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Identification of genes and proteins involved in the regulation of orchid mycorrhiza

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Thesis presented to obtain the degree of Doctor in Science. Area: Soil Science and Plant Nutrition

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To my grandparents,

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To my parents,

Moacir Valadares and Jane Mar Borges da Silva, for their unconditional love

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EPIGRAPH

"Two roads diverged in a wood, and I,

I took the one less traveled by.

And that has made all the difference"

Robert Frost

CONTENT

RESUMO

Identificação de genes e proteínas envolvidos na regulação de micorrizas de orquídeas

As orquídeas são caracterizadas por produzirem sementes diminutas, que não possuem endosperma. Necessitam, portanto, da interação com fungos micorrízicos para germinação e desenvolvimento do embrião. Algumas orquídeas aclorofiladas se mantêm dependentes dos fungos micorrízicos para a aquisição de carbono, enquanto outras desenvolvem a maquinaria fotossintética. Apesar do significado biológico das micorrizas de orquídeas, alterações na expressão gênica e no acúmulo de proteínas foram altamente negligenciads nos últimos anos. Neste trabalho, foram utilizadas diferentes técnicas sequenciamento e identificação de genes e proteínas em larga-escala para acessar as alterações moleculares responsáveis pela regulação das micorrizas de orquídeas. Uma abordagem baseada em 2D-LC MS/MS acoplada a técnica de quantificação absoluta e relativa iTRAQ, foiutilizada para identificar proteínas com acúmulo diferencial em *Oncidium sphacelatum* em diferentes estágios do desenvolvimento do protocormo (protocormos aclorofilados *versus* protocormos fotossintetizantes), após inoculação com um fungo do gênero *Ceratobasidium*. As análises mostraram que, as alterações esperadas no metabolismo do carbono foram acompanhadas de um acúmulo aumentado de proteínas envolvidas na modulação de espécies reativas de oxigênio, respostas de defesa, biossíntese de fitoalexinas e carotenóides, sugerindo que os protocormos de orquídeas passam por profundas alterações metabolicas durante a transição do metabolismo micoheterotrófico para o fotossintético. Posteriormente foram utilizadas três diferentes técnicas de proteômica quantitativa para explorar alterações fisiológicas em raízes micorrizadas e não-micorrizadas de *Oeceoclades maculata*.Este estudo foi ampliado, pela utilização de uma abordagem transcritômica ao mesmo modelo biológico. Em conjunto, os dados revelaram um forte aumento em respostas relacionadas ao estresse, acompanhadas de alterações em vias de transdução de sinal possivelmente relacionadas ao reconhecimento do simbionte fúngico e estabelecimento de uma interação compatível. Alguns genes com expressão aumentada devem estar envolvidos na reorganização celular, provavelmente ligada a acomodação do simbionte fúngico nas raízes das plantas. Também foi observado o aumento de genes envolvidos no metabolismo do carbono e de açúcares aminados, juntamente a genes relacionados a assimilação de nitrogênio em raízes micorrizadas. A expressão diminuída de genes envolvidas nas vias do jasmonato e ácido abscícico, juntamente a genes-chave que codificam para proteínas anti-fúngicas sugerem fortemente uma atenuação das respostas de defesa da planta em raízes micorrizadas de *Oeceoclades maculata*. No geral, parece que as micorrizas de orquídeas são fisiológicamente mais próximas de uma simbiose compatível do que de uma interação unilateral em favor da planta. Sobretudo, este sistema biológico provou ser promissor para investigação de interações planta-fungo e, próximas pesquisas devem agora ser focadas em funções específicas dos genes que mostraram regulação diferencial neste estudo.

Palavras-chave: Simbiose; Proteômica; Transcritômica; Interação-planta-micro-organismo; Genes de defesa vegetal

ABSTRACT

Identification of genes and proteins involved in the regulation of orchid mycorrhiza

Orchids are characterized by producing minute endosperm-lacking seeds, which depend on mycorrhizal fungi for germination and embryo development. Some aclorophyllous orchids remain dependent on the mycorrhizal association for carbon acquisition during their whole life history, whereasother orchids develop photosynthesis. Despite the biological significance of orchid mycorrhiza, gene expression studies are lacking. We have used different high-throughput approaches in order to understanding the mechanisms regulating orchid mycorrhiza development and functioning. Firstly, we have used a 2D-LC-MS/MS approach coupled to isobaric tagging for relative and absolute quantification (iTRAQ) to identify proteins with differential accumulation in *Oncidium sphacelatum* at different stages of mycorrhizal protocorm development (achlorophyllous and green protocorms) after seed inoculation with a *Ceratobasidium* sp. isolate. Quantitative analysis showed that the expected changes in carbon metabolism in green protocorms were accompanied by enhanced accumulation of proteins involved in the modulation of reactive oxygen species homeostasis, defense related responses, phytoalexins and carotenoid biosynthesis, suggesting that orchid protocorms undergo profound metabolic changes during the switch from the fully mycoheterotrophic to the photosynthethic stage. Secondly, three different proteomic techniques were carried out in independent experiments aiming to identify changes in protein accumulation in mycorrhizal roots of the terrestrial orchid *Oeceoclades maculata*.Finally, *O. maculata*mycorrhizal roots were used for transcriptome analyses. The data revealed a strong increase in general stress responses, accompanied by changes in signaling pathways possibly related to fungal recognition and establishment of a compatible interaction. Some of the up-regulated genes may be involved in the reorganization of cell structure, likely related to accommodation of the fungal symbiont in the plant roots. We have also observed in mycorrhizal roots up-regulation of genes involved in carbon metabolism, including glycolysis/gluconeogenesis and amino sugars metabolism, as well as genes involved innitrogen assimilation. The down-regulation of genes involved in the jasmonate and ABA transduction pathways, and key genes encoding anti-fungal proteins, such as chitinase and a mannose-specific binding lectin, strongly suggests an alleviation of plant defense responses in *O. maculata* mycorrhizal roots. In general, our data suggest that the physiology of an orchid mycorrhiza is more similar to a compatible interaction than to an arm-race between plant and fungi. Overall orchid mycorrhiza have proved to be a promising model for investigating plant-fungal interactions and further studies should now address the specific roles of the genes showing differential regulation in this study.

Keywords: Symbiosis; Proteomics; Transcriptomics; Plant-microbe interactions; Plant defense-genes

1 INTRODUCTION

The symbioses between plant roots and mycorrhizal fungi are the most common interaction between plant and microrganisms (PARNISKE, 2008). The physiological regulation of such interaction is a complex process that involves cross-talking mechanisms between organisms, in order to achieve the nutritional demands of both symbionts (GENRE; BONFANTE, 2010; GOMEZ; HARRISON, 2009; KOGEL; FRANKEN; HU, 2006; REINHARDT, 2007). In this context, the relationship between orchids and their mycorrhizal fungi is unique: regardless of their carbon nutrition at adult stage, all orchids produce minute, endosperm-lacking seeds and are dependent on fungal colonization for germination and growth into an heterotrophic, achlorophyllous stage called protocorm (RASMUSSEN, 2002). A great part of orchid species, even being photosynthetic during the adult stage, keep high colonization rates during their whole life cycle, specially the terrestrial and rock dwelling ones (VALADARES et al., 2012).

For many years it was acknowledged that orchids interact mostly, if not only, with members of the ‘rhizoctonia’ complex. This assemblage contains a polyphyletic grouping of three unrelated basidiomycetous genera: Sebacinales, Ceratobasidiaceae and Tulasnellaceae (DEARNALEY, 2007). Recent research has highlighted the diverse ecology of these three ‘rhizoctonia’ taxa (CHEN; WANG; GUO, 2011; GIRLANDA et al., 2011; OTERO; BAYMAN; ACKERMAN, 2005; RASMUSSEN, 2002; ZETTLER; HOFER, 1998). While some species are known to be parasitic, such as in the Ceratobasidiaceae, or are suspected to be saprotrophic (GONZÁLES-GARCÍA; ONCO; SUSAN, 2006), this classical view is now challenged for at least some species. Sebacinales encompasses two major groups that occur as endophytes in the roots of many plant species and part of them are able to form ectomycorrhizal association on trees whilst forming orchid mycorrhiza (OM) with mycoheterotrophic orchids (SELOSSE; ROY, 2009).

Environmental fungi colonize through embryo suspensor tissues or epidermal hairs and enter the cortical cells (PETERSON; UETAKE; ZELMER, 1998). Colonizing fungal hyphae do not breach the cortical cell membrane but ramify in the space between cell wall and membrane, forming elaborate coiled structures known as pelotons (RASMUSSEN, 2002). Pelotons provide the greatest part, if not all the energy demanded for orchid germination and, during the adult stage, they can be used as supplementary source of carbon, nutrients and water (DEARNALEY, 2007). The major part of nutrient flux probably occurs when the

peloton is digested; however, nutrient exchange between symbionts may also occur when peloton surface is still intact (Y. UETAKE, personal communication). The main feature that differentiates both symbioses is the direction of nutrient flow between partners. Whilst it is acknowledged that, in arbuscular mycorrhiza, the fungi transport mineral nutrients to the plant and receives photoassimilates in return, nutrient exchange between orchids and their mycorrhizal fungi is still a matter of discussion (CAMERON; LEAKE; READ, 2006; RASMUSSEN; RASMUSSEN, 2009).

The mechanisms by which orchids might identify, attract and select their fungi, regulate intracellular hyphal proliferation and accomodate the fungal symbiont, leading to a compatible interaction are largely unknown (RASMUSSEN; RASMUSSEN, 2009), but it is likely that similar events to those occurring in other plant-fungal interactions also occurs in orchid mycorrhiza. These must include the recognition of an elicitor, signal transduction, changes in cell architecture and, finally, the modulation of biotic stress and defense responses, just as it occurs in other types of endomycorrhiza (BONFANTE; REQUENA, 2011).

Enzymatic assays involving orchid mycorrhiza were performed firstly by Blakeman *et al*. (1976). In this work, the activity of poliphenol oxidase, ascorbic acid oxidase and catalase were measured in *Dactylorhiza purpurella* protocorms and a *Cymbidium* hybrid. The authors observed that these enzymes had enhanced activity in symbiotic protocorms (comparing to assymbiotic ones) and that their peak activity usually coincided with the peak of respiration and peloton digestion, except for a peroxidases, which was more active in the first contact with mycorrhizal fungi. Salomé-Pais and Barroso (1983) used cytochemical localization to detect poliphenol oxidase activities in *Ophrys lutea*, during the intra-cellular colonization with *Rhizoctonia repens.* They found large amounts of the product in fungal cytoplasm during the first steps of colonization and, subsequently this great part of molecules were found in the plant-fungal interface, suggesting that these fungal-derived enzymes could act in fungal protection against phenolic compounds produced by the host plant.

Still concerning poliphenol metabolism, Beyrle et al. (1995) observed that the activity of phenylalanine ammonia lyase (PAL) and the concentration of the phytoalexin orchinol were higher in symbiotic protocorms of *Anacamptis*(*Orchis*) *morio,* as compared to non-mycorrhizal protocorms, suggesting a role of the plant defense system in OM regulation. Similarly, the enhanced biosynthesis of the phytoalexins has also been observed in orchid protocorms during symbiotic germination with a non-pathogenic *Rhizoctonia* (SHIMURA et al., 2007).

It is noteworthy that, despite all biological significance involving OM, gene expression studies have been largely neglected over the years (DEARNALEY, 2007). Watkinson and Welbaum (2003) have addressed this topic by using the amplification fragment length polymorphism (AFLP). Unfortunately, the greatest part of differentialy expressed fragments were identified as ribossomal genes and only two coding sequences were identified in that oportunity, being nucleotide binding protein (up-regulated) and a trehalose-phosphate phosphatase (down-regulated). Only recently, Zhao et al. (2013) used suppression subtractive hybridization (SSH) to identify genes expressed in symbiotically germinated seeds of *Dendrobium officinale*. However, with the recent advances in sequences techniques(WANG; GERSTEIN; SNYDER, 2009)), it is now possible to yield a very large number of gene sequences, identify and predict their role in symbiosis through semi-automated biological function and cellular location prediction tools (ASHBURNER et al., 2000). Proteomic techniques have been also successfully used to investigate plant response to arbuscular mycorrhizal fungi (SCHENKLUHN et al., 2010) but proteome changes in orchid mycorrhiza have never been adressed.

Understanding the regulation of OMhas broad ecological, scientific and biotechnological implications. OM are essential for orchid propagation and survival in natural ecosystems (BATTY et al., 2006), since it has similarities with either pathogenic or mutualistic plant-fungal interactions (RASMUSSEN; RASMUSSEN, 2009), and orchid mechanisms of fungal control could be target of new products discovery for agroindustry (WANG et al., 2001). By studying molecular changes in the OM process, it will be possible to infer if this symbiosis is closer to a mutualistic interaction or if the hypothesis of an ‘arm-race’ between symbionts (RASMUSSEN; RASMUSSEN, 2009)is supported. By understanding how orchids and fungi are able to form a partnership,where orchidsare the dominant partner, it will be possible to assess the evolution of plant fungal interactions, gaining greater insights into symbiotic and pathogenic interactions.

We have used state-of-the-art proteomic and transcriptomic high-throughput techniques in order to address changes in plant metabolism in response to mycorrhizal fungi: In the first chapter we show changes in *Oncidium sphacelatum* proteome during different trophic stage of its symbiotic germination. Proteins were isolated from dark-grown protocorms and subsequently from green protocorms, to represent the transition from the fully mycoheterotrophic metabolism to photosynthesis that occurs during orchid germination. Proteins were quantified and molecular changes were analyzed with emphasis on changes in

primary metabolism, redox homeostasis and secondary metabolism. In the second chapter we have used three different proteomic techniques to address changes in the proteome of *Oeceoclades maculata* within its mycorrhizal and non-mycorrhizal roots. Finally, in the last chapter, we expanded our analysis by using deep sequencing of the transcriptome (RNAseq) on the same biological model used before.

References

ASHBURNER, M. BALL, C.A.; BLAKE, J.A.; BOTSTEIN, D.; BUTLER, H.; CHERRY, J.M.; DAVIS, A.P.; DOLINSKI, K.; DWIGHT, S.S.; EPPIG, J.T.; HARRIS, M.A.; HILL, D.P.; ISSEL-TARVER, L.; KASARSKIS, A.; LEWIS, S.; MATESE, J.C.; RICHARDSON, J.E.; RUBIN, G.M.; SHERLOCK, G. Gene ontology: tool for the unification of biology. **Nature Genetics**, New York, v. 25, n. 1, p. 25–29, 2000.

BATTY, A.L.; BRUNDRETT, M.C.; DIXON, K.W.; SIVASITHAMPARAM, K. New methods to improve symbiotic propagation of temperate terrestrial orchid seedlings from axenic culture to soil. **Australian Journal of Botany,** Melbourne**,** v. 54, n. 4, p. 367-374, 2006.

BEYRLE, H.F.; SMITH, S.E.; PETERSON, R.L.; FRANCO, C.M.M. Colonization of *Orchis morio* protocorms by a mycorrhizal fungus: effects of nitrogen nutrition and glyphosate in modifying the responses. **Canadian Journal of Botany,** Ottawa, v. 73, p.1128–1140, 1995.

BLAKEMAN, J.P.; MOKAHEL, M.A.; HADLEY, G. Effect of mycorrhizal infection on respiration and activity of some oxidase enzymes of orchid protocorms. **New Phytologist,** Cambridge, v. 77, n. 3, p. 697–704, 1976.

BONFANTE, P.; REQUENA, N. Dating in the dark: how roots respond to fungal signals to establish arbuscular mycorrhizal symbiosis. **Current Opinion in Plant Biology**, London, v. 14, n. 4, p. 451–457, 2011.

CAMERON, D.D.; LEAKE, J.R.; READ, D.J. Mutualistic mycorrhiza in orchids: evidence from plant-fungus carbon and nitrogen transfers in the green-leaved terrestrial orchid *Goodyera repens*. **New Phytologist**, Cambridge, v. 171, n. 2, p. 405–416, 2006.

CHEN, J.; WANG, H.; GUO, S.-X. Isolation and identification of endophytic and mycorrhizal fungi from seeds and roots of *Dendrobium* (Orchidaceae). **Mycorrhiza**, Berlin, v. 22, n. 4, 297-307, 2011.

DEARNALEY, J.D.W. Further advances in orchid mycorrhizal research. **Mycorrhiza**, Berlin, v. 17, p. 475–486, 2007.

GENRE, A.; BONFANTE, P. The making of symbiotic cells in arbuscular mycorrhizal roots. In: KOLTAI, H.; KAPULNIK, Y. (Ed.). **Arbuscular mycorrhizas:** physiology and function. New York: Springer–Verlag, 2010. p. 57-71.

GIRLANDA, M.; SEGRETO, R.; CAFASSO, D.; LIEBEL, H.K.; RODDA, M.; ERCOLE, E.; COZZOLINO, S.; GEBAUER, G.; PEROTTO, S. Photosynthetic Mediterranean meadow orchids feature partial mycoheterotrophy and specific mycorrhizal associations. **American Journal of Botany**, Columbus, v. 98, n. 7, p. 1148–1163, 2011.

GOMEZ, S.K.; HARRISON, M.J. Laser microdissection and its application to analyze gene expression in arbuscular mycorrhizal symbiosis. **Pest Management Science**, Sussex, v. 65, n. 5, p. 504–511, 2009.

GONZÁLES-GARCÍA, V.; ONCO, M.A.P.; SUSAN, V.R. Review. Biology and systematics of the form genus Rhizoctonia. **Spanish Journal of Agricultural Research**, Madrid, v. 4, n. 1, p. 55–79, 2006.

KOGEL, K.H.; FRANKEN, P.; HÜCKELHOVEN, R. Endophyte or parasite – what decides? **Current Opinion in Plant Biology**, Saint Louis,v. 9, n. 4, p. 358–363, 2006.

OTERO, J.T.; BAYMAN, P.; ACKERMAN, J.D. Variation in mycorrhizal performance in the epiphytic orchid *Tolumnia variegata in vitro*: the potential for natural selection. **Evolutionary Ecology**, London, v. 19, p. 29–43, 2005.

PARNISKE, M. Arbuscular mycorrhiza: the mother of plant root endosymbioses. **Nature Reviews**, New York, v. 6, n. 10, p. 763–775, 2008.

PETERSON, R.L.; UETAKE, Y.; ZELMER, C. Fungal symbiosis with orchid protocorms. **Symbiosis**, Rehovot, v. 25, n. 1/3, p. 29–55, 1998.

RASMUSSEN, H.N. Recent developments in the study of orchid mycorrhiza. **Plant and Soil**, The Hague, v. 244, p. 149–163, 2002.

RASMUSSEN, H.N.; RASMUSSEN, F.N. Orchid mycorrhiza: implications of a mycophagous life style. **Oikos**, Copenhagen, v. 118, n. 3, p. 334–345, 2009.

REINHARDT, D. Programming good relations-development of the arbuscular mycorrhizal symbiosis. **Current Opinion in Plant Biology**, Saint Louis,v. 10, n. 1, p. 98–105, 2007.

SALOMÉ-PAIS, M.; BARROSO, J. Localization of polyphenol oxidases during establishment of *Ohrys lutea* endomycorrhizas. **New Phytologist**, Cambridge, v. 95, p. 219-222, 1983.

SCHENKLUHN, L.; HOHNJEC, N.; NIEHAUS, K.; SCHMITZ, U.; COLDITZ, F. Differential gel electrophoresis (DIGE) to quantitatively monitor early symbiosis- and pathogenesis-induced changes of the *Medicago truncatula* root proteome. **Journal of Proteomics**, Valencia,v. 73, n. 4, p. 753–768, 2010.

SELOSSE, M.A.; ROY, M. Green plants that feed on fungi: facts and questions about mixotrophy. **Trends in Plant Science**, Oxford, v. 14, n. 2, p. 64–70, 2009.

SHIMURA, H.; MATSUURA, M.; TAKADA, N.; KODA, Y. An antifungal compound involved in symbiotic germination of *Cypripedium macranthos* var. *rebunense* (Orchidaceae). **Phytochemistry**, Oxford, v. 68, n. 10, p. 1442–1447, 2007.

VALADARES, R.B.S.; PEREIRA M.C.; OTERO, J.T. CARDOSO, E.J.B.N. Narrow fungal mycorrhizal diversity in a population of the orchid *Coppensia doniana*. **Biotropica**, Lawrence, v. 44, n. 1, p. 114–122, 2012.

WANG, X.; WANG, X.; BAUW, G.; VAN DAMME, E.J.; PEUMANS, W.J.; CHEN, Z.L.; VAN MONTAGU, M.; ANGENON, G.; DILLEN, W. Gastrodianin-like mannose-binding proteins: a novel class of plant proteins with antifungal properties. **Plant Journal: for Cell and Molecular Biology**, Oxford, v. 25, n. 6, p. 651–661, 2001.

WANG, Z.; GERSTEIN, M.; SNYDER, M. RNA-Seq: a revolutionary tool for transcriptomics. **Nature Reviews**, New York, v. 10, n. 1, p 57-63, 2009.

WATKINSON, J.I.; WELBAUM, G.E. Characterization of gene expression in roots of *Cypripedium parviflorum* var. *pubescens* incubated with a mycorrhizal fungus. **Acta Horticulturae**, The Hague, v. 624, p. 463–470, 2003.

ZETTLER, L.W.; HOFER, C.J. Propagation of the little club-spur orchid (*Platanthera clavellata*) by symbiotic seed germination and its ecological implications. **Environmental and Experimental Botany**, Elmsford, v. 39, p. 189-195, 1998.

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,**London, v. 8, n. 8, e72705, 2013.

