannose-binding lectins (*SvLect3* and *SvLect5*).

In LMD protocorm cells, *SvNod1* transcripts accumulated only in plant cells containing fungal hyphae (**Fig. 1**). *SvNod1* was also one of the most up-regulated genes in mycorrhizal protocorm tissues.[9] *SvNod1* codes for a protein containing a plastocyanin-like domain identified in early nodulin-like proteins from several legumes, where a common feature is the loss of the copper-binding site.[13,14] Some of these early nodulins may be membrane bound arabinogalactan-like proteins,[15,16] likely involved in cell surface interactions during microbial infection.[17] Although the role of these proteins is still unclear, the strong up-regulation in the *S. vomeracea* mycorrhizal protocorm cells suggests a role in symbiosis.

Figure 1. One-step RT-PCR analysis of *S. vomeracea* genes in 3 populations of microdissected cells: C, protocorm cells containing viable coils; CC, protocorm cells containing collapsed coils; NM, non-colonized cells from colonized protocorms. Briefly, microdissected cells were isolated using an AS LMD system (Leica) from paraffin protocorm sections prepared as described in Pérez-Tienda et al.[27]; RNA was then extracted from each cell-type using the ARCTURUS PicoPure RNA Isolation Kit (Life Technologies). Specific primers for the housekeeping gene *SvEF-1α* amplified a fragment of the expected size from all samples. A fragment of the expected size was also amplified with specific primers for the plant genes *SvNod1, SvPR10, SvLect3, SvLect5*. *SvNod1* transcripts were found only in RNA extracted from colonized cells (C and CC).



The other 3 plant genes assessed on LMD protocorm cells (*SvPR10*, *SvLect3* and *SvLect5*) were previously found to be upregulated in mycorrhizal protocorm tissues, although upregulation was significant only for the 2 lectin genes.[9] One-step RT-PCR identified the corresponding transcripts in RNA extracted from all protocorm cell types (**Fig. 1**). However, primers to the fungal housekeeping gene *TcEF-1α* suggest the presence of fungal structures not visible at microscopic inspection also in non-colonized cells, where they yielded a weak band (**Fig. 1**). *SvPR10* transcripts were not detectable in all replicates of non-colonised LMD cells, suggesting a low level of expression in this cell type.

Two fungal ESTs coding for putative metalloproteases, *TcMprot1* and *TcMprot2*, were found in mycorrhizal protocorms.[9] Here, we have identified the complete genomic sequence of these transcripts by Blastn search against the *T. calospora* genome (MycoCosm JGI portal, http://genome.jgi.doe.gov/Tulca1/Tulca1.home.html).

A single genomic sequence homologous to an insulin-degrading enzyme (*Fomitiporia mediterranea*, e-value 0.0, 46% identity, 98% coverage), and related to STE23-metalloprotease,[18] showed similarity with *TcMprot2* (Protein ID 16860). *TcMprot2* belongs to the peptidase family M16, and its expression in *S. vomeracea* mycorrhizal protocorms was assessed by RNA-Seq (IGA Technologies, Udine, Italy). No changes in gene expression were found in the mycorrrhizal protocorms, as compared with the free living mycelium (Fold Change = 1.286, *P*-value = 0.340).

Two different genomic sequences, both coding for putative M36 metallopeptidases (Protein ID 246057 and 82347 in **Table 1**), showed similarities with *TcMprot1*. The phylogenetic tree in **Figure 2** confirmed that the corresponding proteins clustered together, inside the M36 metallopeptidase family. M36 metallopeptidases (also known as fungalysins) are known to promote fungal pathogenicity both in animals and in plants.[19,20,21,22] We identified all members of this protein

family in the *T. calospora* genome and assessed their expression in *S. vomeracea* mycorrhizal protocorms. Ten genes coding for putative fungalysins (**Table 1**) were found, that clustered in different phylogenetic groups inside this family (**Fig. 2**). An exception was Protein ID 30548, that clustered on a separate branch, mirroring the fact that no conserved domain was recognized in this sequence. A signal peptide could been recognized in the proteins with sequence ID 246057, 82347, 29547, 77986, 222757, thus indicating that they are secreted. Most *T. calospora* fungalysins did not change their expression in symbiosis, as compared with the free living mycelium (**Table 1**). However, one of the secreted fungalysins (protein ID 29547) was significantly up-regulated in mycorrhizal protocorms, whereas another (protein ID 77986) was significantly down-regulated (**Table 1**).

Table 1. M36 Metallopeptidases in the *Tulasnella calospora* genome. The nearest protein sequence identified by BlastP is indicated, together with the corresponding organism. Conserved domain analysis has been carried out by InterPro and Pfam

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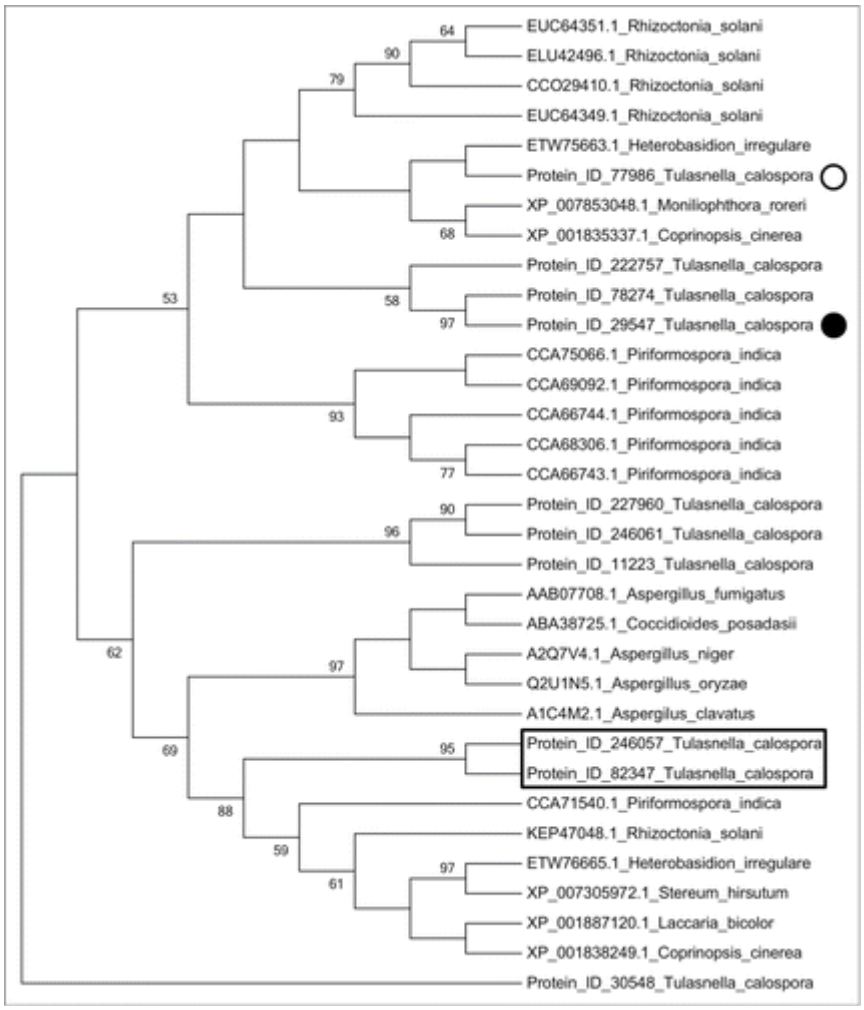
Figure 2. Phylogenetic relationships between fungal M36 metallopeptidases, based on deduced amino acid sequences. The sequences were aligned using Muscle and the unrooted tree was constructed with the maximum likelihood method, using the MEGA software.[28] Numbers indicate bootstrap values, and are indicated only when ≥50%. *T. calospora* sequences corresponding to the EST used in the previous gene expression analysis are boxed.[9] The open circle marks the most downregulated M36 gene, while the closed circle marks the most upregulated M36 gene in symbiotic protocorms.