2 PROTEOME CHANGES IN *Oncidium sphacelatum* (ORCHIDACEAE) AT DIFFERENT TROPHIC STAGES OF SYMBIOTIC GERMINATION

Abstract

Mutualistic symbioses between plants and fungi are a widespread phenomenon in nature. Particularly in orchids, association with symbiotic fungi is required for seed germination and seedling development. During the initial stages of symbiotic germination, before the onset of photosynthesis, orchid protocorms are fully mycoheterotrophic. The molecular mechanisms involved in orchid symbiotic germination and development are largely unknown, but it is likely that changes in plant energy metabolism and defense related responses play a central role in these processes. We have used 2D-LC-MS/MS coupled to isobaric tagging for relative and absolute quantification (iTRAQ) to identify proteins with differential accumulation in *Oncidium sphacelatum* at different stages of mycorrhizal protocorm development (achlorophyllous and green protocorms) after seed inoculation with a *Ceratobasidium* sp. isolate. We identified and quantified 88 proteins, including proteins putatively involved in energy metabolism, cell rescue and defense, molecular signaling and secondary metabolism. Quantitative analysis showed that the expected changes in carbon metabolism in green protocorms were accompanied by enhanced accumulation of proteins involved in the modulation of reactive oxygen species homeostasis, defense related responses, and phytoalexins and carotenoid biosynthesis. Our results suggest profound metabolic changes in orchid protocorms during the switch from the fully mycoheterotrophic to the photosynthethic stage. Part of these changes may be also related to the obligatory nature of the interaction with the endomycorrhizal fungus.

Keywords:Orchid mycorrhiza; iTRAQ; Proteomics; Symbiosis

2.1 Introduction

The Orchidaceae is one of the most diverse plant families and is comprised of more than 27,000 accepted species (THE PLANT LIST, 2010), representing roughly 10% of all flowering plant species. In nature, orchids are fully mycoheterotrophic (dependent on symbiotic fungi for carbon supply) during the achlorophyllous protocorm stage that follows seed germination (RASMUSSEN; RASMUSSEN, 2009). Although some orchid species remain achlorophyllous, and therefore mycoheterotrophic, throughout their life cycle (RASMUSSEN; RASMUSSEN, 2009), protocorms exposed to light usually develop photosynthetic tissues. Green protocorms are likely mixotrophic, assimilating carbon derived from both photosynthesis and mycorrhizal symbiosis (SELOSSE; ROY, 2009).

Photosynthetic orchids form endomycorrhiza mainly with heterobasidiomycetes that were previously related to *Rhizoctonia*, a polyphyletic anamorphic genus with a variety of teleomorphs belonging to Sebacinaceae, Tulasnellaceae and Ceratobasidiaceae. The *Rhizoctonia* form-genus may also include plant pathogens, saprotrophs and ectomycorrhizal fungi (GONZÁLES-GARCÍA; ONCO; SUSAN, 2006). Orchid mycorrhiza (OM) are characterized by the presence of hyphal coils (pelotons) within the parenchyma orchid cells, which are surrounded by the plant plasma membrane (PETERSON et al., 1996).

The mechanisms underlying nutrient flow between the two symbionts are still unknown. Although it has been suggested that enzymatic degradation of pelotons is necessary for nutrient acquisition by the host (RASMUSSEN; RASMUSSEN, 2007,2009), some studies indicate the occurrence of nutrient exchange through intact cell membranes (see in DEARNALEY et al., 2012). During the achlorophyllous stages of their life cycle, orchids receive mostly C from the mycobiont, even though phosphorus (P), nitrogen (N) and water may also be transferred from the fungus to the plant (CAMERON et al., 2007; PETERSON; UETAKE; ZELMER, 1998; RASMUSSEN; RASMUSSEN, 2007). Despite the evidence of carbon © transfer from plant to fungus in photosynthetic orchids (CAMERON et al., 2008; CAMERON; LEAKE; READ, 2006), the benefit of the symbiosis for the fungus is still a matter of discussion (RASMUSSEN; RASMUSSEN, 2009).

Enhanced activity of anti-oxidant enzymes in symbiotic orchid protocorms has been previously reported. For example, polyphenol oxidase, ascorbic acid oxidase, peroxidase and catalase activities were greatly increased in symbiotic protocorms of *Dactylorhiza purpurella* and of a *Cymbidium* hybrid (BLAKEMAN; MOKAHEL; HADLEY, 1976). Cytochemical localization of polyphenol oxidases in *Ophrys lutea* colonized by *Rhizoctonia repens* showed higher levels of activity in the fungal cytoplasm during the first stages of colonization, and at the plant-fungus interface during later stages of symbiosis development (SALOMÈ-PAIS; BARROSO, 1983). It has been suggested that polyphenol oxidases in OM may be of fungal origin and could protect the hyphae at the plant-fungus interface.

Beyrle et al. (1995) observed that the activity of phenylalanine ammonia lyase (PAL) and the concentration of the phytoalexin orchinol were higher in symbiotic protocorms of *Anacamptis*(*Orchis*) *morio,* as compared to non-mycorrhizal protocorms, suggesting a role of the plant defense system in OM regulation. Similarly, the enhanced biosynthesis of the phytoalexins lusianthrin and chrysin has also been observed in orchid protocorms during symbiotic germination with non-pathogenic *Rhizoctonia* (SHIMURA et al., 2007). Orchid

phytoalexins may inhibit the growth of mycorrhizal fungi *in vitro* (FISCH et al., 1973; SHIMURAet al., 2007), and are likely involved in restraining fungal growth in the plant tissues.

More recently, Zhao et al. (2013) used suppression subtractive hybridization (SSH) to identify genes expressed in symbiotically germinated seeds of *Dendrobium officinale*. Comparing symbiotic germinated protocorms with non-germinated seeds, this work provided the first global overview of genes putatively involved in orchid symbiotic seed germination. However additional studies for determining changes in gene expression, protein or metabolite accumulation during protocorm development are essential for better understanding both OM regulation and the metabolic changes occurring as the protocorm acquires photosynthetic capabilities. As a model system, we used achlorophyllous (*i.e.*, mycoheterotrophic) and green (*i.e.*, mixotrophic or photoautotrophic) protocorms of *Oncidium sphacelatum*, germinated with a compatible *Ceratobasidium* sp. isolate, to identify proteins with differential accumulation in orchid symbiotic seed development, using a quantitative shotgun proteomic approach.

2.2 Material and Methods

Seeds sampling and storage

Seeds were collected in the orchidarium of the Genetics Department of the University of São Paulo (Piracicaba, Brazil). Each mature fruit was surface sterilized in 70% ethanol and rinsed with distilled water. Seeds were stored in filter paper packets inside glass flasks containing $CaCl_2$, at 4°C for approximately two months.

Symbiotic germination and growth conditions

One fruit of *Oncidium sphacelatum* was used for our study. Approximately 400 µg of seeds were surface sterilized by immersion in sodium hypochlorite 20% for 8 minutes, rinsed (4 times in sterile distilled water) and placed in 50 ml of sterile distilled water. Three aliquots of 200 µL of the seed suspension were dispensed onto oatmeal agar plates (5% oat meal and 7.5% agar) containing a 9mm plug of a colony of *Ceratobasidium sp.* (BNR MType2*;* GI:260181603), previously isolated and described by (VALADARES et al., 2012). Plates were sealed with plastic film and incubated in the darkness at 26 °C.

After 50 days, approximately 1.5 g of achlorophyllous protocorms at stage 3 (OTERO; BAYMAN; ACKERMAN, 2005; ZETTLER; HOFER, 1998) were removed from the plates, frozen in liquid nitrogen and stored in microtubes at -80 °C (sample 1). The remaining protocorms were further incubated for additional 60 days under a photoperiod of 12/12 hour (light/dark), and approximately 5.0 g of fresh material was collected (sample 2). At this stage, all protocorms were fully green and the first leaves were developing.

Protein extraction and analyses

Samples were ground to a fine powder in liquid nitrogen and resuspended in extraction buffer containing 50 mM Tris-HCl, 10% sucrose, 2 mM DTT, 4 mM EDTA, 0.1% Brij-58 (Sigma-Aldrich, St. Louis, MO, USA), 0.1 mM PMSF and 0.1% protease inhibitor cocktail (P2714, Sigma-Aldrich, St. Louis, MO, USA). Samples were then subjected to seven consecutive freezing/thawing cycles by immersion in liquid nitrogen and 37 °C water bath, and were finally sonicated for 30 seconds. Proteins were precipitated overnight in 3 volumes of acetone at -20 °C and solubilized in 0.1 mM Tris-HCl pH 7.6. Proteins were quantified using a Qubit® fluorimeter (Qiagen, Gaithersburg, MD, USA).

Protein extracts were subjected to reduction and cysteine blocking and digestion with trypsin. Peptides were tagged with isobaric tags using iTRAQ^{tm} reagents (Applied Biosystems, São Paulo, Brazil), according to the manufacturer's instructions. Peptides purified from achlorophyllous protocorms (sample 1) were tagged with isobaric reagents mass 116 Da, whereas proteins from green protocorms (sample 2) were tagged with isobaric reagents mass 117 Da.

Mass spectrometry and bioinformatics

Peptide samples were separated in 5 fractions using an off-line Sep-Pak Light cartridge (Waters Inc., Milford, MA, USA) in order to increase polar organic content, according to the manufacturer's instructions. Peptides in each fraction were then separated by Ultra Performance Liquid Chromatography (UPLC) using a C18 reverse-phase pre-column (SentryTM Guard, Waters Inc., Milford, MA, USA) and a BEH 130-C18 column (1.7 μm particles, 100 μm id × 100 mm, Waters Inc., Milford, MA, USA), and eluted from the second column using a gradient of 5% acetonitrile/water (v/v) containing 0.1% formic acid (v/v) and 95% acetonitrile/water (v/v) containing 0.1% formic acid (v/v). Samples were analyzed using a quadrupole time-of-flight mass spectrometer (QTOF MS) (Synapt, Waters Inc., Milford,

MA, USA). The flow rate was 5 µL min^{-1} during the first 15 min, 2 µL min^{-1} between 15 and 40 min and 5 µL min^{-1} during the last five minutes. The spectra were acquired in the MS/MS mode in the 50-2000 m/z range. A green fluorescent protein (GFP) peptide was used as standard to correct variations in equipment calibration. All parameters were defined using the Mass Lynx v 2.1 software (Waters® Mass-Lynx, Waters Inc., Milford, MA, USA).

Database search was performed using the software MassMatrix v. 2.4.0 (XU; FREITAS, 2009). Fixed modifications were: iTRAQ-N, iTRAQ-K, and Methylthio. Variable modifications were: iTRAQ-Y, methionin oxidation and deamidation. Precursor mass and fragment mass tolerance were 1.4 and 0.8 Da, respectively. The probability identity threshold was set to $p<0.05$ and the relative abundance probability was set to $p<0.001$.

The peptide dataset was compared to a custom database restricted to *Oncidium* (Uniprot/ Swissprot, 2012-01-27), and the non-identified peptides were compared to *Orchidaceae* (Uniprot/ Swissprot, 2011-07-24) and Viridiiplantae databases (Uniprot/Swissprot, 2011-05-31). Sorting of the redundant proteins was performed manually, within and between the results of each search. Relative quantification was also performed using MassMatrix software v. 2.4.0 with the quantification method set to iTRAQ 4-plex and statistical quantification to arithmetic mean. Protein abundance was normalized to overcome quantification problems imposed by the strong presence of ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCo) in the sample 2 (exposed to light), according to Herbrich et al. (2013), *i.e.*, [new ratio for protein i] = [ratio for protein i] / [median of all ratios]. Proteins were functionally classified according to the MIPS functional catalogue database (Ruepp *et al*. 2004; Recorbet *et al*. 2009). Metabolic pathways were drawn using the software yED 3.10.2 (<http://www.yworks.com>); enzymes abbreviations followed the International Union of Biochemistry and Molecular Biology (http://www.chem.qmul.ac.uk/iubmb/).

2.3 Results and Discussion

Proteins were extracted from symbiotic *Oncidium sphacelatum* protocorms at two different stages of development: achlorophyllous protocorms kept in complete darkness and harvested 50 days post inoculation, representing a fully mycoheterotrophic stage, and light exposed protocorms, harvested 110 days post inoculation. The development of green tissues in the second stage suggests the onset of photosynthesis, and these protocorms may represent a mixotrophic or photoautotrophic stage. After purification and digestion, peptides from the

two developmental stages were labeled with iTRAQ reagents, fractionated by LC and analyzed by tandem mass spectrometry. From the 2,166 tandem MS spectra obtained, 724 showed matches to known peptides (33%). Using the MassMatrix algorithm, we were able to identify 267 proteins, when comparing to the orchids database (2,136 entries), and additional 15 proteins when searching against the Viridiiplantae database. This set of 282 proteins was manually verified, according to the nomenclature and parsimony criteria described by Nesvizhskii and Aebersold (2005), in order to exclude redundancies, resulting in a final set of 88 unique proteins.

The identified proteins and their relative abundances, sequence coverage and molecular weights are shown in Table 1. Proteins mirroring changes in carbon metabolism in the protocorm samples are expected to be among the most up- or down-regulated proteins in Table 1. Proteins that may have important roles in OM regulation, as part of key processes involved in C fluxes or in plant-fungus interaction are also expected to be represented in this list.

According to the MIPS functional categorization, “Metabolism” was the most represented category (28.4%), followed by "Energy" (22.7%), "Cell cycle and DNA processing" (13.6%), "Cell rescue and defense" (11.4%), "Protein synthesis and fate" (12.5%), "Signaling" (9.1%) and "Cell wall modification" (2.3%). On the basis of previous proteomic studies, proteins were considered to have suppressed or induced accumulation when their iTRAQ ratios were < 0.8 or > 1.2, respectively (HILL et al., 2008). Assuming these thresholds, 52 proteins (59% of the dataset) showed differential accumulation under the conditions of our study, with 27 being more abundant in mycoheterotrophic protocorms and 25 with enhanced accumulation in green protocorms.

Table 2.1 - Proteins identified in the symbiotic protocorms of*Oncidium sphacelatum* in association with a *Ceratobasidium* sp. isolate using MassMatrix software($p< 0.05$). 117/116 Ratiorepresent the relative abundance of the two iTRAQ tags used to label proteins extracted from green (iTRAQ 117) and achlorophyllous (iTRAQ 116) *O. sphacelatum* protocorms

(continues)

Most similar acession	Organism	Description	Score	Coverage	# of Peptides	Mass (Monoisotopic)	117/116 Ratio
		Cell cycle and DNA processing					
G2XK93	*Galeola falconeri*	B-class MADS-box protein AP3-1	65	37%	8	29.282	1.03
G2XKA8	*Oncidium hybrid cultivar*	B-class MADS-box protein AP3-4 (Fragment)	22	25%	5	26.517	0.91
F2VJ32	*Colmanara hybrid cultivar*	DNA-directed RNA polymerase subunit alpha	47	26%	9	36.861	1.04
D9IFM3	*Oncidium Gower Ramsey*	MADS box transcription factor	27	26%	9	30.545	1.09
D9IFM1	*Oncidium Gower Ramsey*	MADS box transcription factor 1	20	14%	4	30.369	**0.78**
D9IFM4	*Oncidium Gower Ramsey*	MADS box transcription factor 10	32	16%	9	31.029	**0.75**
D9IFL8	*Oncidium Gower Ramsey*	MADS box transcription factor 5	19	26%	8	29.545	**0.77**
D9IFM0	*Oncidium Gower Ramsey*	MADS box transcription factor 8	50	26%	7	27.986	**2.03**
D9IFL9	*Oncidium Gower Ramsey*	MADS box transcription factor 9	42	11%	5	28.293	1.12
A1XJ22	*Oncidium sphacelatum*	Maturase (Fragment)	123	18%	15	72.557	**0.70**
Q84U45	*Dendrobium sp.*	MYB9	68	15%	3	18.872	**0.71**
		Cell wall metabolism					
B6V9S5	*Oncidium Gower Ramsey*	Pectinesterase	60	23%	13	61.507	**1.20**
P85413	*Phoenix dactylifera*	alpha 1,4 glucan protein synthase	16	17%	2	29.359	**3.51**
		Cell rescue and defense					
B8XF08	*Oncidium Gower Ramsey*	Ascorbate peroxidase	25	4%	2	30.011	**1.51**
P18122	*Zea mays*	Catalase	117	28%	18	60.857	0.92
Q9SES7	*Hordeum vulgare*	Glutathione-S-transferase	139	47%	14	25.040	0.86
O65719	*Oryza sativa*	Heat shock 70Kda	140	10%	5	71.288	**1.98**
Q0J4P2	*Oryza sativa*	Heat shock 81 Kda	98	7%	5	80.197	**1.92**

Table 2.1 -Proteins identified in the symbiotic protocorms of *Oncidium sphacelatum* in association with a *Ceratobasidium* sp. isolate using MassMatrix software ($p < 0.05$). 117/116 Ratiorepresent the relative abundance of the two iTRAQ tags used to label proteins extracted from green (iTRAQ 117) and achlorophyllous (iTRAQ 116) *O. sphacelatum* protocorms

(continuation)

Most similar acession	Organism	Description	Score	Coverage	# of Peptides	Mass (Monoisotopic)	117/116 Ratio
P84882	*Bauhinia rufa*	Kunitz-type trypsin inhibitor	16	13%	2	20.332	**0.72**
F5ANJ9	*Solanum tuberosum*	Late blight resistance protein	26	1%	2	161.050	**1.61**
B8XF12	*Oncidium Gower Ramsey*	Monodehydroascorbate reductase	96	37%	15	51.672	**1.58**
A1XK95	*Oncidium sphacelatum*	RNA polymerase beta chain (Fragment)	147	28%	24	100.265	1.13
Q8LEP0	*Arabidopsis thaliana*	Superoxide dismutase [Cu-Zn] 1	30	4%	2	28.329	**2.10**
		Energy					
F2VJ72	*Oncidium hybrid cultivar*	Acetyl-CoA carboxylase carboxyltransferase beta subunit (Fragment)	33	17%	6	32.213	1.19
A1XIS8	*Oncidium sphacelatum*	ATP synthase subunit alpha (Fragment)	29	17%	8	42.873	0.90
P14578	*Oryza sativa*	Cytochrome c oxidase subunit 1 (Fragment)	26	20%	2	21.314	**0.79**
P42895	*Zea mays*	Enolase	27	8%	2	53.694	**0.52**
P08440	*Zea mays*	Fructose bisphosphate aldolase	85	17%	6	38.604	**0.55**
Q8LJT3	*Oncidium Goldiana*	Glucose-1-phosphate adenylyltransferase	22	19%	12	61.401	**1.31**
C7E4Z8	*Phragmipedium schlimii*	Glyceraldehyde-3-phosphate dehydrogenase (Fragment)	71	39%	10	24.923	1.15
AQ9SE26	*Dendrobium crumenatum*	Isocitrate lyase	24	17%	10	67.975	**0.59**
D5HQ13	*Oncidium Gower Ramsey*	NAD(P)H-quinone oxidoreductase subunit 4L	28	12%	2	11.539	1.06
P92253	*Cypripedium acaule*	NADH dehydrogenase subunit	44	35%	10	46.140	1.16
A1XJZ7	*Oncidium sphacelatum*	NADH-ubiquinone oxidoreductase chain 5 (Fragment)	22	8%	2	41.027	1.09
Q9M3H4	*Epidendrum stamfordianum*	Phosphoenolpyruvate carboxylase (Fragment)	24	14%	7	45.337	1.08
D5HQ30	*Oncidium Gower Ramsey*	Photosystem I assembly protein ycf3	23	12%	3	20.483	**0.78**
Q3BAP0	*Phalaenopsis aphrodite*	Photosystem I P700 chlorophyll a apoprotein A1	21	3%	4	85.965	**6.19**

Table 2.1 -Proteins identified in the symbiotic protocorms of *Oncidium sphacelatum* in association with a *Ceratobasidium* sp. isolate using MassMatrix software ($p < 0.05$). 117/116 Ratiorepresent the relative abundance of the two iTRAQ tags used to label proteins extracted from green (iTRAQ 117) and achlorophyllous (iTRAQ 116) *O. sphacelatum* protocorms

(continuation)

Most similar acession	Organism	Description	Score	Coverage	# of Peptides	Mass (Monoisotopic)	117/116 Ratio
B8YHX7	*Oncidium andradeanum*	Photosystem I subunit B (Fragment)	39	5%	7	59.504	0.99
D5HQ34	*Oncidium Gower Ramsey*	Ribulose bisphosphate carboxylase large chain	261	34%	20	57.506	**7.09**
Q8H1Y1	*Oncidium Goldiana*	Sucrose phosphate synthase	171	19%	32	127.748	1.16
D5HQ34	*Oncidium Gower Ramsey*	Ribulose bisphosphate carboxylase large chain	261	34%	20	57.506	**7.09**
Q8H1Y1	*Oncidium Goldiana*	Sucrose phosphate synthase	171	19%	32	127.748	1.16
		Secondary Metabolism					
Q52QS5	*Oncidium Gower Ramsey*	9-*cis*-epoxycarotenoid dioxygenase	26	22%	6	29.190	**1.82**
Q96413	*Dendrobium crumenatum*	ACC synthase	20	5%	3	52.684	**4.48**
C3VEQ1	*Oncidium Gower Ramsey*	Beta-carotene hydroxylase	24	18%	3	36.016	**0.79**
Q2MV11	*Oncidium Gower Ramsey*	Beta-mannosidase 2	29	16%	11	61.479	1.01
Q2MV12	*Oncidium Gower Ramsey*	Beta-mannosidase 3	40	23%	14	60.947	0.95
Q9ZRS2	*Bromheadia finlaysoniana*	Bibenzyl synthase	28	4%	2	46.324	**0.72**
C3VEQ0	*Oncidium Gower Ramsey*	Carotene desaturase	19	16%	12	67.347	1.00
C3VEQ4	*Oncidium Gower Ramsey*	Carotenoid cleavage dioxygenase	16	21%	14	70.085	**0.74**
A7KTI4	*Oncidium Gower Ramsey*	Chalcone isomerase	16	29%	6	28.015	0.83
A7KTI3	*Oncidium Gower Ramsey*	Chalcone synthase	31	24%	7	46.010	**1.56**
B4F6G1	*Oncidium Gower Ramsey*	Fibrillin-like protein	31	9%	3	37.604	**0.64**
B2LU34	*Oncidium Gower Ramsey*	Chromoplast specific carotenoid associated protein	31	5%	2	37.413	**0.64**
B8XF1	*Oncidium Gower Ramsey*	Galacturonate reductase	63	43%	11	39.347	**0.47**
B9W015	*Oncidium Gower Ramsey*	GDP-D-mannose pyrophosphorylase (Fragment)	23	11%	4	21.590	1.19
Q4W8D0	*Oryza sativa*	Glutamine synthetase	61	34%	12	46.404	1.07
B9UP05	*Oncidium hybrid cultivar*	Hydroxymethylbutenyl diphosphate reductase	32	14%	7	58.519	**0.69**

Table 2.1 - Proteins identified in the symbiotic protocorms of *Oncidium sphacelatum* in association with a *Ceratobasidium* sp. isolate using MassMatrix software ($p < 0.05$). 117/116 Ratiorepresent the relative abundance of the two iTRAQ tags used to label proteins extracted from green (iTRAQ 117) and achlorophyllous (iTRAQ 116) *O. sphacelatum* protocorms

(continuation)

Most similar acession	Organism	Description	Score	Coverage	# of Peptides	Mass (Monoisotopic)	117/116 Ratio
D9IL24	*Oncidium Gower Ramsey*	Lycopene zeta-cyclase	29	19%	8	61.867	**2.09**
B8XF09	*Oncidium Gower Ramsey*	Mitochondrial galactono-1,4-lactone dehydrogenase	40	23%	13	68.074	0.59
Q43741	*Bromheadia finlaysoniana*	Naringenin 3-dioxygenase	16	2%	2	45.153	**0.74**
Q42609	*Bromheadia finlaysoniana*	Phenylalanine ammonia-lyase	26	18%	10	83.400	**1.95**
C3VEP9	*Oncidium Gower Ramsey*	Phytoene desaturase	40	24%	15	72.258	**0.25**
Q6RIC0	*Oncidium Gower Ramsey*	Phytoene synthase	21	18%	9	51.030	**1.24**
Q52QW3	*Oncidium hybrid cultivar*	Prolycopene isomerase 1, chloroplastic	40	25%	15	71.826	**2.24**
C3VEQ5	*Oncidium Gower Ramsey*	Short-chain dehydrogenase/reductase	16	27%	8	35.885	1.11
C3VEQ2	*Oncidium Gower Ramsey*	Zeaxanthin epoxidase, chloroplastic	32	15%	13	79.692	**0.75**
		Protein synthesis and fate					
D5HQ50	*Oncidium Gower Ramsey*	30S ribosomal protein S12, chloroplastic	18	20%	4	15.596	**0.32**
D5HQ24	*Oncidium Gower Ramsey*	30S ribosomal protein S14, chloroplastic	21	14%	2	13.524	1.08
D5HQ48	*Oncidium Gower Ramsey*	30S ribosomal protein S18, chloroplastic	18	22%	3	13.540	**1.39**
D5HQ81	*Oncidium Gower Ramsey*	30S ribosomal protein S2, chloroplastic	17	32%	8	29.586	0.91
D5HQ62	*Oncidium Gower Ramsey*	30S ribosomal protein S3, chloroplastic	28	38%	8	27.858	**1.43**
D5HQ60	*Oncidium Gower Ramsey*	50S ribosomal protein L14, chloroplastic	45	36%	4	14.538	0.81
D5HQ49	*Oncidium Gower Ramsey*	50S ribosomal protein L20, chloroplastic	22	32%	6	16.095	0.81
P34824	*Hordeum vulgare*	Elongation factor 1-alpha (Fragment)	57	19%	6	30.379	0.97
E5DMC0	*Oncidium sphacelatum*	Ribosomal protein S3 (Fragment)	23	10%	7	61.336	**0.69**
F8SVM5	*Vanilla planifolia*	Ubiquitin-like protein (Fragment)	51	28%	3	11.980	1.10

Table 2.1 -Proteins identified in the symbiotic protocorms of *Oncidium sphacelatum* in association with a *Ceratobasidium* sp. isolate using MassMatrix software ($p < 0.05$). 117/116 Ratiorepresent the relative abundance of the two iTRAQ tags used to label proteins extracted from green (iTRAQ 117) and achlorophyllous (iTRAQ 116) *O. sphacelatum* protocorms

(conclusion)

Most similar acession	Organism	Description	Score	Coverage	# of Peptides	Mass (Monoisotopic)	117/116 Ratio
D5HQ50	*Oncidium Gower Ramsey*	30S ribosomal protein S12, chloroplastic	18	20%	4	15.596	**0.32**
		Signalling					
Q9SP07	*Lilium longiflorum*	14-3-3-like protein	43	10%	7	29.252	0.89
B2KL91	*Phalaenopsis amabilis*	Calcium-dependent protein kinase 1 (Calmodulin)	20	9%	5	72.358	**1.31**
Q71SV5	*Oncidium Gower Ramsey*	Ethylene receptor	89	18%	11	75.603	**1.49**
G0Y290	*Oncidium Gower Ramsey*	Ethylene insensitive 3-like protein	101	21%	14	73.244	1.18
Q9AT80	*Brassica napus*	GF14 nu (Fragment)	37	18%	2	18.184	0.90
B2LYE8	*Arabidopsis thaliana*	G-type lectin S-receptor-like serine/threonine-protein kinase	133	26%	22	103.590	**0.72**
Q9XFJ0	*Bauhinia variegata*	Lipoxygenase	35	4%	2	104.519	**2.28**
B9SSY3	*Ricinus comunis*	Type II Inositol 5 phosphatase	23	1%	2	82.359	**0.78**

*Values in bold indicate that the protein was considered to be up- or down- regulated in this study.

Changes in orchid carbon metabolism during protocorm development

Given the lack of storage reserves typical of the minute orchid "dust-seeds" (DEARNALEY, 2007), all carbon needed to sustain embryo and protocorm development during the achlorophyllous stage must be supplied by the endomycorrhizal fungus, which is able to degrade complex substrates and provide organic carbon to the orchid embryo (ZETTLER et al., 1997). The catabolism of fungal provided-organic carbon in our study is inferred by the enhanced accumulation of fructose-bisphosphate aldolase and enolase, from the glycolytic pathway, and isocitrate lyase from the Krebs cycle, during the mycoheterotrophic stage. By contrast, the most up-regulated proteins in the green protocorms were RuBisCO and Photosystem I P700 chlorophyll a apoprotein A1 (Table 2.1). These data likely mirror the switch from fully mycoheterotrophic to mixotrophic or fully autotrophic metabolism as protocorms were exposed to light. A possible role of enzymes from the glycolytic pathway in general plant responses to fungal infection may also be considered. Activities of all enzymes of the glycolytic pathway and concentration of their products increase in the pathogenic interaction between *Oryza sativa* and *Rhizoctonia solani* Khun (MUTUKU; NOSE, 2012). In addition, these changes were accompanied by the activation of the phenylpropanoid pathway (DANSON; WASANO; NOSE, 2000).

The enhanced accumulation of a glucose-1-phosphate adenylyl-transferase (Table 2.1) and the higher levels of sucrose phosphate synthase in green protocorms suggest enhanced starch biosynthesis with the onset of photosynthesis. Although there is no experimental evidence, the higher accumulation of invertases in our study may suggest a flow of carbon from the host plant to the fungus. In contrast, phosphoenolpyruvate carboxylase accumulated at approximately the same levels in both developmental stages, corroborating the hypothesis that dark CO_2 fixation may supplement carbon acquisition during protocorm development (ARDITTI, 1992). The core reactions of the energy metabolism and redox homeostasis highlighting proteins that were identified in our experiment are shown in Figure 2.1.

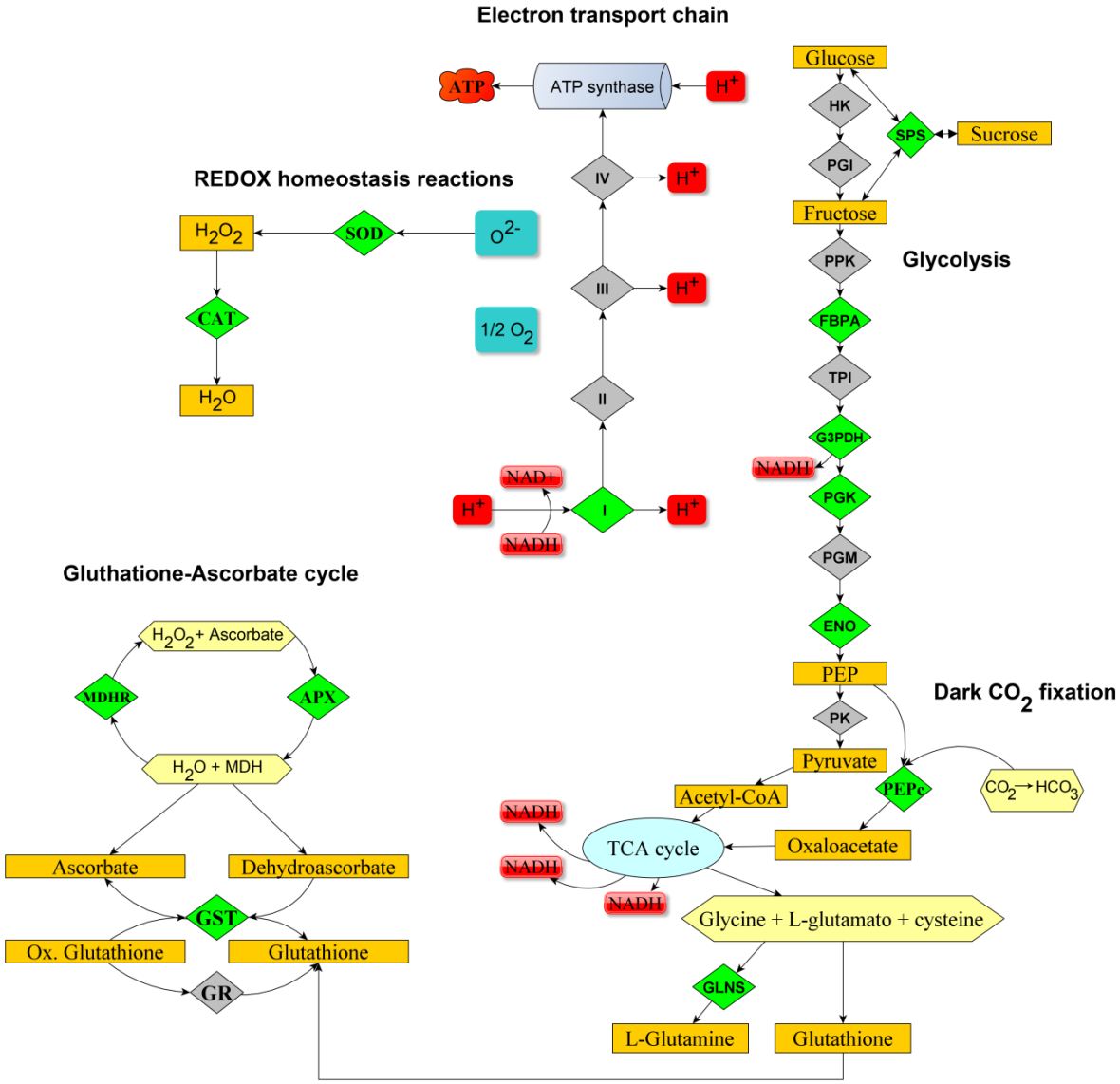

Figure 2.1 -Core reactions of the energetic metabolism and associated redox homeostasis mechanisms involving proteins identified in the symbiotic protocorms of *Oncidium sphacelatum* in associationwith a *Ceratobasidium* sp. isolate. Proteins represented inside green diamonds were identified in this study. Abbreviations used: SPS - sucrose phosphate synthase; HK - hexokinase; PGI - phosphoglugoisomerase; PPK - phosphofrutokinase; FBPA - fructose-1,6-bisphosphate aldolase; TPI triosephosphate isomerase; G3PDH - glyceraldehyde 3-phosphate dehydrogenase; PGK - phosphoglycerate kinase; PGM phosphoglycerate mutase; ENO - enolase; PK - pyruvate kinase; PEP - phosphoenolpyruvate; PEPc - phosphoenolpyruvate carboxylase; GLNS - glutamine synthetase; GR - glutathione reductase; GST - glutathione-S-transferase; MDH - monodehydroascrobate; MDHR - monodehydroascorbate reductase; APX - ascorbate peroxidase; CAT - catalase; SOD - superoxide dismutase; I, II, III e IV refers to complexes of the electron transport chain (ETC); not all reactions of ETC are represented

Secondary metabolism and phytohormones

Among plant secondary metabolites, flavonoids/isoflavonoids may contribute to protecting plants against biotic and abiotic stresses, and may also be involved in signaling during plant symbiotic interactions with mycorrhizal fungi (ALOUI et al., 2011; LAROSE et al., 2002; VOLPIN et al., 1994, 1995) and nodulating bacteria as well (HASSAN; MATHESIUS, 2012). The higher phenyalanine ammonia lyase (PAL) activity in symbiotic protocorms of *Anacamptis* (*Orchis*) *morio*, as compared to non-mycorrhizal protocorms (BEYRLEet al., 1995), suggests a role of phenolic compounds in plant-fungus interactions, and strengthens the similarities between OM and arbuscular mycorrhiza (AM).

Among the proteins with differential accumulation in *O. sphacelatum* protocorms, we detected enzymes involved in flavonoid biosynthesis, such as PAL, chalcone synthase (CHS) and naringenin 3-dioxygenase. In particular, the abundance of PAL and CHS were higher in green protocorms, as compared to dark-grown protocorms, but the levels of CHI showed no significant difference between the two developmental stages, and naringenin 3-dioxygenase was suppressed. In OM of green protocorms, the changes in PAL, CHS and CHI expression, as compared to achlorophyllous protocorms, were similar to those observed in *M. truncatula* roots colonized by *G. versiforme* when compared to non-mycorrhizal roots(HARRISON; DIXON, 1994). However, whether PAL, CHS and CHI have the same functions in OM and AM is not known. The enhanced accumulation of PAL and CHS in green protocorms may also be associated with the effect of light on the regulation of the flavonoid biosynthetic genes (KUBASEK et al., 1992). Alternatively, the enhanced accumulation of these proteins may be associated with the biosynthesis of defense molecules, such as phytoalexins, as well as signal molecules, as it occurs in other plant-microbe interactions (SCHENKLUHN et al., 2010). It has been suggested that phytoalexins may be associated with restriction of fungal growth in the orchid photosynthesizing tissues (SHIMURA et al., 2007). We therefore suggest that the increased accumulation of PAL and CHS in green protocorms may be related to the fungal colonization pattern observed at that stage of protocorm development, where cells belonging to the protoderm and those that will originate the apical meristem are rarely colonized (PEREIRA et al., 2005), and the proportion of colonized to non-colonized cells decreases.

Other secondary metabolites associated with several physiological processes in plants are the carotenoids. As accessory pigments, carotenoids participate in photo-induced electron transfer processes and also protect cells against excessive light, by quenching both singlet and triplet chlorophylls states (TAIZ; ZEIGER, 2010). Photosynthetic development and

carotenoid biosynthesis are known to be coordinately regulated by phytochromes, through phytochrome–interacting transcription factors (SHIN et al., 2009). Carotenoids in plants are synthesized via the plastidic methylerythritol phosphate (MEP) pathway, a non-mevalonate alternative route that is also strongly up-regulated in AM roots (WALTER; FESTER; STRACK, 2000). Indeed, specific carotenoids (C_{14} mycorradicin and C_{13} cyclohenenone) accumulate in roots during AM development (FESTER et al., 2002; KLINGNER et al., 1995; WALTER; FLOSS; STRACK, 2010). In our study, green protocorms showed induced accumulation of several enzymes involved in carotenoid biosynthesis, such as phytoene synthase (PSY), prolycopene isomerase (PLIso), lycopene zeta cyclase (LCY-z) and 9-cis-epoxycarotenoid dioxygenase (NCED) (Table 2.1, Figure2.2). In contrast, suppressed accumulation of 4-hydroxy-3-methyl-but-2-enylpyrophosphate (HMB-PPr), phytoene desaturase (PSD), carotenoid cleavage dioxygenase (CCD), beta-carotene hydroxylase (BCH) and zeaxanthin epoxidase (ZXE) was observed (Table 2.1, Figure 2.2).

Carotenoid-derived molecules such as the phytohormones abscisic acid (ABA) and strigolactones may also have a role in signaling and signal transduction in plant-microbe interactions (AKIYAMA; MATSUZAKI; HAYASHI, 2005; BOUVIER et al., 2005; MARTÍN-RODRÍGUEZ et al., 2011; MATUSOVA et al., 2005). In our study, 9-cis-epoxycarotenoid dioxygenase (NCED) and carotenoid cleavage dioxygenase (CCD), key enzymes involved in the biosynthesis of ABA and strigolactones, respectively, have been detected and showed altered levels of accumulation in protocorms. NCED showed induced accumulation in green protocorms, whereas the CCD showed suppressed accumulation (Table 2.1, Figure 2.2). ABA is important for arbuscule development and functionality, and for promoting sustained colonization of the plant roots in AM (HERRERA-MEDINA et al., 2007; MARTÍN-RODRÍGUEZ et al., 2011). However, the roles of ABA in OM are not known. Strigolactones are able to induce fungal hyphal branching and root colonization in AM (AKIYAMA; MATSUZAKI; HAYASHI, 2005; BESSERER et al., 2006; KRETZSCHMAR et al., 2012), and might also be important in the regulation of fungal growth in OM.

Proteins involved in the biosynthesis and perception of ethylene, a key phytohormone involved in the regulation of plant development (SCHALLER, 2012) and plant-microbe interactions (FRACETTO et al., 2013; PENMETSA et al., 2008; RIEDEL; GROTEN; BALDWIN, 2008; ZSÖGÖN et al., 2008), have also been detected in our study. The enzyme 1-aminocyclopropane-1-carboxylate (ACC) synthase, which catalyzes the first committed step

in the ethylene biosynthesis (KENDE; ZEEVAART, 1997), and an ethylene receptor showed enhanced accumulation in green protocorms, as compared to achlorophyllous protocorms (Table 2.1). It is known that high concentrations of ethylene inhibit the intraradical growth of arbuscular mycorrhizal fungi (AMF) and that tomato mutants overproducing ethylene have reduced levels of AM colonization (FRACETTO et al., 2013; MARTÍN-RODRÍGUEZ et al., 2011; ZSÖGÖN et al., 2008). Given the multiple roles of ethylene in plants, the significance of this finding in OM is unclear. Besides its effects on fungal growth, ethylene might also be involved in photosynthesis regulation in green protocorms, since tobacco mutants insensitive to ethylene have shown down-regulation of RuBisCO expression and photosynthetic capacity (THOLEN *e*t al., 2007), and the application of ethephon to *Brassica juncea*increases the activity of nitrate reductase and ATP sulfurylase, resulting in increased photosynthetic responses (IQBAL et al., 2012).

In addition, our data also suggest that jasmonic acid (JA) may also be involved in the regulation of OM. In plants, lipoxygenases catalyzes the biosynthesis of oxylipins, which will further generate an extensive family of metabolites, including JA. JA signaling is well known for its role in plant defense against necrotrophic pathogens and herbivores, and several studies indicate that JA- and ethylene-signaling often operate synergistically to activate the expression of defense related genes after pathogen infection (BARI; JONES, 2009; PENNINCKX et al., 1998; THOMMA; PENNINCKX; BROEKAERT, 2001). Even though no attempts to determine changes in JA concentration in OM have been made, it is possible that the higher accumulation of lipoxygenase in green protocorms, as compared to achlorophyllous protocorms, may result in enhanced biosynthesis of JA at this stage.

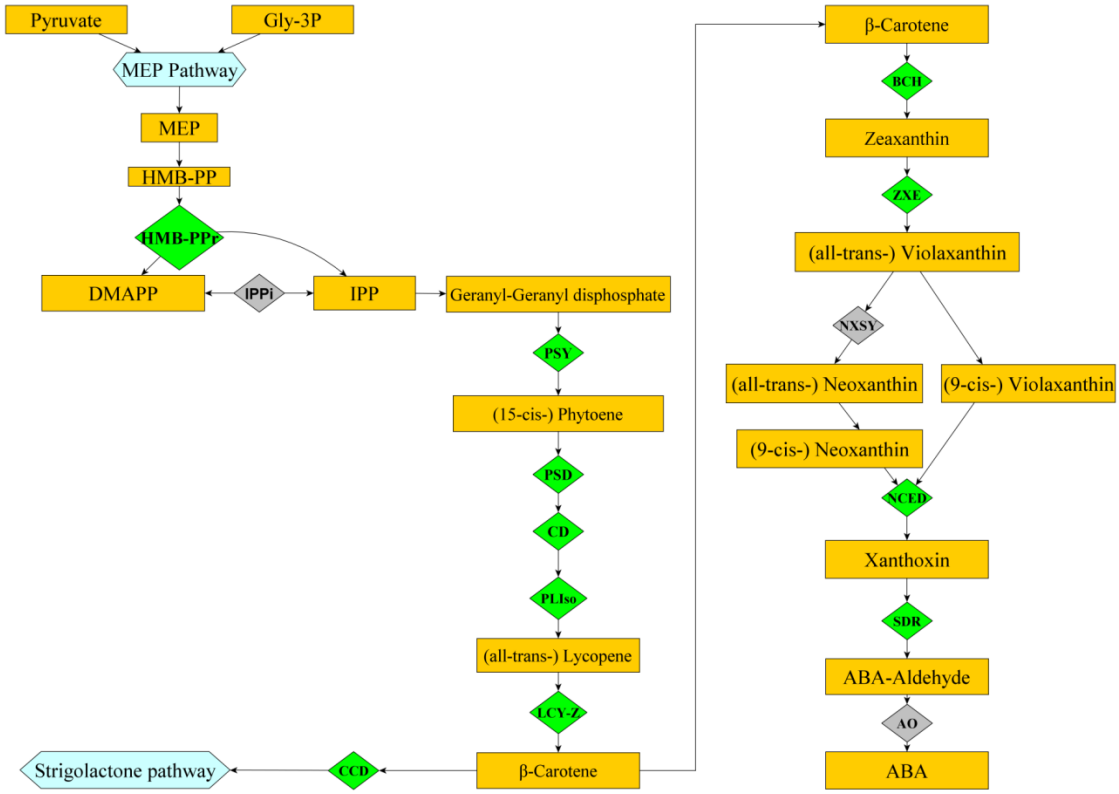

Figure 2.2 - Proteins involved in the biosynthesis of carotenoids, ABA and strigolactone identified in the symbiotic protocorms of *Oncidium sphacelatum* in associationwith a *Ceratobasidium* sp. isolate. Proteins represented inside green diamonds were identified in this study. Abbreviations used: Gly-3P - glyceraldehyde-3-phosphate; MEP - 2-C-methyl-D-erythritol 4-phosphate; HMB-PP - 4-Hydroxy-3-methyl-but-2-enyl pyrophosphate; HMB-PPr - HMB-PP reductase; DMAPP - Dimethylallyl pyrophosphate; IPP - Isopentenyl-diphosphate; IPPi - IPP isomerase; PSY - phytoene synthase; PSD - phytoene desaturase; CD - carotenoid desaturase; PLIso - Prolycopene isomerase; LCY-Z - Lycopene zeta cyclase; CCD - carotenoid cleavage dioxygenase; BCH - betacaroteno hydroxylase, ZXE - zeaxanthin epoxidase, NXSY - Neoxanthin synthase, NCED - 9-cis-epoxycarotenoid dioxygenase, SDR - short chain dehydrogenase/reductase, AO aldehyde oxidase

Reactive oxygen species homeostasis and stress-related responses

Photosynthesis and respiration generate high levels of reactive oxygen species (ROS), whose homeostasis is controlled by a network of enzymatic and non-enzymatic components, including catalase, superoxide dismutase (SOD) and enzymes from the glutathione-ascorbate cycle (FOYER; NOCTOR, 2011). A Cu,Zn-SOD, ascorbate peroxidase and monodehydroascorbate reductase were significantly up-regulated at the photosynthetic stage, as compared to the fully mycoheterotrophic stage (Table 2.1), in our study. Ascorbate peroxidase and Cu,Zn-SOD play major roles in the removal of ROS produced during photosynthesis (DANNA et al., 2003; GILL; TUTEJA, 2010), which may explain their up-regulation in green protocorms.