Fungalysins are a family of zinc metallopeptidases (M36) found in many human and animal pathogens (e.g. *Aspergillus fumigatus, Coccidioides immitis, Cryptococcus neoformans, Microsporum canis,* dermatophytic fungi), where they help fungi during host tissue invasion either because they degrade structural components of the host extracellular matrix,[19,20] or because they degrade immunodominant cell surface antigens, thus escaping host recognition.[23] This gene family has expanded in the Onygenales, that includes several animal pathogens,[24] with a rapid functional divergence of the duplicated proteins. The high number of fungalysin genes identified in *T. calospora* indicates expansion in this endomycorrhizal fungus. Interestingly, expansion of the M36 metallopeptidase family was also reported in other fungi interacting with plants, like the endophytic fungus *Piriformospora indica* and the ectomycorrhizal fungus *Laccaria bicolor*.[25,26]
Few studies have addressed the role of fungalysins in plant-fungal interactions. Gene expression studies in *Colletotrichum graminicola*, an initially biotrophic pathogen that later switches to necrotrophy, showed maximum accumulation of fungalysin mRNA simultaneously with the switch to the necrotrophic lifestyle.[22] In *P. indica,* it has been suggested that fungalysins could be involved in plant tissue degradation during the colonization of dead roots.[25] An interesting function was discovered in the plant pathogen *Fusarium verticillioides*, where a fungalysin was found to truncate 2 maize chitinases, that are part of the plant defense response.[21] As a chitinase was found to be expressed in *S. vomeracea* mycorrhizal protocorms,[9] it would be intriguing to understand whether any of the fungalysins identified in the *T. calospora* genome may have a similar role in orchid mycorrhiza.
In conclusion, we have just started to unravel the molecular bases of plant-fungus interactions in orchid mycorrhiza. More powerful approaches, such as next generation sequencing of plant and fungal transcripts, will be needed to provide a more comprehensive view of this fascinating but still poorly understood mycorrhizal symbiosis.

Disclosure of Potential Conflicts of Interest

Acknowledgments

We are grateful to F. Martin, A. Kohler and the Mycorrhizal Genomics Initiative consortium for access to unpublished genome and transcriptome data. The genome sequence data were produced by the US Department of Energy Joint Genome Institute in collaboration with the user community. We also thank E. Ercole and M. Rodda for their expertise on orchid symbiotic seed germination.

References

1. Smith SE, Read DJ. Mycorrhizal Symbiosis, Academic Press, London, UK; 2008. 800 p.
2. McCormick MK, Jacquemyn H. What constrains the distribution of orchid populations? New Phytol 2014; 202:392-400; http://dx.doi.org/10.1111/nph.12639
3. Smith SE. Carbohydrate translocation in orchid mycorrhizal fungi. New Phytol 1967; 66:371-8; http://dx.doi.org/10.1111/j.1469-8137.1967.tb06016.x
4. Burges A. The defensive mechanism in orchid mycorrhizas. New Phytol 1939; 38:273-83; http://dx.doi.org/10.1111/j.1469-8137.1939.tb07105.x
5. Rasmussen HN. Terrestrial Orchids: From Seed to Mycotrophic Plant. Cambridge: Cambridge University Press; 1995. 312 p.
6. Shimura H, Matsuura M, Takada N, Koda Y. An antifungal compound involved in symbiotic germination of Cypripedium macranthos var. rebunense (Orchidaceae). Phytochemistry 2007; 68:1442-7; PMID: 17445846; http://dx.doi.org/10.1016/j.phytochem.2007.03.006

7. Selosse MA, Roy M. Green plants that feed on fungi: facts and questions about mixotrophy. Trends Plant Sci 2009; 14:64-70; PMID: 19162524; http://dx.doi.org/10.1016/j.tplants

8. Selosse MA, Boullard B, Richardson D. Noël Bernard (1874-1911): orchids to symbiosis in a dozen years, one century ago. Symbiosis 2011; 54: 61-8. http://dx.doi.org/10.1007/s13199-011-0131-5

9. Perotto S, Rodda M, Benetti A, Sillo F, Ercole E, Rodda M. Girlanda M, Murat C, Balestrini R. Gene expression in mycorrhizal orchid protocorms suggests a friendly plant–fungus relationship. Planta 2014; 239:1337-49; PMID: 24760407; http://dx.doi.org/10.1007/s00425-014-2062-x

10. Watkinson JO, Welbaum GE. Characterization of gene expression in roots of Cypripedium parviflorum var. pubescens incubated with a mycorrhizal fungus. Acta Hort 2003; 624:463-70

11. Dearnaley JDW. Further advances in orchid mycorrhizal research. Mycorrhiza 2007; 17:475-86; PMID: 17582535; http://dx.doi.org/10.1007/s00572-007-0138-1

12. Zhao M-M, Zhang G, Zhang D-W, Hsiao Y-Y, Guo S-X. ESTs Analysis reveals putative genes involved in symbiotic seed germination in Dendrobium officinale. PLoS One 2013; 8:e72705; PMID: 23967335; http://dx.doi.org/10.1371/journal.pone.0072705

13. Greene EA, Erard M, Dedieu A, Barke DGB. MtENOD16–20 are members of a family of phytocyanin-related early nodulins. Plant Mol Biol 1998; 36:775-83; PMID: 9526510; http://dx.doi.org/10.1023/A:1005916821224