The generation of ROS is also a hallmark of symbiotic interactions(both mutualistic and pathogenic) and is one of the earliest defense responses in plants. It is well known that ROS can interact with other molecules and phytohormones playing multifaceted signaling roles, or simply accumulate in specific sub-cellular compartments acting as antimicrobials during the oxidative burst (GARG; MANCHANDA, 2009; TORRES, 2010). Differential expression of antioxidant enzymes has been observed in AM (BLILOU et al., 2000; FRACETTO et al., 2013; LAMBAIS; RIOS-RUIZ; ANDRADE, 2003) and rhizobium-legume interactions (PAULY; PUCCIARIELLO; MANDON, 2006; TORRES, 2010). Staining techniques showed that intracellular accumulation of H_2O_2in AM is higher in the plant cytoplasm close to intact and collapsing fungal structures, whereas intercellular H_2O_2 was located on the surface of fungal hyphae(FESTER; HAUSE, 2005). In OM, Blakeman et al. (1976) showed that catalase, peroxidase, polyphenol oxidase and ascorbate oxidase activities are greatly increased in mycorrhizal protocorms, and that peak activities often coincide with peak oxygen uptake and digestion of pelotons.

In our study, whereas SOD and ascorbate peroxidase showed increased accumulation in green protocorms, catalase accumulation was not affected, as compared to achlorophyllous protocorms, suggesting enhanced accumulation of H_2O_2 in at least part of green protocorm cells. This is in agreement with previous studies showing accumulation of H_2O_2 in interactions between legumes and different AMF (LANFRANCO et al., 2005; SALZER; CORBIERE; BOLLER, 1999), and also with the induction of a late-blight resistance protein and two heat shock proteins(70 and 81 KDa) that may be up-regulated by H_2O_2(GUSUI et al., 1995; NEILL et al., 2002). In contrast to other defense related proteins, a *Kunitz*-1 type proteinase inhibitor, which was also highly induced in *Medicago truncatula* roots colonized by *Glomus intraradices* (SCHENKLUHN et al., 2010), showed suppressed accumulation in green protocorms. Suppressed accumulation of a xanthine dehydrogenase, which is a potent producer of superoxide anions via its NADH oxidase activity in response to biotic or abiotic stimuli (HESBERG et al., 2004; SAGI; FLUHR; LIPS, 1999; YESBERGENOVA et al., 2005; ZAREPOUR et al., 2010), and a bibenzyl synthase, known to catalyze the biosynthesis of phytoalexins in orchids (REINECKE; KINDL, 1994), was also observed in green protocorms (Table 2.1), suggesting that the regulation of OM may occur at the cell level.

Enhanced accumulation of an actin-like protein and enzymes involved in cell wall modifications (*i.e.*, pectinesterase, alpha 1,4 glucan synthase) in green protocorms also suggests that cell wall modifications and reorganization of cytoskeleton, necessary to

accommodate the fungal symbiont (UETAKE; PETERSON, 1996), were taking place in OM. Other defense-related proteins, such as defensins and serine proteases, have also been identified, but were removed from the dataset due to their single peptide identification. With the present protein dataset it is still not clear which defense strategies are employed in each stage of protocorm development, but we can speculate that cell-specific defense responses should take place, modulating fungal growth and peloton formation in symbiotic cells, while repressing hyphal spread in meristematic and photosynthesizing tissues.

Signaling and signal transduction

Several proteins involved in the signal transduction showed differential accumulation in our study (Table 2.1), although their significance in the regulation of OM interactions is currently unknown. The presence of a calmodulin in this dataset is noteworthy, due its central role in the transduction of calcium signaling (YANG; POOVAIAH, 2003). Increase in intracellular calcium concentration has been observed after a range of biotic and abiotic stimuli, and it might be involved in the cross-talking of different signaling pathways (BOWLER; FLUHR, 2000). In fact, intracellular calcium levels are highly modulated during plant-microbe interactions, and calcium spiking is in the core of the common symbiosis signaling pathway in AM and legume-Rhizobium interactions (ROBERTS et al., 2013). Recently, Zhao et al. (2013) described two calmodulin genes putatively involved in symbiotic protocorm development. Also related to calcium signaling, the suppressed accumulation in green protocorms of an inositol-5-phophatase, which has been described to be involved in IP3 hydrolysis (CHEN et al., 2008) and up-regulated in response to ABA in *Arabdopsis thaliana*(BURNETTE; GUNESEKERA; GILLASPY, 2003), points to a role of calcium in the regulation of OM.

2.4 Conclusions

The 2D LC-MS/MS coupled to iTRAQ approach used in this study has provided the first quantitative large-scale analysis of the proteome of OM. Our results suggest that profound changes occur in symbiotic orchid protocorms as they switch from the fully mycoheterotrophic to the mixotrophic or fully autotrophic metabolism. Some differentially regulated proteins may be directly or indirectly involved in protocorm development and changes in carbon metabolism that follows the onset of photosynthesis. On the other hand, the differential accumulation of several proteins may suggest specific regulatory mechanisms

controlling OM. Phytohormone and secondary metabolites, ROS, and defense-related proteins are well known to play multiple roles in the regulation of plant-microbe interactions, and some of the responses observed in our study were also described in AM, suggesting that both symbioses may share at least part of their regulatory mechanisms.

References

AKIYAMA, K.; MATSUZAKI, K.; HAYASHI, H. Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. **Nature**, London, v. 435, n. 7043, p. 824–827, 2005.

ALOUI, A.; RECORBET, G.; ROBERT, F.; SCHOEFS, B.; BERTRAND, M. Arbuscular mycorrhizal symbiosis elicits shoot proteome changes that are modified during cadmium stress alleviation in *Medicago truncatula*. **BMC Plant Biology**, v. 11, n. 1, p. 75, 2011.

BARI, R.; JONES, J.D.G. Role of plant hormones in plant defence responses. **Plant Molecular Biology**, Dordrecht, v. 69, n. 4, p. 473–488, 2009.

BESSERER, A.; PUECH-PAGÈS, V.; KIEFER, P. Strigolactones stimulate arbuscular mycorrhizal fungi by activating mitochondria. **Plos Biology**, London,v. 4, n. 7, e226, 2006.

BEYRLE, H.F.; SMITH, S.E.; PETERSON, R.L.; FRANCO, C.M.M. Colonization of *Orchis morio* protocorms by a mycorrhizal fungus: effects of nitrogen nutrition and glyphosate in modifying the responses. **Canadian Journal of Botany**, Ottawa, v. 73, p. 1128–1140, 1995.

BLAKEMAN, J.P.; MOKAHEL, M.A.; HADLEY, G. Effect of mycorrhizal infection on respiration and activity of some oxidase enzymes of orchid protocorms. **New Phytologist**, Cambridge, v. 77, n. 3, p. 697–704, 1976.

BLILOU, I.; BUENO, P.; OCAMPO, J.A.; A-GARRIDO, J.M.G. Induction of catalase and ascorbate peroxidase activities in tobacco roots inoculated with the arbuscular mycorrhizal *Glomus mosseae*. **Mycological Research**, New York, v. 104, n. 6, p. 722–725, 2000.

BOUVIER, F.; ISNER, J.-C.; DOGBO, O.; CAMARA, B. Oxidative tailoring of carotenoids: a prospect towards novel functions in plants. **Trends in Plant Science**, Oxford, v. 10, n. 4, p. 187–194, 2005.

BOWLER, C.; FLUHR, R. The role of calcium and activated oxygens as signals for controlling. **Trends in Plant Science**, Oxford, v. 5, n. 6, p. 241–246, 2000.

BURNETTE, R.N.; GUNESEKERA, B.M.; GILLASPY, G.E. An *Arabidopsis* inositol 5-phosphatase gain-of-function alters abscisic acid signaling. **Plant Physiology**, Minneapolis, v. 132, n. 6, p. 1011–1019, 2003.

CAMERON, D.; LEAKE, J.; READ, D. Mutualistic mycorrhiza in orchids: evidence from plant–fungus carbon and nitrogen transfers in the green leaved terrestrial orchid *Goodyera repens*. **New Phytologist**, Cambridge, v. 171, p. 405–416, 2006.

CAMERON, D.D.; JOHNSON, I.; LEAKE, J.R.; READ, D.J. Mycorrhizal acquisition of inorganic phosphorus by the green-leaved terrestrial orchid *Goodyera repens*. **Annals of Botany**, London, v. 99, n. 5, p. 831–834, 2007.

CAMERON, D.D.; JOHNSON, I.; READ, D.J.; LEAKE, J.R. Giving and receiving: measuring the carbon cost of mycorrhizas in the green orchid, *Goodyera repens*. **New Phytologist**, Cambridge, v. 180, n. 1, p. 176–184, 2008.

CHEN, X.; LIN, W.H.; WANG, Y.; LUAN, S.; XUE, H.W. An inositol polyphosphate 5-phosphatase functions in PHOTOTROPIN1 signaling in *Arabidopis* by altering cytosolic Ca^{2+}. **Plant Cell**, Rockville, v. 20, n. 2, p. 353–366, 2008.

DANNA, C.H.; BARTOLI, C.G.; SACCO, F.; INGALA, L.R.; SANTA-MARI, G.E.; GUIAMET, J.J.; UGALDE, R.A. Thylakoid-bound ascorbate peroxidase mutant exhibits impaired electron transport and photosynthetic activity. **Plant Physiology**, Minneapolis, v. 132, n. 8, p. 2116–2125, 2003.

DANSON, J.; WASANO, K.; NOSE, A. Infection of rice plants with the sheath blight fungus causes an activation of pentose phosphate and glycolytic pathways. **European Journal of Plant Pathology**, Dordrecht, p. 555–561, 2000.

DEARNALEY J.D.W.; MARTOS, F.; SELOSSE, M.A.; Orchid mycorrhizas: molecular ecology, physiology, evolution and conservation aspects. In: HOCK, B. (Ed.). **The mycota IX: Fungal associations**. New York: Springer-Verlag, 2012. p. 207-230.

FESTER, T.; HAUSE, G. Accumulation of reactive oxygen species in arbuscular mycorrhizal roots. **Mycorrhiza**, Berlin, v. 15, n. 5, p. 373–379, 2005.

FESTER, T.; SCHMIDT, D.; LOHSE, S.; WALTER, M.H.;GIULIANO, G.;BRAMLEY, P.M.;FRASER, P.D.;HAUSE, B.;STRACK. D. Stimulation of carotenoid metabolism in arbuscular mycorrhizal roots. **Planta**, Berlin, v. 216, n. 1, p. 148–154, 2002.

FISCH, M.H.; FLICK, B.H.; ARDITTI, J. Structure and antifungal activity of hircinol, loroglossol and orchinol. **Phytochemistry**, Oxford, v. 12, p. 437–441, 1973.

FOYER, C.H.; NOCTOR, G. Ascorbate and glutathione: the heart of the redox hub. **Plant Physiology**, Minneapolis, v. 155, n. 1, p. 2–18, 2011.

FRACETTO, G.G.M.; PERES, L.E.P.; MEHDY, M.C.; LAMBAIS, M.R. Tomato ethylene mutants exhibit differences in arbuscular mycorrhiza development and levels of plant defense-related transcripts. **Symbiosis**, Rehovot, v. 60, n. 3, p. 155–167, 2013.

GARG, N.; MANCHANDA, G. ROS generation in plants: boon or bane? **Plant Biosystems**, Rome, v. 143, n. 1, p. 81–96, 2009.

GILL, S.S.; TUTEJA, N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. **Plant Physiology and Biochemistry**, New Delhi, v. 48, n. 12, p. 909–930, 2010.

GONZÁLES-GARCÍA, V.; ONCO, M.A.P.; SUSAN, V.R. Biology and systematics of the form genus *Rhizoctonia*. **Spanish Journal of Agricultural Research**, Madrid, v. 4, n. 1, p. 55–79, 2006.

GUSUI, W.; SHORTT, B.J.; LAWRENCE, E.B.; LEVINE, E.B.; FITZSIMMONS, K.C. Disease resistance conferred by expression of a gene encoding H202-generating glucose oxidase in transgenic potato plants. **Plant Cell**, Rockville, v. 7, n. 9, p. 1357–1368, 1995.

HARRISON, M.J.; DIXON, R.A. Spatial patterns of expression of flavonoid/isoflavonoid pathway genes during interactions between roots of *Medicago truncatula* and the mycorrhizal fungus *Glomus versiforme*. **Plant Journal**, Oxford, v. 6, p. 9–20, 1994.

HASSAN, S.; MATHESIUS, U. The role of flavonoids in root-rhizosphere signalling: opportunities and challenges for improving plant-microbe interactions. **Journal of Experimental Botany**, Oxford, v. 63, n. 9, p. 3429–3444, 2012.

HERRERA-MEDINA, M.J.; STEINKELLNER, S.; VIERHEILIG, H.; OCAMPO BOTE, J.A.; GARCÍA-GARRIDO, J.M. Abscisic acid determines arbuscule development and functionality in the tomato arbuscular mycorrhiza. **New Phytologist**, Cambridge, v. 175, n. 3, p. 554–564, 2007.

HERBRICH, S.M.; COLE, R.N.; WEST, K.P. Statistical inference from multiple iTRAQ experiments without using common reference standards. **Journal of Proteome Research**, Washington, v. 12, p. 594-604, 2013.

HESBERG, C.; HÄNSCH, R.; MENDEL, R.R.; BITTNER, F. Tandem orientation of duplicated xanthine dehydrogenase genes from *Arabidopsis thaliana*: differential gene expression and enzyme activities. **Journal of Biological Chemistry**, Bethesda, v. 279, n. 14, p. 13547–13554, 2004.

HILL, E.G.; SCHWACKE, J.H.; COMTE-WALTERS, S.; SLATE, E.H.; OBERG, A.L.; ECKEL-PASSOW, J.E.; THERNEAU, T.M.; SCHEY, K.L. A statistical model for iTRAQ data analysis research articles. **Journal of Proteome Research**, Washington, v. 7, p. 3091–3101, 2008.

IQBAL, N.; KHAN, N.A.; NAZAR, R.; SILVA, J.A. Ethylene-stimulated photosynthesis results from increased nitrogen and sulfur assimilation in mustard types that differ in photosynthetic capacity. **Environmental and Experimental Botany**, Elmsford, v. 78, p. 84–90, 2012.

KENDE, H.; ZEEVAART, J.A.D. The five classical plant hormones. **Plant Cell**, Rockville, v. 9, n. 7, p. 1197–1210, 1997.

KLINGNER, A.; BOTHE, H.; WRAY, V.; MARNER, F. Identification of a yellow pigment formed in maize roots. **Phytochemistry**, Oxford, v. 38, n. 1, p. 53–55, 1995.

KRETZSCHMAR, T.; KOHLEN, W.; SASSE, J.; BORGHI, L.; SCHLEGEL, M.; BACHELIER, J.B.; REINHARDT, D.; BOURS, R.; BOUWMEESTER, H.J.; MARTINOIA, E. A petunia ABC protein controls strigolactone-dependent symbiotic signalling and branching. **Nature**, London, v. 483, n. 7389, p. 341–344, 2012.

KUBASEK, W.L.; SHIRLEY, B.W.; MCKILLOP, A.; GOODMAN, H.M.; BRIGGS, W.; AUSUBEL, F.M. Regulation of flavonoid biosynthetic genes in germinating *Arabidopsis* seedlings. **Plant Cell**, Rockville, v. 4, p. 1229-1236, 1992.

LAMBAIS, M.R.; RIOS-RUIZ, W.F.; ANDRADE, R.M. Antioxidant responses in bean (*Phaseolus vulgaris*) roots colonized by arbuscular mycorrhizal fungi. **New Phytologist**, Cambridge, v. 160, n. 2, p. 421–428, 2003.

LANFRANCO, L.; NOVERO, M.; BONFANTE, P. The mycorrhizal fungus *Gigaspora margarita* possesses a CuZn superoxide dismutase that is up-regulated during symbiosis with legume hosts. **Plant Physiology**, Minneapolis, v. 137, n. 4l, p. 1319–1330, 2005.

LAROSE, G.; CHÊNEVERT, R.; MOUTOGLIS, P.; GAGNÉ, S.; PICHÉ, Y.; VIERHEILIG, H. Flavonoid levels in roots of *Medicago sativa* are modulated by the developmental stage of the symbiosis and the root colonizing arbuscular mycorrhizal fungus. **Journal of Plant Physiology**, Stuttgart, v. 159, p. 1329–1339, 2002.

MARTÍN-RODRÍGUEZ, J.; LEÓN-MORCILLO, R.; VIERHEILIG, H.; OCAMPO J.A.; MULLER J.L.; GARCIA-GARRIDO J.M. Ethylene-dependent/ethylene-independent ABA regulation of tomato plants colonized by arbuscular mycorrhiza fungi. **New Phytologist**, Cambridge, p. 193–205, 2011.

MATUSOVA, R.; RANI, K.; VERSTAPPEN, F.W.A.; FRANSSEN M.H.B.; BOUWMEESTER, H.J. The strigolactone germination stimulants of the plant-parasitic *Striga* and *Orobanche* spp. are derived from the carotenoid pathway. **Plant Physiology**, Minneapolis, v. 139, n. 11, p. 920–934, 2005.

MUTUKU, J.M.; NOSE, A. Changes in the contents of metabolites and enzyme activities in rice plants responding to *Rhizoctonia solani* Kuhn infection: activation of glycolysis and connection to phenylpropanoid pathway. **Plant and Cell Physiology**, Kyoto, v. 53, n. 6, p. 1017–1032, 2012.

NEILL, S.J.; DESIKAN, R.; CLARKE, A.; HURST, R.D.; HANCOCK, J.T. Hydrogen peroxide and nitric oxide as signalling molecules in plants. **Journal of Experimental Botany**, Oxford, v. 53, n. 372, p. 1237–1247, 2002.

NESVIZHSKII, A.I.; AEBERSOLD, R. Interpretation of shotgun proteomic data: the protein inference problem. **Molecular & Cellular Proteomics**, San Francisco, v. 4, n. 10, p. 1419–1440, 2005.

OTERO, J.T.; BAYMAN, P.; ACKERMAN, J.D. Variation in mycorrhizal performance in the epiphytic orchid *Tolumnia variegata in vitro*: the potential for natural selection. **Evolutionary Ecology**, London, v. 19, p. 29–43, 2005.

PAULY, N.; PUCCIARIELLO, C.; MANDON, K.; INNOCENTI, G.; JAMET, A.; FRENDO, P.; PUPPO, A. Reactive oxygen and nitrogen species and glutathione: key players in the legume–*Rhizobium* symbiosis. **Journal of Experimental Botany**, Oxford, v. 57, n. 8, p. 1769–1776, 2006.

PENMETSA, R.V.; URIBE, P.; ANDERSON, J.; LICHTENZVEIG, J.; GISH, J.; NAM, Y.; ENGSTROM, E.; XU, K.; SCKISEL, G.; PEREIRA, M.; BAEK, J.M.; LOPEZ-MEYER, M.; LONG, S.R.; HARRISON, M.J.; SINGH, K.B.; KISS,G.B.; COOK, D.R. The *Medicago truncatula* ortholog of *Arabidopsis* EIN2, sickle, is a negative regulator of symbiotic and pathogenic microbial associations. **Plant Journal**, Oxford, v. 55, n. 4, p. 580–595, 2008.

PENNINCKX, I.A.; THOMMA, B.P.; BUCHALA, A; MÉTRAUX, J.P.; BROEKAERT, W.F. Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in *Arabidopsis*. **Plant Cell**, Rockville, v. 10, n. 12, p. 2103–2113, 1998.

PEREIRA, O.L.; KASUYA, M.C.M.; ROLLEMBERG, C.D.L.; BORGES, A.C. Indução *in vitro* da germinação de sementes de *Oncidium flexuosum* (Orchidaceae) por fungos micorrízicos rizoctonióides. **Revista Brasileira de Ciência do Solo**, Viçosa, v. 29, n. 2, p. 199–206, 2005.

PETERSON, R.L.; UETAKE, Y.; ZELMER, C. Fungal symbiosis with orchid protocorms. **Symbiosis**, Rehovot, v. 25, n. 1-3, p. 29–55, 1998.

PETERSON, R.L.; UETAKE, Y.; BONFANTE, P.; FACCIO, A. The interface between fungal hyphae and orchid protocorm cells. **Canadian Journal of Botany**, Ottawa, v. 74, n. 12, p. 1861–1870, 1996.

THE PLANT LIST: a working list of all plant species. The Plant List, version 1. Disponível em: <http://www.theplantlist.org>. Acesso em: 15 jan. 2013.

RASMUSSEN, H.N.; RASMUSSEN, F.N. Trophic relationship in orchid mycorrhiza: diversity and implications for conservation. **Lankesteriana**, Cartago, v. 7, n. 1, p. 334–341, 2007.

_____. Orchid mycorrhiza: implications of a mycophagous life style. **Oikos**, Copenhagen, v. 118, n. 3, p. 334–345, 2009.

RECORBET, G.; ROGNIAUX, H.; GIANINAZZI-PEARSON, V.; DUMAS-GAUDOT, E. Fungal proteins in the extra-radical phase of arbuscular mycorrhiza: a shotgun proteomic picture. **New Phytologist**, Cambridge, v.181, p. 248–260, 2009.

REINECKE, T.; KINDL, H. Inducible enzymes of the 9,10-dihydro-phenanthrene pathway. Sterile orchid plants responding to fungal infection. **Molecular Plant Microbe Interactions**, Saint Paul, v. 7, p. 449-454, 1994.

RIEDEL, T.; GROTEN, K.; BALDWIN, I.T. Symbiosis between *Nicotiana attenuata* and *Glomus intraradices*: ethylene plays a role, jasmonic acid does not. **Plant, Cell & Environment**, Oxford, v. 31, n. 9, p. 1203–1213, 2008.

ROBERTS, N.J.; MORIERI, G.; KALSI, G.; ROSE, A.; STILLER, J.; EDWARDS, A.; XIE, F.; GRESSHOFF, P.M.; OLDROYD, G.E.D.; DOWNIE, J.A; ETZLER, M.E.; RHIZOBIAL AND mycorrhizal symbioses in Lotus japonicus require lectin nucleotide phosphohydrolase, which acts upstream of calcium signaling. **Plant Physiology**, Minneapolis, v. 161, n. 1, p. 556–567, 2013.

RUEPP, A.; ZOLLNER, A.; MAIER, D.; ALBERMANN, K.; HANI. J.; MOKREJS, M.; TETKO, I.; GÜLDENER, U.; MANNHAUPT, G.; MÜNSTERKÖTTER, M; MEWES, H.W. The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. **Nucleic Acids Research**, New York, v. 14, p. 5539–5545, 2004.

SAGI, M.; FLUHR, R.; LIPS, S. Aldehyde oxidase and xanthine dehydrogenase in a flacca tomato mutant with deficient abscisic acid and wilty phenotype. **Plant Physiology**, Minneapolis, v. 120, n. 2, p. 571–578, 1999.

SALOMÉ-PAIS, M.; BARROSO, J. Localization of polyphenol oxidases during stablishment of *Ohrys lutea* endomycorrhizas. **New Phytologist,** Cambridge, v. 95, p. 219-222, 1983.

SALZER, P.; CORBIERE, H.; BOLLER, T. Hydrogen peroxide accumulation in *Medicago truncatula* roots colonized by the arbuscular mycorrhiza-forming fungus *Glomus intraradices*. **Planta**, Berlin, v. 208, p. 319–325, 1999.

SCHALLER, G.E. Ethylene and the regulation of plant development. **BMC Biology**, Leiden, v. 10, p. 9, 2012.

SCHENKLUHN, L.; HOHNJEC, N.; NIEHAUS, K.; SCHMITZ, U.; COLDITZ, F. Differential gel electrophoresis (DIGE) to quantitatively monitor early symbiosis- and pathogenesis-induced changes of the *Medicago truncatula* root proteome. **Journal of Proteomics**, Valencia,v. 73, n. 4, p. 753–768, 2010.

SELOSSE, M.A.; ROY, M. Green plants that feed on fungi: facts and questions about mixotrophy. **Trends in Plant Science**, Oxford, v. 14, n. 2, p. 64–70, 2009.

SHIMURA, H.; MATSUURA, M.; TAKADA, N.; KODA, Y. An antifungal compound involved in symbiotic germination of *Cypripedium macranthos* var. *rebunense* (Orchidaceae). **Phytochemistry**, Oxford, v. 68, n. 10, p. 1442–1447, 2007.

SHIN, J.; KIM, K.; KANG, H.; ZULFUGAROV, I.S.; BAE, G; LEE, C.H.; LEE, D.; CHOI, G. Phytochromes promote seedling light responses by inhibiting four negatively-acting phytochrome-interacting factors. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, v. 106, n. 18, p. 7660–7665, 2009.

TAIZ, L.; ZEIGER, E. **Plant physiology**. 5thed. Sunderland: Sinauer Associates, 2010. 690 p.

THOLEN, D.; PONS, T.L.; VOESENEK, L.A.; POORTER, H. Ethylene insensitivity results in down-regulation of rubisco expression and photosynthetic capacity in tobacco. **Plant Physiology**, Minneapolis, v. 144, n. 3, p. 1305–1315, 2007.

THOMMA, B.P.H.J.; PENNINCKX, I.A.; BROEKAERT, W.F. The complexity of disease signaling in *Arabidopsis*. **Current Opinion in Immunology**, London, v. 13, p. 63–68, 2001.

TORRES, M.A. ROS in biotic interactions. **Physiologia Plantarum**, Copenhagen, v. 138, n. 4, p. 414–429, 2010.

UETAKE, Y.; PETERSON, L.R. Changes in actin filament arrays in protocorm cells of the orchid species, *Spiranthes sinensis*, induced by the symbiotic fungus *Ceratobasidium cornigerum*. **Canadian Journal of Botany**, Ottawa v. 75, p. 1661–1669, 1996.

VALADARES, R.B.S.; PEREIRA, M.C.; OTERO, J.T.; CARDOSO, E.J.B.N. Narrow fungal mycorrhizal diversity in a population of the orchid *Coppensia doniana*. **Biotropica**, Lawrence, v. 44, n. 1, p. 114–122, 2012.

VOLPIN, H.; ELKIND, Y.; OKON, Y.; KAPULNIK, Y. A vesicular arbuscular mycorrhizal fungus (*Glomus intraradix*) induces a defense response in alfalfa roots. **Plant Physiology**, Minneapolis, v. 104, n. 2, p. 683–689, 1994.

VOLPIN, H.; PHILLIPS, D.A.; OKON, Y.; KAPULNIK, Y. Suppression of an isoflavonoid phytoalexin defense response. **Plant Physiology**, Minneapolis, v. 108, n. 1995, p. 1449–1454, 1995.

WALTER, M.H.; FESTER, T.; STRACK, D. Arbuscular mycorrhizal fungi induce the non-mevalonate methylerythritol phosphate pathway of isoprenoid biosynthesis correlated with accumulation of the "yellow pigment" and other apocarotenoids. **Plant Journal: for Cell and Molecular Biology**, Oxford, v. 21, n. 6, p. 571–578, 2000.

WALTER, M.H.; FLOSS, D.S.; STRACK, D. Apocarotenoids: hormones, mycorrhizal metabolites and aroma volatiles. **Planta**, Berlin, v. 232, n. 1, p. 1–17, 2010.

XU, H.; FREITAS, M.A. MassMatrix: a database search program for rapid characterization of proteins and peptides from tandem mass spectrometry data. **Proteomics**, Weinheim, v. 9, n. 6, p. 1548–1555, 2009.

YANG, T.; POOVAIAH, B.W. Calcium/calmodulin-mediated signal network in plants. **Trends in Plant Science**, Oxford, v. 8, n. 10, p. 505–512, 2003.

YESBERGENOVA, Z.; YANG, G.; ORON, E.; SOFFER, D.; FLUHR, R.; SAGI, M.The plant Mo-hydroxylases aldehyde oxidase and xanthine dehydrogenase have distinct reactive oxygen species signatures and are induced by drought and abscisic acid. **Plant Journal: for Cell and Molecular Biology**, Oxford, v. 42, n. 6, p. 862–876, 2005.

ZAREPOUR, M.; KASPARI, K.; STAGGE, S.; RETHMEIER, R.; MENDEL, R.R.; BITTNER, F. . Xanthine dehydrogenase AtXDH1 from Arabidopsis thaliana is a potent producer of superoxide anions via its NADH oxidase activity. **Plant Molecular Biology**, Dordrecht, v. 72, n. 3, p. 301–310, 2010.

ZETTLER L.W. Terrestrial orchid conservation by symbiotic seed germination: techniques and perspectives. **Selbyana**, Sarasota, v. 18, p. 188–194, 1997.

ZETTLER, L.W.; HOFER, C.J. Propagation of the little club-spur orchid (*Platanthera ellata*) by symbiotic seed germination and its ecological implications. **Environmental and Experimental Botany**, Elmsford, v. 39, p. 189–195, 1998.

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**, London, v. 8, n. 8, e72705, 2013.

ZSÖGÖN, A.; LAMBAIS, M.R.; BENEDITO, V.A.; FIGUEIRA, A.V.O.; PERES, L.E.P. Reduced arbuscular mycorrhizal colonization in tomato ethylene mutants. **Scientia Agricola**, Piracicaba, v. 65, n. 3, p. 259–267, 2008.

3 DIFFERENTIAL ACCUMULATION OF PROTEINS IN MYCORRHIZAL ROOTS OF *Oeceoclades maculata* (ORCHIDACEAE)

Abstract

The differential accumulation of proteins in orchid mycorrhizal roots has never been investigated using high throughput technologies. We have used three different proteomic approaches to identify molecular changes occurring in mycorrhizal roots of the terrestrial green orchid *Oeceoclades maculata*. Protein accumulation in mycorrhizal and non-mycorrhizal roots was compared in three independent experiments with three biological replicates for each condition. For each biological replicate, we pooled material from 2 to 4 plants, depending on the assay. After extraction, proteins were quantified and identified by LC–tandem mass spectrometry, using both unlabeled and iTRAQ labeled samples, and an ESI–Q-ToF MS/MS mass spectrometer. A 2D-DiGE was also performed and differentially expressed proteins were identified using Maldi Q-ToF MS/MS. All protein searches were performed using either Mascot or Protein Pilot software with the NCBInr green plants database (07/2012). Using the label free approach, 188 proteins were identifiedof which 94 proteins showed differential regulation. Among the 168 proteins identified using the iTRAQ approach 86 were differentially expressed being 10 below the statistical threshold (p <0.05). Using 2D-DIGE, 60 spots with differential accumulation were detected, but 15were identified. Our data suggest differential regulation of plant defense responses, redox homeostasis, increased proteases biosynthesis, carbohydrate metabolism and water transport in *O. maculata* mycorrhizal roots, and represent an important step towards a better understanding of the mechanisms involved in the regulation of orchid mycorrhizal symbiosis.

Keywords: Orchid mycorrhiza; Proteomics; iTRAQ; Label-free; 2D-DIGE

3.1 Introduction

With more than 27,000 accepted species (THE PLANT LIST, 2010), the Orchidaceae represents the largest flowering plant family and account for roughly 10% of the plant kingdom diversity. All orchids produce endosperm-lacking seeds, and are dependent on mycorrhizal fungi for seed germination and seedling establishment (RASMUSSEN; RASMUSSEN, 2009).

Despite the significance of the mycorrhizal interactions in the biology and ecology of orchids, studies on gene expression in orchid mycorrhiza (OM) are sparse, as compared to other mycorrhizal associations. Watkinson and Welbaum (2003) were the first to show, using differential display of reverse transcripts, the up-regulation of a nucleotide binding protein and down-regulation of a trehalose-phosphate phosphatase in *Cypripedium parviflorum* var. *pubescens* mycorrhizal roots, and only recently, Zhao et al. (2013) were able to identify

several genes expressed only in symbiotically germinated seeds of *Dendrobium officinale*, using suppression subtractive hybridization (SSH).

Oeceoclades maculata (Lindley) Lindley is a terrestrial invasive orchid, first described in a Brazilian orchid collection in 1827. This species has since spread at an accelerated rate through tropical and subtropical areas (AGUIAR et al., 2012). Its large roots allow the easy detection of mycorrhizal colonization and therefore rapid screening for mycorrhizal and non-mycorrhizal root fragments. Besides being a widespread orchid, our observations indicates that *O. maculata*have in general only one or two fully colonized roots per individual, whereas the remaining roots are not colonized, and may accumulate large amounts of starch. Thus, *O. maculata* is a suitable experimental system to investigate the regulation of gene expression (mRNA and protein profiling) in orchid mycorrhiza, mostly because it allows the comparison of mycorrhizal and non-mycorrhizal roots from the same individual, but also because it provides plenty of biological material for molecular analyses, allowing the use of technical and biological replicates.

Two-dimensional electrophoresis (2-DE) is a common technique used for protein analyses in proteomic research (RABILLOUDet al.,2010). This technique consists on the separation of proteins based on their isoelectric points by isoelectric focusing (IEF), and then based on their molecular masses, under denaturing conditions, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). However, the analyses of differential accumulation using conventional 2-D gel-based depends on analyzing each test sample in a single gel, which can lead to dramatic bias as a result of gel-to-gel variation. In order to improve reproducibility when comparing samples, the DIGE technique was developed (ÜNLÜ et al., 1997). DIGE is based on the separation of two or more samples differentially labeled with fluorescent dyes (*i.e.*, Cy3 and Cy5) in the same gel. On the other hand, gel-free proteomic techniques, which are based on the separation of peptides by liquid chromatography prior to tandem mass spectrometry (MS/MS), can also be used to analyze proteomes, with the advantage of using more straight-forward pipelines and reduced amounts of samples. In the later case, protein quantification can be done using label-free (spectral counting) or labeling techniques such as Isobaric Tags for Relative and Absolute Quantification (iTRAQ).

We have collected mycorrhizal and non-mycorrhizal roots fragments of*O. maculata* and analyzed them using different and complementary proteomic approaches in order to identify proteins involved in the regulation of OM. The relevance of our results and the suitability of

these proteomic techniques for a better understanding of the molecular basis of OM symbiosis are discussed.

3.2 Material and Methods

Sampling

Adult plants of *Oeceoclades maculata* were harvested in the dry season of 2012, in the Piracicaba *campus* of the University of Sao Paulo (Brazil). Plants had their roots cut and washed with distilled water. Roots were then screened for the presence or absence of mycorrhizal fungi. Three biological replicates of each treatment (mycorrhizal or non-mycorrhizal roots) were used for RNA and protein extractions, each one consisting of a pool of roots from two plants, in the case of iTRAQ and RNAseq, and from four plants, in the case of Label-free protein quantification and 2D-Dige. Samples were immediately frozen in liquid nitrogen and stored at -80^{o}C, for further DNA and protein extractions.

Protein extraction

Proteins were extracted following the procedure described by Wang (2006) for recalcitrant plant tissues with modifications. Roots were ground in liquid N_2 using a mortar and pestle. The powdered tissue was placed in microtubes (0.2 g tissue powder per 2.0 mL microtubes) and then resuspended in 1.5 mL cold acetone with PMSF 1%, β-mercaptoethanol 2% e PVPP 1%. After sonication (5 times for 20 s on ice), the tubes were centrifuged at 10,000*g* for 3 min (4ºC). The pellet was washed twice with cold acetone and three times with cold 10% TCA in acetone or until it was colorless, then twice with cold aqueous 10% TCA, and finally twice with cold 80% acetone. After each washing, the pellet was resuspended completely by vortexing and then centrifuged as above. The final pellet was dried at room temperature and used for phenolic protein extraction.

Dried pellet was resuspended in 0.8 mL phenol (Tris-HCl, pH 8.0) and0.8 mL SDS buffer (30% sucrose, 2% SDS, 0.1 M Tris-HCl, pH 8.0, 5% 2-mercaptoethanol) in a 2.0 mL microtube. The mixture was vortexed thoroughly for 30 s and the phenol phase was separated by centrifugation at 10,000*g* for 3 min. The upper phenol phase was transferred to fresh microtubes. Five volumes of 0.1 M ammonium acetate in cold methanol were added to the phenol phase and the proteins precipitated at -80ºC for 30 min. Precipitated proteins were recovered after centrifugation at10.000*g* for 5 min, and were washed with cold 80% acetone twice and 70% ethanol. The final pellet was dried and proteins dissolved in 2-DE rehydration

buffer (8 M urea, 4% CHAPS, 2% IPG buffer, 20 mM dithiothreitol) for in gel applications, or in a detergent-free buffer (8M urea,7 M thiourea) for in-solution mass spectrometry.

In-solution sample preparation and differential digestion

Two sets of samples were prepared. The first one was used directly for Label-free protein quantification by mass spectrometry, and the second was used for iTRAQ (Applied Biosystems Canada, Streetsville, ON) relative quantification.

For iTRAQ analysis, samples containing 50 µg of proteins were processed using the iTRAQ kit (GE Healthcare, São Paulo, Brazil),according to the manufacturer's instructions. Proteins were desalted using 1CC sep pak cartridges (Waters), precipitated overnight using 100% acetone, and dissolved in denaturing buffer containing 5 µmol DTT and 2 µL Iodoacetamide, and incubated at 60°C for 1 h, after vortexing. An amount of 1 µL of cysteine blocking solution (MMTS) was then added to each sample, followed by incubation at room temperature for 10 min. Protein samples were differential digested overnight with trypsin (Promega, Madison, WI, USA) at 37°C as described in (WOJCECHOWSKYJ et al., 2011). The differential digestion was stopped with formic acid (2.5 % final concentration) and samples were centrifuged at 20,000 *g* for 20 min. iTRAQ tags were added in a pairwise arrangement as detailed in Table 3.1. Samples for each biological replicate were pooled together and incubated at room temperature for 1 h prior to mass spectrometry analysis.

Table 3.1 -iTRAQ labeling scheme for forward and reverse tagging of three biological replicates of *Oeceoclades maculata* roots

	Sample	**Mycorrhizal**	**Non-mycorrhizal**
Biological replicate 1	A	114	115
	B (reverse)	115	114
Biological replicate 2	C	114	115
	D (reverse)	115	114
Biological replicate 3	E	116	117
	F (reverse)	117	116

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

An aliquot containing 5 µg of peptides (4.5 µL) of either iTRAQ or Label-free samples was run through a C18 1.7 µm BEH 130 (100 µm x 100 mm) RP-UPLC (nanoAcquity UPLC,

Waters) analytical column coupled to a nano-electrospray tandem Q-Tof PREMIER API mass spectrometer (MicroMass/ Waters) at a flow rate of 600 nL /min. The gradient was 2-90% acetonitrile in 0.1% formic acid for 60 min. The instrument was operated in MS positive mode, data continuum acquisition from m /z 100 –2,000Da, at a scan rate of 1 sec $^{-1}$ and an interscan delay of 0.1 s.

Database searching

Tandem mass spectra were extracted and charge state deconvoluted by Mascot Distiller version 2.4.3. Deisotoping was not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.3.02) and X! Tandem (TheGPM, thegpm.org; version Cyclone 2010.12.01.1). Mascot was set up to search the NCBInr greenplants (07/2012; 2,229,089 entries) assuming trypsin digestion. X! Tandem was set up to search a subset of the same database also assuming trypsin differential digestion. Mascot and X! Tandem were searched with a fragment ion mass and parent ion tolerances of 0.100 Da. Carbamidomethylation of cysteine was specified in Mascot and X! Tandem as a fixed modification. Deamidation of asparagine and glutamine and oxidation of methionine were specified as variable modifications. For the iTRAQ samples, iTRAQ-K, iTRAQ-N were set as fixed modifications and iTRAQ-Y and oxidation of methionine were set as variable modifications.

Criteria for protein identification

Scaffold (version 4.0.6.1, Proteome Software Inc., Portland, OR) was used to validate MS /MS based peptide and protein identifications. Peptide identifications were accepted if they could be established with more than 99.0% probability, to achieve an FDR <1.0%. Peptide Probabilities from X! Tandem were assigned by the Peptide Prophet algorithm (KELLER et al., 2002) with Scaffold delta-mass correction. Protein identifications were accepted if they could be established with more than 95.0% probability, to achieve an FDR <5.0%. Protein probabilities were assigned by the Protein Prophet algorithm (NESVIZHSKII; AEBERSOLD, 2005). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins were annotated with GO terms from NCBI (downloaded 17/07/2013) using the Blast2Go software (ASHBURNER et al., 2000).

Sample preparation for 2D-DIGE

Analytical gels: 100µg of each protein sample was used for labeling reaction with CyDye™ Fluor minimal labeling reagents (Lumiprobe, Hallandale Beach, FL, USA). Protein samples were resuspended in a minimum volume of CyDye labeling-compatible lysis buffer containing 40 mMTris–HCl, 8 M urea, 4% (w/v) CHAPS at pH 8.5. Protein samples of each biological replicate were labeled as following: gels 1 and 3:Cy3™ - mycorrhizal, Cy5™ - non-mycorrhizal roots; gel 2: Cy3™ - non-mycorrhizal,Cy5™ - mycorrhizal. Labeling reactions were performed according to the 2D-Dige minimal labeling protocol. The labeled samples for each experiment were mixed together and volume was adjusted to 340 µL with rehydration buffer to a final solution containing 8M urea, 2% (w/v) CHAPS, 0.5% DTT, 0.5% IPGbuffer 4-7 (GE Healthcare, São Paulo, Brazil).

Preparative gels: For protein identification by MS analysis (Maldi TOF-TOF), one additional gel for each treatment was prepared with the same sample composition as described for 2D-DIGE, but 250 µg of each protein sample was loaded.