14. Khan JA, Wang Q, Sjolund RD, Schulz A, Thompson GA. An early nodulin-like protein accumulates in the sieve element plasma membrane of Arabidopsis. Plant Physiol 2007; 143:1576-89; PMID: 17293437; http://dx.doi.org/10.1104/pp.106.092296

15. Frühling M, Albus U, Hohnjec N, Geise G, Pühler A, Perlick AM. A small gene family of broad bean codes for late nodulins containing conserved cysteine clusters. Plant Sci 2000; 152:67-77; http://dx.doi.org/10.1016/S0168-9452(99)00219-8

16. Mashiguchi K, Asami T, Suzuki Y. Genome-wide identification, structure and expression studies, and mutant collection of 22 early nodulin-like protein genes in Arabidopsis. Biosci Biotechnol Biochem 2009; 73:2452-9; PMID: 19897921; http://dx.doi.org/10.1271/bbb.90407

17. Scheres B, van Engelen F, van der Knaap E, van de Wiel C, van Kammen A, Bisseling T. Sequential induction of nodulin gene expression in the developing pea nodule. Plant Cell 1990; 2:687-700; PMID: 2152123; http://dx.doi.org/10.1105/tpc.2.8.687

18. Adames N, Blundell K, Ashby MN, Boone C. Role of yeast insulin-degrading enzyme homologs in propheromone processing and bud site selection. Science 1995; 270:464-7; PMID: 7569998; http://dx.doi.org/10.1126/science.270.5235.464

19. Markaryan A, Morozova I, Yu H, Kolattukudy PE. Purification and characterization of an elastinolytic metalloprotease from Aspergillus fumigatus and immunoelectron microscopic evidence of secretion of this enzyme by the fungus invading the murine lung. Infect Immun 1994; 62:2149-57; PMID: 8188335

20. Brouta F, Descamps F, Monod M, Vermout S, Losson B, Mignon B. Secreted metalloprotease gene family of Microsporum canis. Infect Immun 2002; 70:5676-83; PMID: 12228297; http://dx.doi.org/10.1128/IAI.70.10.5676-5683.2002

21. Naumann TA, Wicklow DT, Price NP. Identification of a chitinase-modifying protein from Fusarium verticillioides: truncation of a host resistance protein by a fungalysin metalloprotease. J Biol Chem 2011; 286:35358-66; PMID: 21878653; http://dx.doi.org/10.1074/jbc.M111.279646

22. Vargas WA, Sanz Martın JM, Rech GE, Rivera LP, Benito EP, Dıaz-Mınguez JM, Thon MR, Sukno SA. Plant defense mechanisms are activated during biotrophic and necrotrophic development of Colletotricum graminicola in maize. Plant Physiol 2012; 158:1342-58; PMID: 22247271; http://dx.doi.org/10.1104/pp.111.190397

23. Hung C-Y, Seshan KR, Yu J-J, Schaller R, Xue J, Basrur V, Gardner MJ, Cole G. A metalloproteinase of Coccidioides posadasii contributes to evasion of host detection. Infect Immun 2005; 73:6689-703; PMID: 16177346; http://dx.doi.org/10.1128/IAI.73.10.6689-6703.2005
24. Li J, Zhang K-Q. Independent expansion of zincin metalloproteinases in Onygenales fungi may be associated with their pathogenicity. PLoS One 2014; 9:e90225; PMID: 24587291; http://dx.doi.org/10.1371/journal.pone.0090225
25. Zuccaro A, Lahrmann U, Guldener U, Langen G, Pfiffi S, Biedenkopf D, Wong P, Samans B, Grimm C, Basiewicz M, Endophytic life strategies decoded by genome and transcriptome analyses of the mutualistic root symbiont Piriformospora indica. PLoS Pathog 2011; 7:e1002290; PMID: 22022265; http://dx.doi.org/10.1371/journal.ppat.1002290
26. Zuccaro A, Lahrmann U, Langen G. Broad compatibility in fungal root symbioses. Curr Op Plant Biol 2014; 20:135-45; PMID: 24929298; http://dx.doi.org/10.1016/j.pbi.2014.05.013
27. Pérez-Tienda J, Testillano PS, Balestrini R, Fiorilli V, Azcón-Aguilar C, Ferrol N. GintAMT2, a new member of the ammonium transporter family in the arbuscular mycorrhizal fungus Glomus intraradices. Fungal Genet Biol 2011; 48:1044-55; PMID: 21907817; http://dx.doi.org/10.1016/j.fgb.2011.08.003
28. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011; 28:2731-9; PMID: 21546353; http://dx.doi.org/10.1093/molbev/msr121