2D Electrophoresis

Samples prepared for analytical and preparative gels were submitted to IEF and 2D electrophoresis in a single run using an Etan Dalt Six electrophoresis system (GE Healtchare, Sao Paulo, Brazil). Samples were added to IPG immobiline dry stripspH 4-7 (GE Healthcare, São Paulo, Brazil) and rehydrated for 12 h. Voltage settings for IEF were 250 V for 4 h; 300 V, 500 V, 1000 V, 3000 V, and 5000 V for 1 h each; and 8000 V until achieve a total of 40 kVh. For the second dimension, the gel strips were incubated with equilibration buffer 1 (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 1% DTT) and equilibration buffer 2 (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS,0.002% bromophenol blue, 2.5% iodoacetamide) for 30 min each, and subsequently placed onto 12.5% polyacrylamide gel (26 x 32 cm) with a Tris-glycine buffer system as described by Laemmli (1970). Strips were overlaid with an agarose sealing solution (0.25 M Tris-HCl, 1.92 M glycine, 1% SDS, 0.5% agarose, 0.002% bromophenol blue). The initial 2D eletrophoresis setting was 5 W (constant and maximal 20 mA), followed by a 6 h at 30 W per gel (constant and maximal 50 mA). The two preparative gels were fixed overnight in 40% methanol and 10% acetic acid and then washed three times for 30 min with distilled water prior to staining with 0.1% Coomassie brilliant blueCBB-G250 for 48 h. Analytical gels were

scanned on a Typhoon Trio Fluorescence Scanner (GE Healthcare, São Paulo, Brazil) at 300 micron resolution using excitation wavelengths of 532 nm for Cy3™ and 633 nm for Cy5™.

Gel analysis of the multiplexed 2D-Dige images was performed with the Progenesis SameSpots V 4.5 software (NonLinear Dynamics). Spot volumes were normalized by a ratiometric approach to centre the standardized abundance values to one, where standardized abundance is the Cy3 normalized spot volume divided by the Cy5 normalised spot volume, thus eliminating the necessity of a internal standard (as recommended for standard 2D-Dige). Spots that were significantly different between treatments (ANOVA, $p< 0.05$) and fold change > 1.5 were selected to generate a spot picking list.

Protein Digestion and MS Analysis

Protein spots were manually excised from gels and placed in 1.5 mL microtubes, destained with 50% acetonitrile (ACN) and 25 mM ammonium bicarbonate. Gel plugs were dehydrated with 100% ACN and rehydrated with 20 mM DTT; after incubating for 40 min at 56 °C, the supernatant was discarded and replaced with 55 mM iodoacetamide. The tubes were stored in the dark for 30 min, and the gel pieces were dehydrated again with 100% ACN and allowed to air dry after solvent removal. The gel pieces were rehydrated with 10 ng/ml trypsin solution (Promega) in 25 mM ammonium bicarbonate, and the tubes were incubated for 12 h at 37 °C. To extract the peptides, the gel pieces were incubated twice with 50 µl of 60% v/v ACN and 1% v/v formic acid (FA) and once with 50 µL of ACN. The pellets containing the peptides were resuspended in 0.1% v/v FA for MS analysis.

3.3 Results

We have used different and complementary proteomic techniques to investigate plant-fungus interactions in OM. For this purpose, plants of the terrestrial orchid *Oeceoclades maculata* were collected and roots screened for the presence of mycorrhizal fungi under a stereoscopic microscope. Mycorrhizal colonization was easily detected in the thick *O. maculata* roots (Figure 3.1).

Mycorrhizal (Myc) and non-mycorrhizal (Non-Myc) roots collected from 2-4 plants were pooled together in order to have one Myc and one Non-Myc analytical sample, respectively. Three separate biological replicates were used for DNA and protein extractions. DNA was used for direct amplification of the fungal ITS region, cloning and sequencing, in order to identify the main fungal symbionts. Proteins were extracted and processed using

three different proteomic approaches: one gel-based (2D-DIGE) and two gel-free (iTRAQ® and Label-free LC-MS/MS).

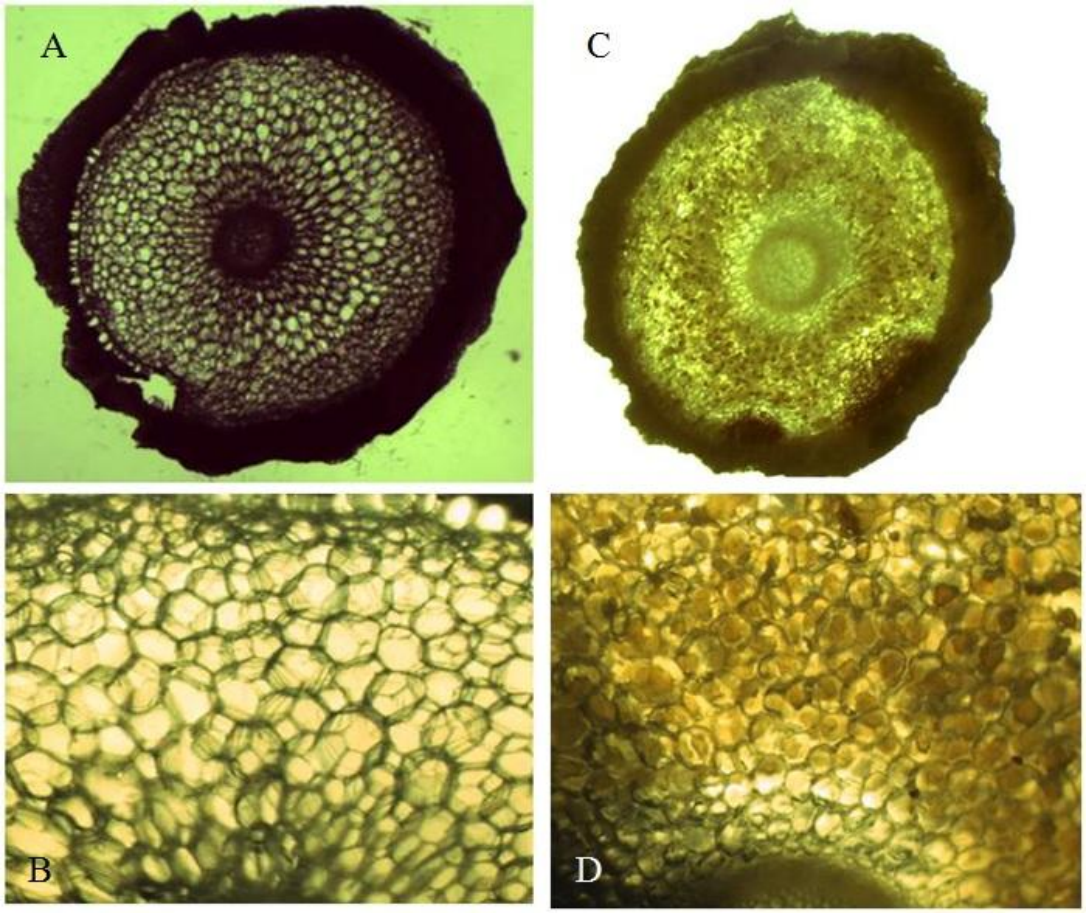

Figure 3.1 -A: Transversal segment of a non-mycorrhizal root of *Oeceoclades maculata* under stereoscopic microscope. B: Non-mycorrhizal roots under light microscopy (2X). C: Tranversal mycorrhizal root segment under stereoscopic microscope. D: Mycorrhizal root under light microscopy (2x)

2D-DIGE

Using 2D-DIGE, 749 protein spots were detected (Figure 3.2). After image analyses and statistical comparisons, 60 protein spots were found to be differentially accumulated (fold change >1.5; $p<0.05$) in Myc roots of *O. maculata*. These protein spots were excised from the gels and analyzed by Maldi Q-ToF MS/MS. Sixteen proteins were identified, including 6 up- and 6 down-regulated plant proteins in Myc roots, as well as 4 fungal proteins unique to Myc roots (Table 3.2). The relatively low number of identified proteins could be explained by the low amount of protein used in the preparative gels (approximately 250 µg for either, Myc and Non-Myc gels), which might have been limiting for obtaining a high score protein identification. On the other hand, it is also possible that most of the isolated proteins represented novel plant and/or fungus proteins, which are not in the public databases.

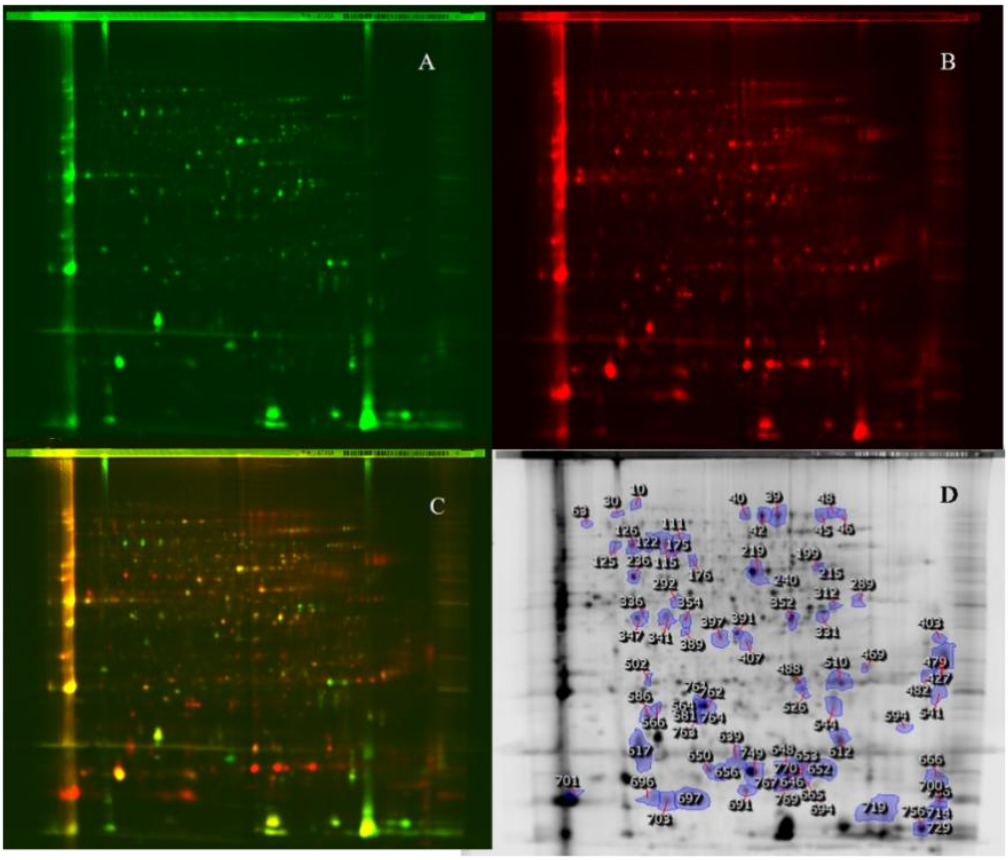

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Figure 3.2 -2D-DIGE of proteins isolated from *Oeceoclades maculata* mycorrhizal andnon-mycorrhizal roots. All images refer to the same gel but scanned with different wavelengths **A:** Proteins from non-mycorrhizal roots stained with Cy3. **B:** Proteins from mycorrhizal roots stained with Cy5. C: Overlaid images of A and B. **D:** Spot picking list

Table 3.2 -Significant protein hits from differentially accumulated spots of mycorrhizal and non-mycorrhizal roots of *O. maculata* (fold change >1,5; $p < 0.05$), observed with the 2D-DIGE technique

Plant Proteins						
Spot ID	**Anova**	**Fold Change**	**Best Protein Accession**	**Best Protein Mass**	**Best Protein Score**	**Best Protein Description**
586	0.009	5.1	ref|XP_002277861.1|	2630529	156	Chaperonin
762	0.003	5.2	gb|ABD98056.1|	2184808	90	quinone-oxidoreductase QR2
42	0.013	5.4	ref|XP_002518324.1|	7130398	87	heat shock protein
391	0.004	4.3	ref|XP_001419026.1|	2372541	77	ABC(binding protein) family transporter
469	0.048	3.7	emb|CAN60148.1|	2643814	73	Dienelactone hydrolase family
199	0.019	2.9	F2YP47_VANPL	5004591	77	Beta-tubulin
594	0.029	-2	gb|ABC55649.1|	1912855	98	translationally controlled tumor protein
666	0.013	-1.6	ref|XP_002866880.1|	2034206	90	lipid-associated family protein
219	0.003	-1.6	sp|P84541.1|ENO_POPEU	274941	76	Enolase
336	0.015	-1.8	MDHC_BETVU	3581036	67	Malate dehydrogenase
354	0.015	-1.6	G9FP71_9ASPA	3134526	63	Glyceraldehyde 3P dehydrogenase
352	0.002	-1.6	LGUL_BRAOG	3174010	94	Lactoylglutathione lyase
Fungal Proteins						
Spot ID	**Anova**	**Fold Change**	**Best Protein Accession**	**Best Protein Mass**	**Best Protein Score**	**Best Protein Description**
612	0,012	4.3	ACK1_YEAST	6995384	58	Activator of C kinase protein
236	0,005	3.5	ref|XP_002911568.1|	5305201	146	Histidine phosphatase superfamily
650	0,001	4.9	ref|XP_961813.1|	204088	63	pyrABCN

Fold Change = Mycorrhizal /Non-mycorrhizal normalized peak intensity values.

Gel Free LC MS/MS differential protein expression

Using a label-free approach, we were able to identify a total of 188 proteins in *O. maculata* roots. In the Scaffold software, 51 and 50 proteins were detected exclusively in Myc and Non-Myc roots, respectively (data not shown). In order to reduce the possibility of assigning a protein hit to a sample category due to random identification bias, we only considered hits that were identified in at least two biological replicates.

After filtering, 33 proteins were only found and 15 proteins were up-regulated Myc roots, and 39 proteins only found in Non-Myc roots and 8 were down regulated, totalizing 95 proteins with differential expression.These hits arelisted in Table 3; protein names written in bold indicate that theirfold change values are significant among biological replicates (Student *t*-test;p <0.05).

iTRAQ

Using the iTRAQ approach, 168 proteins were identified, and 86 showed differential accumulation (fold change >1.5). In this analysis, we have focused only in proteins that were statiscally significant (Mann-Whitney test, p <0.05) among replicates. One technical replicate (S1-F) was removed from the dataset due to technical problems.

Comparing both gel free LC-MS/MS techniques, 86 proteins were found with either iTRAQ or Label-free approach, 102 proteins were identified only with the Label-free whilst other 82 were identified only with iTRAQ.

Table 3.3 -Proteins differentially expressed in mycorrhizal roots of *O. maculata* using the Label free-LC MS/MS approach

(continue)

Protein ID	Accession	MW	FC	Myc1	Myc2	Myc3	Ref	Ref	Ref
jasmonate ZIM-domain protein 2	gi|196259696	32 kDa	**Myc**	0	95%	98%	0	0	0
cucumisin-like	gi|359486591	81 kDa	**Myc**	100%	100%	100%	0	0	0
NADH-dependent enoyl-ACP reductase*	gi|75225229	47 kDa	**Myc**	100%	100%	100%	0	0	78%
cyclophilin	gi|192910744	18 kDa	**Myc**	100%	100%	100%	0	0	0
hypothetical protein SORBIDRAFT_01g050550	gi|241919896	81 kDa	**Myc**	0	95%	94%	0	0	0
cysteine proteinase	gi|148927394	51 kDa	**Myc**	93%	100%	100%	0	0	0
alpha-glucosidase	gi|15239154	101 kDa	**Myc**	100%	100%	100%	0	0	0
NADH dehyd. [ubiquinone] Fe-Su protein 7*	gi|259491355	23 kDa	**Myc**	99%	99%	99%	0	0	0
Cell division cycle protein 27 homolog B	gi|75330104	81 kDa	**Myc**	84%	100%	99%	0	0	0
lysosomal alpha-mannosidase-like	gi|357156617	113 kDa	**Myc**	65%	100%	100%	0	0	0
nitrile-specifier protein 5	gi|225447524	35 kDa	**Myc**	100%	100%	100%	0	0	0
Hypothetical protein	gi|300138377	80 kDa	**Myc**	85%	100%	95%	0	0	0
Oryzain beta chain	gi|109939735	50 kDa	**Myc**	100%	99%	100%	0	0	0
LRR receptor-like ser/thr-protein kinase *	gi|125581346	111 kDa	**Myc**	85%	81%	98%	0	0	0
vacuolar 69 kDa subunit	gi|1049253	62 kDa	**Myc**	99%	53%	100%	0	0	0
glyceraldehyde-3-phosphate dehydrogenase 1	gi|258642941	37 kDa	**Myc**	47%	97%	100%	0	0	0
arabinosidase ARA-1	gi|16417958	74 kDa	**Myc**	100%	100%	0	0	0	0
RUBISCO large subunit	gi|156454192	51 kDa	**Myc**	95%	96%	0	0	0	0

Table 3.3 -Proteins differentially expressed in mycorrhizal roots of *O. maculata* using the Label free-LC MS/MS approach

(continuation)

Protein ID	Accession	MW	FC	Myc1	Myc2	Myc3	Ref	Ref	Ref
hypothetical protein ARALYDRAFT_354856	gi|297313807	12 kDa	**Myc**	68%	99%	75%	0	0	0
Aspartic proteinase*	gi|78099760	54 kDa	**Myc**	96%	97%	11%	0	0	0
predicted protein	gi|162662890	31 kDa	**Myc**	100%	0	83%	0	0	0
Cucumisin*	gi|297321645	40 kDa	**Myc**	96%	42%	0	0	0	0
Actin-1, putative, expressed	gi|108710772	42 kDa	**Myc**	100%	100%	100%	0	0	0
Hypo. SORBIDRAFT_09g019152	gi|241946316	32 kDa	**Myc**	99%	98%	0	0	0	0
ATPase alpha subunit (mitochondrion)	gi|388890762	55 kDa	**Myc**	0	76%	99%	0	0	0
eukaryotic translation initiation factor 3	gi|356524672	105 kDa	**Myc**	0	100%	70%	0	0	0
Glucan endo-1,3-beta-glucosidase 11	gi|75154301	35 kDa	**Myc**	64%	99%	0	0	0	0
ATP synthase alpha subunit	gi|20146556	43 kDa	**Myc**	100%	0	100%	0	0	0
cysteine proteinase	gi|595986	47 kDa	**Myc**	0	92%	100%	0	0	0
putative plasma membrane intrinsic protein	gi|27527694	31 kDa	**Myc**	98%	0	83%	0	0	0
Patellin-1*	gi|78099065	17 kDa	**Myc**	0	79%	98%	0	0	0
Disease resistance protein RPP13*	gi|29839653	105 kDa	**Myc**	47%	95%	0	0	0	0
hypothetical protein VOLCADRAFT_105607	gi|300262036	330 kDa	**Myc**	0	98%	42%	0	0	0
glutathione reductase	gi|1370285	54 kDa	**6.7**	0	100%	100%	100%	100%	0
cytosolic ascorbate peroxidase	gi|120969450	27 kDa	**5.5**	45%	100%	96%	100%	0	0
predicted protein	gi|326502400	59 kDa	**5.3**	0	100%	100%	100%	0	52%
fructose-bisphosphate aldolase	gi|226316441	39 kDa	**5.2**	96%	96%	99%	84%	99%	90%

Table 3.3 -Proteins differentially expressed in mycorrhizal roots of *O. maculata* using the Label free-LC MS/MS approach

(continuation)

Protein ID	Accession	MW	FC	Myc1	Myc2	Myc3	Ref	Ref	Ref
vacuolar H+-pyrophosphatase	gi|336112676	80 kDa	**5**	0	0	100%	51%	100%	0
aquaporin	gi|1212923	25 kDa	3.2	100%	100%	100%	100%	100%	0
NADP malic enzyme4	gi|162463047	72 kDa	3	100%	0	0	76%	0	95%
Glyceraldehyde-3-phosphate dehydrogenase	gi|120669	37 kDa	2.9	100%	99%	99%	99%	99%	100%
pathogenesis-related protein	gi|359840826	17 kDa	2.9	96%	64%	97%	66%	42%	99%
ATPase subunit 1 (mitochondrion)	gi|340549482	55 kDa	2.6	11%	100%	100%	100%	0	100%
Pyruvate kinase*	gi|2497538	57 kDa	2.5	100%	100%	100%	100%	100%	100%
Elongation factor EF-2	gi|355485214	94 kDa	2.5	0	100%	100%	100%	100%	100%
Core histone H2A/H2B/H3/H4 family protein	gi|108707495	44 kDa	2.3	100%	100%	100%	100%	100%	100%
Enolase	gi|1169534	48 kDa	2.2	100%	100%	100%	100%	100%	100%
Glyceraldehyde-3-phosphate dehydrogenase	gi|462137	37 kDa	2.1	100%	100%	100%	100%	100%	100%
elongation factor 1-alpha	gi|327164449	49 kDa	-2	99%	100%	0	100%	100%	100%
Malate dehydrogenase*	gi|18202485	36 kDa	-2	0	100%	100%	100%	100%	100%
Elongation factor 2*	gi|6015065	94 kDa	-2,5	100%	0	100%	100%	100%	100%
heat shock protein 90	gi|110270498	80 kDa	-3,3	100%	0	0	100%	80%	96%
Enolase	gi|1169534	48 kDa	-5	100%	0	5%	100%	100%	100%
homocysteine methyltransferase*	gi|8134566	85 kDa	-5	100%	100%	100%	100%	100%	100%
Chaperonin CPN60-2	gi|355480690	61 kDa	-5	100%	0	0	100%	100%	100%
heat shock protein 70	gi|189380223	75 kDa	-5	100%	100%	100%	100%	100%	100%
lipoxygenase, partial	gi|384407029	49 kDa	Non-myc	0	0	0	100%	100%	100%

Table 3.3- Proteins differentially expressed in mycorrhizal roots of *O. maculata* using the Label free-LC MS/MS approach

(continuation)

Protein ID	Accession	MW	FC	Myc1	Myc2	Myc3	Ref	Ref	Ref
methionine synthase protein, partial	gi|374256013	33 kDa	**Non-myc**	0	0	0	100%	100%	100%
FLORICAULA/LEAFY	gi|67515298	6 kDa	**Non-myc**	66%	21%	5%	100%	95%	96%
alpha-tubulin	gi|134035494	50 kDa	**Non-myc**	0	0	0	100%	100%	100%
homocysteine methyltransferase, putative	gi|223535009	85 kDa	**Non-myc**	0	0	0	100%	100%	100%
L-ascorbate peroxidase 1*	gi|122247194	24 kDa	**Non-myc**	0	0	76%	100%	100%	100%
Subtilisin-like protease SDD1	gi|75099062	79 kDa	**Non-myc**	0	0	0	100%	100%	100%
lypoxygenase	gi|165928943	39 kDa	**Non-myc**	0	0	0	100%	100%	100%
FLORICAULA/LEAFY	gi|67515298	6 kDa	**Non-myc**	66%	21%	5%	100%	95%	96%
alpha-tubulin	gi|134035494	50 kDa	**Non-myc**	0	0	0	100%	100%	100%
homocysteine methyltransferase, putative	gi|223535009	85 kDa	**Non-myc**	0	0	0	100%	100%	100%
L-ascorbate peroxidase 1*	gi|122247194	24 kDa	**Non-myc**	0	0	76%	100%	100%	100%
Subtilisin-like protease SDD1	gi|75099062	79 kDa	**Non-myc**	0	0	0	100%	100%	100%
lypoxygenase	gi|165928943	39 kDa	**Non-myc**	0	0	0	100%	100%	100%
L-ascorbate peroxidase 1*	gi|122247194	24 kDa	**Non-myc**	0	0	76%	100%	100%	100%
Subtilisin-like protease SDD1	gi|75099062	79 kDa	**Non-myc**	0	0	0	100%	100%	100%
lypoxygenase	gi|165928943	39 kDa	**Non-myc**	0	0	0	100%	100%	100%
L-ascorbate peroxidase 1*	gi|122247194	24 kDa	**Non-myc**	0	0	76%	100%	100%	100%
Subtilisin-like protease SDD1	gi|75099062	79 kDa	**Non-myc**	0	0	0	100%	100%	100%

Table 3.3 - Proteins differentially expressed in mycorrhizal roots of *O. maculata*using the Label free-LC MS/MS approach

(continuation)

Protein ID	Accession	MW	FC	Myc1	Myc2	Myc3	Ref	Ref	Ref
lypoxygenase	gi|165928943	39 kDa	**Non-myc**	0	0	0	100%	100%	100%
L-ascorbate peroxidase 1*	gi|122247194	24 kDa	**Non-myc**	0	0	76%	100%	100%	100%
Subtilisin-like protease SDD1	gi|75099062	79 kDa	**Non-myc**	0	0	0	100%	100%	100%
hypothetical protein VITISV_041925	gi|147809484	26 kDa	**Non-myc**	0	0	0	100%	100%	100%
malate dehydrogenase	gi|344190166	36 kDa	**Non-myc**	0	0	0	100%	100%	100%
60S ribosomal protein L3-1*	gi|27735225	63 kDa	**Non-myc**	0	0	0	98%	100%	100%
conserved hypothetical protein	gi|223519414	8 kDa	**Non-myc**	0	0	0	100%	100%	93%
fructokinase	gi|1052973	35 kDa	**Non-myc**	0	0	0	100%	100%	0
Fructose-bisphosphate aldolase*	gi|1168410	28 kDa	**Non-myc**	0	0	0	100%	98%	0
GDP-mannose 3',5'-epimerase	gi|110740559	43 kDa	**Non-myc**	0	0	0	97%	100%	100%
60S ribosomal protein L11*	gi|1173055	20 kDa	**Non-myc**	54%	0	0	100%	100%	0
Allene oxide synthase 3*	gi|73619654	54 kDa	**Non-myc**	0	0	0	100%	100%	0
S-adenosylmethionine synthetase	gi|100801600	43 kDa	**Non-myc**	0	0	0	100%	100%	0
Clathrin heavy chain 1*	gi|122223702	193 kDa	**Non-myc**	0	0	0	100%	100%	0
26S proteasome subunit 11*	gi|75264101	46 kDa	**Non-myc**	0	0	0	0	100%	97%
adenine nucleotide translocator	gi|1297066	36 kDa	**Non-myc**	0	83%	0	0	99%	98%
Elongation factor 1-alpha*	gi|6015058	49 kDa	**Non-myc**	44%	0	0	0	100%	100%
phosphoglycerate kinase, putative	gi|102139814	42 kDa	**Non-myc**	0	0	0	100%	0	100%
Peroxidase 51*	gi|26397925	36 kDa	**Non-myc**	0	0	0	100%	0	100%
ubiquitin monomer protein	gi|73747822	17 kDa	**Non-myc**	0	0	0	100%	100%	0

Table 3.3 -Proteins differentially expressed in mycorrhizal roots of *O. maculata*using the Label free-LC MS/MS approach

(conclusion)

Protein ID	Accession	MW	FC	Myc1	Myc2	Myc3	Ref	Ref	Ref
allene oxide synthase	gi|375004669	45 kDa	**Non-myc**	0	0	0	99%	100%	0
PREDICTED: RING-H2 finger protein	gi|357127651	23 kDa	**Non-myc**	0	0	0	49%	99%	0
Isocitrate dehydrogenase*	gi|1708402	47 kDa	**Non-myc**	0	0	0	28%	100%	0
40S ribosomal protein S14*	gi|131772	16 kDa	**Non-myc**	0	0	0	0	100%	93%
pentatricopeptide repeat-containing protein	gi|297336922	63 kDa	**Non-myc**	0	0	0	87%	95%	0

FC =Fold change in relation to the reference category; MW= Predicted molecular weight. Myc1, Myc2 and Myc3 values refers to the protein identification probability in each biological replicate. REF refers to the same values in reference samples (in this case, non-mycorrhizal roots). Myc and Non-Myc (in the FC columns) refers to proteins which are assumed to be only expressed in mycorrhizal or non mycorrhizal roots respectively. * pBLAST best hit with e-value < 10e-5. Fold change values written in bold are statistically significant (p <0.05).

Table 3.4 -Relative quantification of proteins with significant differential accumulation in mycorrhizal and non-mycorrhizal roots of Oeceoclades maculata using iTRAQ

Highest hit in database	Accession Number	MW Test	S1-R	S2-F	S 2-R	S3-F	S3-R	Average	FC
40S ribosomal protein S15 [Arabidopsis thaliana]	gi|10178203	95% (0.021)	0	1.6	1	1.1	1.9	1.12	2.2
PR protein [Musa acuminata AAA Group]	gi|359840826	95% (0.0090)	0.6	0.7	0.8	1	0.9	0.8	1.7
peroxidase 12-like [Brachypodium distachyon]	gi|357157932	95% (0.021)	0.3	0	0.4	1.4	1.3	0.68	1.6
SORBIDRAFT_04g019020 [Sorghum bicolor]	gi|241933653	95% (0.0017)	-	0.5	0.8	0.8	0.5	0.65	1.6
predicted protein [Hordeum vulgare subsp. vulgare]	gi|326527391	95% (0.00016)	1	0.4	0.7	0.4	0.5	0.6	1.5
lypoxygenase [Capsicum annuum]	gi|165928943	95% (0.0090)	-0.8	-0.6	-0.5	-0.5	-0.5	-0.58	-1.5
SELMODRAFT_129301 [Selaginella moellendorffii]	gi|300143068	95% (0.0090)	-1	-1	-0.8	-0.2	-0.2	-0.64	-1.6
PREDICTED: annexin D3-like [Glycine max]	gi|356556843	95% (0.0090)	-0.9	-0.5	-0.3	-0.6	-1	-0.66	-1.6
heat shock protein [Fragaria x ananassa]	gi|54306589	95% (0.021)	0	-1.3	-1.2	-0.4	-0.5	-0.68	-1.6
16.9 kDa class I heat shock protein 1 [Zea mays]	gi|195605946	95% (0.021)	-2.2	-1.3	-1.5	-	-	-1.66	-3.2

S1-F, S2-F and S3-F: biological sample replicates 1, 2 and 3 with forward tagging; S1-R, S2-R and S3-R: biological sample replicates 1, 2 and 3 with reverse tagging. FC= Fold change. MW test = Mann Whittney test (*p*-value). Values from each replicate and Average are expressed as $\log_2$.

Protein categorization

We have used label-free data (more representative dataset) for gene ontology annotation with the Blast2Go software in order determine the putative functions of the proteins identified and their possible roles in OM. Figure 4 shows a multi-level bar chart which was drawn based on automated annotation and a sequence cutoff =15 sequences per category. In the Myc roots, proteins were classified into 10 categories, whereas in Non-Myc roots only 5 categories were represented. In Non-Myc roots, 27.7% of the proteins were assigned to the category carbohydrate metabolic process", 20.5% to "biosynthetic process"; 18.7% to "catabolic process"; 17.9% to "generation of precursors metabolites and energy" and 15.2% to "transport". In Myc roots, the most frequent category was "response to stress", comprising 15.8% of the identified proteins, followed by "catabolic process" (14.9%) and "response to stimulus" (11.1%). Concerning the molecular function assignments, all categories were enhanced in Myc roots, being “nucleotide binding” the most represented (33.9%), followed by “hydrolase activity” (25%), “protein binding” (16.1%), “transport activity” (16.1%) and “nucleic acid binding” (8.87%), which was only represented in mycorrhizal roots.

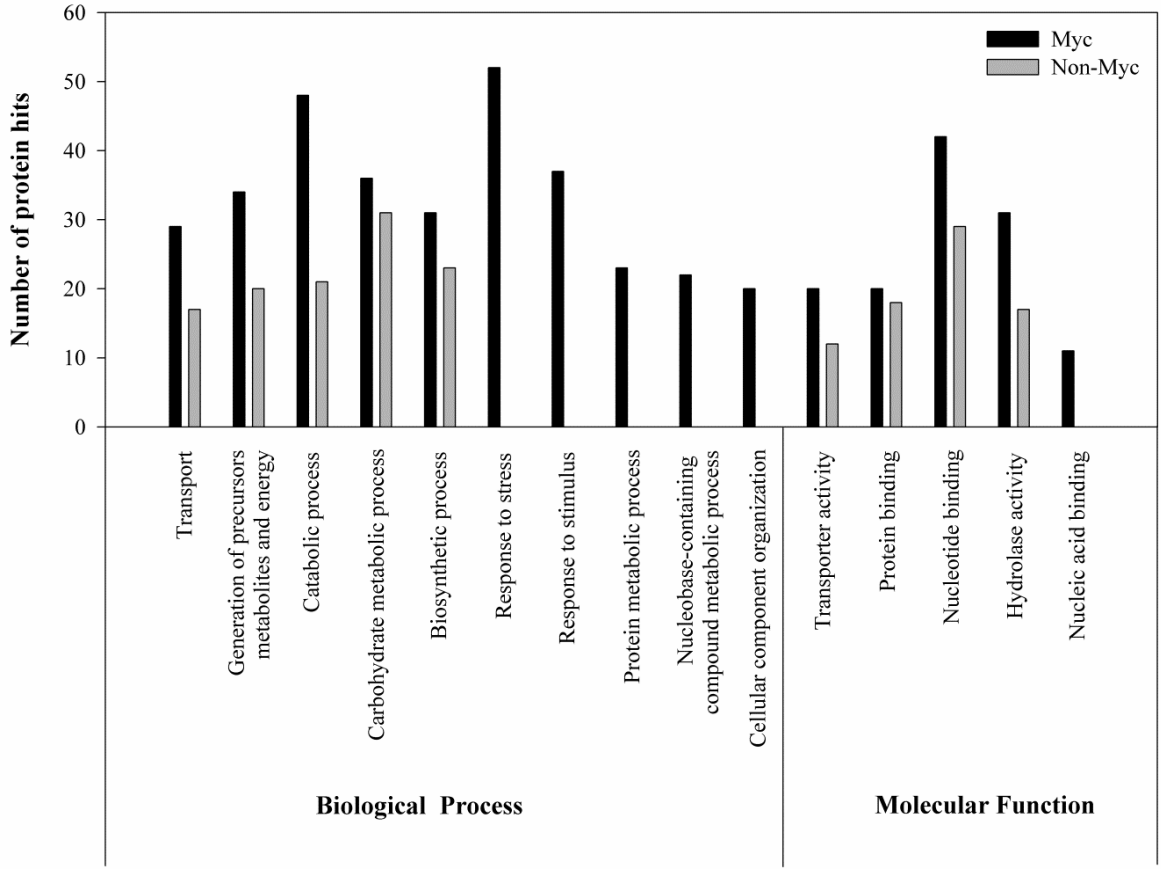


Figure 3.4 -Representation of gene ontology (GO) biological processes and molecular function assignments for plant homologous proteins derived from Label-Free LC-MS/MS proteomics of mycorrhizal (Myc) and non-mycorrhizal (Non-Myc) roots of *Oeceoclades maculata*

3.4 Discussion

Oeceoclades maculata is a widespread orchid in the neotropics, but it was believed that it is orginated from Africa, as it is the only specie of this genera found outside that continent (DOD, 1986; STERN, 1988 apud PESSOAet al., 2012). As it occurs with other orchids, *O. maculata* produces extremelly small seeds lacking energetic reserves, relying on mycorrhizal fungi to germinate. Pessoa et al. (2012) described five different *Rhizoctonia*-like fungi isolated from *O. maculata* in Rio Paranaíba (Minas Gerais, Brazil), together with two other basidiomycetes and one *Fusarium sp*. Surprisingly, the two basidiomycetes, not related to *Rhizoctonia* were the best on promote *O. maculata* in vitro germination, suggesting that this orchid species is generalist and able to recruit fungi outside the *Rhizoctonia* aliance for its germination.

We have cloned and sequenced 18 fungal ITS fragments from O. maculata in the site of this study. One clone is 99% related to a uncultures Basidiomycete clone (NCBI acession EU490155) and to a *Rhizoctonia sp*. 138a (NCBI acession AY586176.1), but 8 clones grouped in a monophyletic clade without close neighboor in the NCBI (data not shown), suggesting that a new orchid mycorrhizal fungal candidate has been found. However, further morphological based studies should address the identity and would characterize the main symbiont of *O. maculata* in the study site.

In this work, we have collected and screened roots of *O. maculata* for the presence or absence of pelotons, which are typical structures of orchid mycorrhiza. We have isolated total proteins and performed three different proteomic techniques in order to address changes in *O. maculata* roots proteome in response to mycorrhizal fungal colonization. Here we discuss our results in the light of recent bibliography concerning plant-microbe interactions, specially focusing in endomycorrhiza symbiosis.

Comparing gel-based versus gel-free proteomic approaches

In the present work we have identified and quantified 188 proteins using a label-free proteomic approach and 168 proteins using iTRAQ. Despite the possibility of enhancing protein identification using fractionation of peptides prior to LC, these numbers are still representative when compared to the 15 proteins identified using2D-DIGE. This gel-based technique has the advantage of high-resolution protein separation and has led to the visualization of 749 spots, 60 of which were differentially accumulated. However, the amount of total protein used in the preparative gels (no more than 250 ug) was limiting for the identification of the excised spots. 2D-DIGE is still a powerful and very suitable technique for studying differential proteomes, especially those of plant-microbe interactions. However, besides the higher requirements of biological material for protein identification, 2D-DIGE it is also much more time consuming than *gel-free* approaches.

Comparing the two *gel-free* techniques we had clearer results with the label-free workflow, being iTRAQ more sensitive to technical bias such as incorrect handling of samples during the preparation of sample procedures. Moreover (WU *et al*., 2006) have shown that iTRAQ is more susceptible to errors in precursor anion isolation, which could be manifested with increasing sample complexity. A representative part of the iTRAQ and Label-free datasets overlaps but the 2D-DIGE have led to the identification of different proteins homologues. Another technical issue that should be commented is that, in the Label-

free approach we identified a number of proteins that were assigned to be exclusive from Myc or Non-Myc samples. This could derive from the fact that we have identified only a small percentage of the proteome or even a strong up- or down- regulation has led to no identification in some of the samples. In order to overcome this problem, we have manually excluded proteins found in only one biological replicate but the exclusivity of proteins to a determined sample group is still an assumption.

Fungal recognition and signaling related proteins

Plant endophytes and pathogens have structural similarities and are recognized by the host's non-self recognition system. Plant cell wall penetration by a fungal intruder is normally accompanied by the release of plant-derived and fungal-derived effector molecules. Hence, endophytes must avoid or overcome non-specific resistance responses to achieve successful penetration and reprogram the invaded cell to accommodate infection structures and to maintain host cell integrity for a long-lasting interaction (KOGEL; FRANKEN; HU, 2006). Accommodation requires sophisticated recognition and for arbuscular mycorrhiza (AM), it is known that the process involves a host receptor-kinase-mediated transmembrane signalling (STRACKE et al., 2002). The recognition of fungal effectors triggers signal transduction cascades that lead to strong defense mobilization (HAMMOND-KOSACK; PARKER, 2003), including general plant responses to microbial infection, such as rapid changes in ion fluxes, production of reactive oxygen species (ROS) and accumulation of antimicrobial compounds (BONFANTE; REQUENA, 2011; CAMPOS-SORIANO; GARCÍA-GARRIDO; SAN SEGUNDO, 2010; GARCÍA-GARRIDO; OCAMPO, 2002), all of them represented somehow in our dataset.

A common class of plant proteins involved in microbe recognition contains a nucleotide-binding site (NBS) and a leucine-rich repeat (LRR) region. LRR domains are typical of peptide-binding motifs involved in protein–protein interaction or ligand binding (KOBE; DEISENHOFER, 1994). For many NBS-LRR class resistance proteins, the LRR domains may be the specific determinants of symbiont recognition (ELLIS; DODDS; PRYOR, 2000). In our dataset we have two candidate fungal recognition receptors, a LRR receptor-like serine/threonine protein kinase and a gastrodianin-4B, which is a mannose-binding lectin with antifungal properties, first isolated from the orchid *Gastrodia elata*(WANG et al., 2001). The LRR receptor has been detected only in *O. maculata*

mycorrhizal roots, but Gastrodianin-4B showed reduced accumulation in the same condition, suggesting that antifungal activities are somehow alleviated in OM roots.

Another protein exclusively found in mycorrhizal roots is a cyclophilin. It belongs to a family of proteins categorized by the ability to bind specific immunosuppressant molecules of fungal origin in eukaryotes (KUMARI et al., 2013). Cyclophilin expression has been shown to be induced by both biotic and abiotic stresses including $HgCl_2$, viral infection, ethephon (an ethylene releaser), salicylic acid, salt stress, heat and cold shock (GODOY; LAZZARO; CASALONGUE, 2000) light (LUAN; ALBERS; SCHREIBER, 1994), drought (SHARMA; SINGH, 2003), wounding, fungal infection, abscisic acid and methyl jasmonate (CHOU; GASSER, 1997). However, the mechanism by which cyclophilins are involved in stress protection is not clear. Most cyclophilins have a peptidyl-prolyl cis/trans isomerase domain, which catalyzes the rate limiting step in protein folding, and might act as chaperones, facilitating the folding of stress related proteins or protecting these proteins from proteolytic degradation or aggregation under stress conditions (KUMARI et al., 2013).

A jasmonic acid (JA) ZIM-domain protein (JAZ2) has also been detected exclusively in mycorrhizal roots. Upon perception of pathogens, JA is synthesized and mediates a signaling cascade resulting in the activation of various plant defense responses. In most cases, necrotrophic fungi have been shown to be the primary activators of JA-dependent defense responses (SIVASANKAR; SHELDRICK; ROTHSTEIN, 2000). Conversely, plants infected with biotrophic fungi have been associated with suppressing JA-mediated responses and, the JAZ proteins acts to repress transcription of jasmonate-responsive genes (BALBI; DEVOTO, 2008). In *O. maculata*, the enzyme allene oxide synthase, which catalyzes the first step in the biosynthesis of jasmonic acid from lipoxygenase-derived hydroperoxides of free fatty acids (SIVASANKAR; SHELDRICK; ROTHSTEIN, 2000), was detected only in Non-Myc roots (with the label free approach). These data suggest that, in *O. maculata* mycorrhizal roots, the suppression of JA biosynthesis and perception acts sinergically to modulate host defense responses.

In fungi and plants, mineral nutrition and growth processes are strongly dependent on H^+ eletrochemical gradients that are primarily generated by proton pumps The generation of an electrochemical gradient across membranes are also a hallmark of plant-microbe interactions (JOLICOEUR et al., 1998) and are required for nutrient transfer across the plasma membrane in AM symbiosis (REQUENA et al., 2003), being one of the major alterations expected in endomycorrhizal systems. Moreover, enhanced electrochemical

potential difference for H^+ across the tonoplast facilitates Na+/H+ antiporter activity, increasing Na^+ sequestration into vacuoles and therefore providing salinity tolerance(PASAPULA et al., 2011; SCHILLING et al., 2013)

We have detected a 5-foldup-regulation of a vacuolar H^+-pyrophosphatase (H^+-PPase) in *O. maculata* Myc roots together with three mitochondrion H^+-ATPases (two were only found and one was up-regulated 2.6 fold in Myc). Ramos et al. (2009) detected a down-regulation of a plasma membrane and a vacuolar H^+-ATPase in the early stages of arbuscular mycorrhizal colonization, together with a concomitant stimulation of the vacuolar H^+-PPase. His data provided evidences for a coordinated regulation of the H^+ pumps, and the activation of root H^+-PPase as an adaptative response to the energetic changes faced by the host during the early stages of the endomycorrhiza symbiosis.

Proteases

In our dataset, at least four proteases (cucumisin, cysteine, aspartic proteases and oryzain beta chain) have been exclusively detected in mycorrhizal roots.In addition, a protein belonging to the dienelactone hydrolase family was 4.3 fold more abundant in Myc roots than in non-myc roots. It is believed that the enzymatic mechanism of dienelactone hydrolases may be similar to that of the thiol and serine proteases(PATHAK; NGAI; OLLIS, 1988). Despite their house keeping roles in degrading non-functional proteins and to release amino acids for recycling, proteases may take part of regulatory systems in plants, such as the recognition of pathogens and the induction of defense responses (VAN DER HOORN; JONES, 2004).

In analogy to other biological systems, there are different hypothesis on how proteases are involved in the perception of fungal symbionts. Proteases may be involved in the release of peptide elicitors from symbiont cell wall or membranes (BOLLER; FELIX, 2009). Secondly, the recognition of specific elicitors may activate proteases, which could trigger downstream signaling components (SERWOLD et al., 2002). Alternatively, binding of specific elicitors to proteases may inhibit their activity, and the elicitor-protease complex or altered proteolytic activity might induce signalling (VAN DER HOORN; JONES, 2004). Thus, proteases may be involved either in the release of positive regulators, in the degradation of negative regulators or in the blocking of signaling pathways (WOLTERING; BENT; HOEBERICHTS, 2002).

Plant proteases may be also directly involved in degrading proteins from the symbiont (VAN DER HOORN; JONES, 2004), and for the senescence of symbiotic structures. The induction of cysteine proteases during nodule senescence is a key process in the later stages of

nodule development leading to proteolysis(KARDAILSKY; BREWIN, 1996; VORSTER et al., 2013). In this sense, it is plausible that, in OM roots, proteases that accumulate to high levels at the site of infection may be involved in restricting fungal colonization in the root cortex or degrading fungal pelotons for further nutrient absorption.

Conserved proteolytic domains are found in a variety of proteins from diferent organisms. As our matches had more similarity with plant proteins, we assume that our proteases are from plant origin. However, due to the putative roles that proteases should have in fungal mycorrhizal symbiosis, specially during the colonization process, the possibility of some of the proteases present in the dataset being from fungal origin cannot be ruled out.

Redox homeostasis

The generation of ROS and the regulation of their homeostasis by anti-oxidant enzymes are frequently observed in plant-microbe interactions, including mutualistic symbioses. ROS can accumulate in specific sub-cellular compartments acting as antimicrobials during the oxidative burst (GARG; MANCHANDA, 2009; TORRES, 2010), and/or interact with other molecules and phytohormones playing multifaceted signaling roles.

Differential expression of antioxidant enzymes has been observed during the colonization process in AM (LAMBAIS et al., 2003) and in the rhizobium-legume interaction (PAULY; PUCCIARIELLO; MANDON, 2006; TORRES, 2010). Staining techniques showed that intracellular accumulation of H_2O_2in AM is higher in the plant cytoplasm close to intact and collapsing fungal structures, whereas intercellular H_2O_2 was located on the surface of fungal hyphae (FESTER; HAUSE, 2005).

In OM roots, we have detected higher levels of a quinone-oxidoreductase (5.1 fold), a peroxidase 12-like protein (1.6 fold), a glutathione reductase (6.7 fold) and a cytosolic ascorbate peroxidase (5.5 fold), as compared to non-mycorrhizal roots. These enzymes are known to be involved in redox homeostasis regulation in response to fungal infection, and may indicate that OM and other plant-fungal interactions such as AM have similar mechanims to alleviate the ROS stress during the colonization process.

The quinone-oxidoreductase belongs to a ubiquitous class of enzymes that catalyze the reduction of quinones using NAD(P)H as electron donor, named cytosolic quinone reductases (QRs). QRs are also known as detoxifying enzymes. In yeast, QRs play a role in protection against quinone-stress by maintaining a balance in ROS levels during stress responses via hydrogen peroxide mediated cell death (SOLLNER et al.,2007, 2009). In plants, QRs have

been associated to salt stress (JIANG et al., 2007) and defense against pathogen infection (GREENSHIELDS et al., 2005). Peroxidases are commonly up-regulated in earlier stages of AM interactions (SPANU; BONFANTE, 1988), and might be related to a non-specific stress response or to a counterattack to a large production of ROS, leading to a peaceful symbiosis (PASSARDI et al., 2005).

Glutathione reductase and ascorbate peroxidase are part of the glutathione-ascorbate cycle, which besides its main role in cellular detoxification, is also related to systemic stress tolerance to biotic and abiotic stresses (WALLER et al., 2005). Based on the function of these proteins, we therefore speculate that the higher accumulation of anti-oxidant enzymes in OM roots, as compared to non-myc roots, might be associated to the need for regulating ROS homeostasis in order to avoid the activation of the plant defense system and development of an incompatible interaction.

Disease and stress related proteins

Disease resistance proteins (R-proteins) are plant proteins that interact directly or indirectly with microbial avirulence effectors triggering several defense responses. These R genes encode proteins that recognize, directly or indirectly, pathogen effector molecules encoded by Avr genes (SHIRASU; SCHULZE-LEFERT, 2003).

We have detected a disease resistance protein (RPP 13) only in Myc roots. In *Arabidopsis thaliana* the gene codingRPP 13 confers resistance to five different isolates of *Peronospora parasitica* (causal agent of downy mildew). RPP 13 is a NBS-LRR type R-protein with a putative amino-terminal leucine zipper (BITTNER-EDDY et al., 2000). In addition to RPP 13, we have detected in *O. maculate*, 3 pathogenesis-related proteins, a chaperonin and one heat-shock proteins, with significant enhanced accumulation in mycorrhizal roots.

As orchids interact mostly with Rhizoctonia-like fungi, depending on the balance of the interaction and fungal lineage, the output of the interaction may vary from mutualistic to pathogenic. During the mutualistic interaction, the fungal symbiont may have to overcome or avoid the plant defense mechanisms. Whether R-proteins are involved in these processes is not known. However, it is likely that the orchid needs to modulate fungal growth, inducing the formation of pelotons and restricting fungal spread to non-photosynthesizing tissues (RASMUSSEN; RASMUSSEN, 2009; SHIMURA et al., 2007).

In this sense, we suggest that, as it occurs in AM, defense mechanisms may be spatially and temporally modulated, being alleviated in cortical cells during the initial stages of colonization, and more intense near root vascular tissues and during the digestion of pelotons, but this hypothesis needs further investigation.

Enhanced transport in Mycorrhizal roots

The higher frequency of proteins of the category "transport" in Myc roots than in non-myc roots (29 proteins, against 17 proteins in Non-Myc) is in line with the expectation of enhanced solute exchange at the plant-fungus interface. Among these proteins, an ABC transporter was 4.3 fold more abundant in Myc roots than in non-myc roots. ABC transporters are driven by ATP hydrolysis and can act as exporters as well as importers.Their function was first related to detoxification processes, but they have later been shown to be required for organ growth, plant nutrition, development, response to abiotic stresses, pathogen resistance and the interaction of the plant with its environment (KANG et al., 2011).

Recently (CAMERON et al., 2006,2008) have shown that part of photosynthesis-derived host carbon is returned to the fungus and that *Goodyera repens* can obtain phosphorous from its fungal partner. We therefore believe that molecular identification and characterization of solute transporters should also contribute to the understanding of the nutrient dynamics between symbionts. It is also expected that many other transporters, that we were not able to detect in the present work should be present in symbiotic cells. Other approaches such as RNAseq (WANG; GERSTEIN; SNYDER, 2009; WILHELM; LANDRY, 2009) may provide a more complete picture of the molecular changes that take place at the plant-fungal interface and, techniques such as immuno-localization, substrate specificity and labeled isotopes assays should address how this transporters works, especially concerning the direction of nutrient flow in different stages of the symbiosis. Moreover, it is still not clear whether solute transport in OMoccurs in digested or intact peloton surface. Y. Uetake (personal communication) has recently demonstrated that carbon fluxes occur in intact pelotons containing cells, but the great part of carbon flux still occurs when the peloton is digested.

We have also detected enhanced accumulation (3 fold) of an aquaporin inMyc roots, which could be involved in increasing water uptake and transport in OM. It would be interesting to test this hypothesis by comparing orchid from sites with different water availabilities and evaluate the contribution of OM fungi to orchid water status.

Putative fungal proteins

Among the proteins identified using the 2D-DIGE technique, 3 of them had their best match against fungal databases (Table 3.5). The first one is an activator of a protein kinase C (PKC). PKCsare activated by signals such as increases in the concentration of diacylglycerol or calcium ions. Hence these enzymes play important roles in several signal transduction cascades. The second protein belongs to the Histidine phosphatase superfamily. This protein family is mainly composed of phosphatases and phytases. Besides their metabolic functions, a large proportion of the family is involved in signalling. Among them, F26BPase and TIGAR are involved in controlling the concentration of intracellular F26BP (fructose 2,6-bisphosphate) and thereby influencing the rates of glycolysis and gluconeogenesis. Finally, the PyrABCN catalyzes the first two steps of the pyrimidine biosynthesis (ALEKSENKO et al., 1999), and its function can be related to the enhanced cytokinesis and nuclei hyperplasia that are observed in symbiotic cells (WATKINSON;WELBAUM, 2003).

3.5 Conclusions

The changes in the proteome of mycorrhizal roots of *Oeceoclades maculata* points to complex mechanisms of mutual recognition, symbiont accommodation, nutrient exchange, and control of the infection. The modulation of redox homeostasis, hormone balance and general plant defense responses, including the biosynthesis of proteases are likely to play a central role in these processes. It is likely that defense responses in OM are somehow modulated to partially restrict fungal colonization in vascular tissues in order to avoid systemic infections but allowing hyphal spread and controlling peloton formation and degradation dynamics in cortical cells. On the other hand, the enhanced accumulation of transporters agrees with the expected exchange of nutrients between symbionts in a functional symbiosis.

Overall, the use of complementary proteomic approaches had led to the identification of molecular processes that may be involved in OM regulation.

References

AGUIAR, J.M.R.B.V.; PANSARIN, L.M.; ACKERMAN, J.D.; PANSARIN, E.R. Biotic *versus* abiotic pollination in *Oeceoclades maculata* (Lindl.) Lindl. (Orchidaceae). **Plant Species Biology**, Hokkaido,v. 27, n. 1, p. 86-95, 2012.

ALEKSENKO, A; LIU, W.; GOJKOVIC, Z.; NIELSEN, J.; PISKUR, J. Structural and transcriptional analysis of the pyrABCN, pyrD and pyrF genes in *Aspergillus nidulans* and the evolutionary origin of fungal dihydroorotases. **Molecular Microbiology**, Salem, v. 33, n. 3, p. 599-611, 1999.

ASHBURNER, M.; BALL, C.A.; BLAKE, J.A.; BOTSTEIN, D.; BUTLER, H.; CHERRY, J.M.; DAVIS, A.P.; DOLINSKI, K.; DWIGHT, S.S.; EPPIG, J.T.; HARRIS, M.A.; HILL, D.P.; ISSEL-TARVER, L.; KASARSKIS, A.; LEWIS, S.; MATESE, J.C.; RICHARDSON, J.E.; RUBIN, G.M.; SHERLOCK, G. Gene Ontology: tool for the unification of biology. **Nature Genetics**, New York, v. 25, n. 1, p. 25-29, 2000.

BALBI, V.; DEVOTO, A. Jasmonate signalling network in *Arabidopsis thaliana*: crucial regulatory nodes and new physiological scenarios. **New Phytologist,** Cambridge, v. 177, n. 2, p. 301-318, 2008.

BITTNER-EDDY, P.D.; CRUTE, I.R.; HOLUB, E.B.; BEYNON, J.L. RPP13 is a simple locus in *Arabidopsis thaliana* for alleles that specify downy mildew resistance to different avirulence determinants in *Peronospora parasitica*. **Plant Journal**, Oxford, v. 21, n. 2, p. 177-188, 2000.

BOLLER, T.; FELIX, G. A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. **Annual Review of Plant Biology**, Palo Alto, v. 60, p. 379-406, 2009.

BONFANTE, P.; REQUENA, N. Dating in the dark: how roots respond to fungal signals to establish arbuscular mycorrhizal symbiosis. **Current Opinion in Plant Biology**, Saint Louis,v. 14, n. 4, p. 451-457, 2011.

CAMERON, D.D.; LEAKE, J.R.; READ, D.J. Mutualistic mycorrhiza in orchids: evidence from plant-fungus carbon and nitrogen transfers in the green-leaved terrestrial orchid *Goodyera repens*. **New Phytologist**, Cambridge, v. 171, n. 2, p. 405-416, 2006.

CAMERON, D.D.; JOHNSON, I.; LEAKE, J.R.; READ, D.J. Mycorrhizal acquisition of inorganic phosphorus by the green-leaved terrestrial orchid *Goodyera repens*. **Annals of Botany**, London, v. 99, n. 5, p. 831-834, 2007.

CAMERON, D.D.; JOHNSON, I.; READ, D.J.; LEAKE, J.R. Giving and receiving: measuring the carbon cost of mycorrhizas in the green orchid, *Goodyera repens*. **New Phytologist**, Cambridge, v. 180, n. 1, p. 176-184, 2008.

CAMPOS-SORIANO, L.; GARCÍA-GARRIDO, J.M.; SAN SEGUNDO, B. Activation of basal defense mechanisms of rice plants by *Glomus intraradices* does not affect the arbuscular mycorrhizal symbiosis. **New Phytologist**, Cambridge, v. 188, n. 2, p. 597-614, 2010.

CHOU, I.T.; GASSER, C.S. Characterization of the cyclophilin gene family of *Arabidopsis thaliana* and phylogenetic analysis of known cyclophilin proteins. **Plant Molecular Biology**, Dordrecht, v. 35, n. 6, p. 873-892, 1997.

DOD, D.D. *Oeceoclades maculata* (Orchidaceae), 1975 a 1985, en una marcha inexorable cruza La Isla Española. **Moscosoa**, Moscosoa, v. 4, p. 203-205, 1986.

ELLIS, J.; DODDS, P.; PRYOR, T. Structure, function and evolution of plant disease resistance genes. **Current Opinion in Plant Biology**, Saint Louis,v. 3, n. 4, p. 278-284, 2000.

FESTER, T.; HAUSE, G. Accumulation of reactive oxygen species in arbuscular mycorrhizal roots. **Mycorrhiza**, Berlin, v. 15, n. 5, p. 373-379, 2005.

GARCÍA-GARRIDO, J.M.; OCAMPO, J.A. Regulation of the plant defence response in arbuscular mycorrhizal symbiosis. **Journal of Experimental Botany**, Oxford, v. 53, n. 373, p. 1377-1386, 2002.

GARG, N.; MANCHANDA, G. ROS generation in plants: boon or bane? **Plant Biosystems**, Rome,v. 143, n. 1, p. 81-96, 2009.

GODOY, A.V; LAZZARO, A.S.; CASALONGUE, C.A. Expression of a *Solanum tuberosum* cyclophilin gene is regulated by fungal infection and abiotic stress conditions. **Plant Science**, Limerick, v. 152, p. 123-134, 2000.

GREENSHIELDS, D.L.; LIU, G.; SELVARAJ, G.; WEI, Y. Differential regulation of wheat quinone reductases in response to powdery mildew infection. **Planta**, Berlin, v. 222, n. 5, p. 867-875, 2005.

HAMMOND-KOSACK, K.E.; PARKER, J.E. Deciphering plant-pathogen communication: fresh perspectives for molecular resistance breeding. **Current Opinion in Biotechnology**, Saint Louis,v. 14, n. 2, p. 177-193, 2003.

JIANG, Y.; YANG, B.; HARRIS, N.S.; DEYHOLOS, M.K. Comparative proteomic analysis of NaCl stress-responsive proteins in *Arabidopsis* roots. **Journal of Experimental Botany**, Oxford, v. 58, n. 13, p. 3591-3607, 2007.

JOLICOEUR, M.; GERMETTE, S.; GAUDETTE, M.; PERRIER, M.; BECARD, G. Intracellular pH in arbuscular mycorrhizal fungi. A symbiotic physiological marker. **Plant Physiology**, Minneapolis, v. 116, n. 4, p. 1279-1288, 1998.

KANG, J.; PARK, J.; CHOI, H.; BURLA, B.; KRETZSCHMAR, T.; LEE, Y.; MARTINOIA, E. Plant ABC transporters. **The Arabidopsis Book**,v. 9, e0153, 2011.

KELLER, A.; NESVIZHSKII, A.I.; KOLKER, E.; AEBERSOLD, R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. **Analytical Chemistry**, Washington, v. 74, n. 20, p. 5383-5392, 2002.

KOBE, B.; DEISENHOFER, J. The leucine-rich repeat: a versatile binding motif. **Trends in Biochemical Science**, Cambridge,v. 19, p. 415-421, 1994.

KOGEL, K.; FRANKEN, P.; HU, R. Endophyte or parasite - what decides? **Current Opinion in Plant Biology**, Saint Louis,v. 9, n. 4, p. 358–363, 2006.

KUMARI, S.; ROY, S.; SINGH, P.; SINGLA-PAREEK, S.L.; PAREEK, A. Cyclophilins: proteins in search of function. **Plant Signalling and Behavior**, Austin,v. 8, n. 1, p. 25-32, 2013.

LAEMMLI, U.K. Most commonly used discontinuous buffer system for SDS electrophoresis. **Nature**, London, v. 227, p. 680-685, 1970.

LAMBAIS, M.R.; RIOS-RUIZ, W.F.; ANDRADE, R.M. Antioxidant responses in bean (*Phaseolus vulgaris*) roots colonized by arbuscular mycorrhizal fungi. **New Phytologist**, Cambridge, v. 160, n. 2, p. 421-428, 2003.

LUAN, S.; ALBERS, M.W.; SCHREIBER, S.L. Light-regulated, tissue-specific immunophilins in a higher plant. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, v. 91, n. 3, p. 984-988, 1994.

NESVIZHSKII, A.I.; AEBERSOLD, R. Interpretation of shotgun proteomic data: the protein inference problem. **Molecular & Cellular Proteomics**, Bethesda, v. 4, n. 10, p. 1419-1440, 2005.

PASSARDI, F.; COSIO, C.; PENEL, C.; DUNAND, C. Peroxidases have more functions than a Swiss army knife. **Plant Cell Reports**, Berlin, v. 24, n. 5, p. 255-265, 2005.

PATHAK, D.; NGAI, K.L.; OLLIS, D. X-ray crystallographic structure of dienelactone hydrolase at 2.8 Å. **Journal of Molecular Biology**, London, v. 204, p. 435-445, 1988.

PAULY, N.; PUCCIARIELLO, C.; MANDON, K. Reactive oxygen and nitrogen species and glutathione: key players in the legume - *Rhizobium* symbiosis. **Journal of Experimental Botany**, Oxford, v. 57, n. 8, p. 1769-1776, 2006.

PESSOA, H.P.; TEIXEIRA, A.F.S.; MIRANDA, L.; PEREIRA, M.C. Caracterização micorrízica de *Oeceoclades maculata* (Lindl.) Lindl. e utilização de seus simbiontes fúngicos na germinação e produção de mudas de orquídeas nativas da região do Alto Paranaíba – MG. **Evolução e Conservação da Biodiversidade**, Rio Paranaíba, v. 3, n. 1, p. 6-13, 2012.

RABILLOUD, R.; CHEVALLET, M.; LUCHE, S.; LELONG, C. Two-dimensional electrophoresis in proteomics: past, present and future. **Journal of Proteomics**, Amsterdam,v. 73, n. 11, p. 2064 - 2077,2010.

RAMOS, A.C.; MARTINS, M.A.; FAÇANHA, A.R. ATPase and pyrophosphatase activities in corn root microsomes colonized with arbuscular mycorrhizal fungi. **Revista Brasileira de Ciência do Solo**, Viçosa, v. 29, n. 2, p. 207-213, 2005.

RASMUSSEN, H.N.; RASMUSSEN, F.N. Orchid mycorrhiza: implications of a mycophagous life style. **Oikos**, Copenhagen, v. 118, n. 3, p. 334-345, 2009.

REQUENA, N.; BREUNINGER, M.; FRANKEN, P.; OCÓN, A. Symbiotic status, phosphate, and sucrose regulate the expression of two plasma membrane H^+-ATPase genes from the mycorrhizal fungus *Glomus mosseae*. **Plant Physiology**, Minneapolis, v. 132, n. 3, p. 1540-1549, 2003.

SERWOLD, T.; GONZALEZ, F.; KIM, J.; JACOB, R.; SHASTRI, N. ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum. **Nature**, London, v. 419, n. 6906, p. 480-483, 2002.

SHARMA, A.D.; SINGH, P. Effect of water stress on expression of a 20 kd cyclophilin-like protein in drought susceptible and tolerant cultivars of *Sorghum*. **Journal of Plant Biochemistry & Biotechnology**, New Dehli,v. 12, n. 1, p. 77-80, 2003.

SHIMURA, H.; MATSUURA, M.; TAKADA, N.; KODA, Y. An antifungal compound involved in symbiotic germination of *Cypripedium macranthos* var. *rebunense* (Orchidaceae). **Phytochemistry**, Oxford, v. 68, n. 10, p. 1442-7, 2007.

SHIRASU, K.; SCHULZE-LEFERT, P. Complex formation, promiscuity and multi-functionality: protein interactions in disease-resistance pathways. **Trends in Plant Science**, Oxford, v. 8, n. 6, p. 252-8, 2003.

SIVASANKAR, S.; SHELDRICK, B.; ROTHSTEIN, S.J. Expression of allene oxide synthase determines defense gene activation in tomato. **Plant Physiology**, Minneapolis,v. 122, n. 4, p. 1335-1342, 2000.

SOLLNER, S.; DURCHSCHLAG, M.; FRÖHLICH, K.U.; MACHEROUX, P. The redox-sensing quinone reductase Lot6p acts as an inducer of yeast apoptosis. **FEMS Yeast Research**, Amsterdam,v. 9, n. 6, p. 885-891, 2009.

SOLLNER, S.; NEBAUER, R.; EHAMMER, H.; PREM, A.; DELLER, S.; PALFEY, B.A.; DAUM, G.; MACHEROUX, P. Lot6p from *Saccharomyces cerevisiae* is a FMN-dependent reductase with a potential role in quinone detoxification. **FEBS Journal**, Amsterdam, v. 274, n. 5, p. 1328-1339, 2007.

SPANU, P.; BONFANTE, P. Cell-wall-bound peroxidase activity in roots of mycorrhizal *Allium porrum*. **New Phytologist**, Cambridge, v. 109, p. 119-124, 1988.

STERN, W.L. The long distance dispersal of *Oeceoclades maculata*.**American Orchid Society Bulletin**, West Palm Beach, v. 57, p. 960-971, 1988.

STRACKE, S.; KISTNER, C.; YOSHIDA, S.; MULDER, L.; SATO, S.; KANEKO, T.; TABATA S.; SANDAL, N.; STOUGAARD, J.; SZCZYGLOWSKI, K.; PARNISKE, M. A plant receptor-like kinase required for both bacterial and fungal symbiosis. **Nature**, London, v. 417, n. 6892, p. 959-962, 2002.

TORRES, M.A. ROS in biotic interactions. **Physiologia Plantarum**, Copenhagen, v. 138, n. 4, p. 414-429, 2010.

VAN DER HOORN, R.A.L.; JONES, J.D.G. The plant proteolytic machinery and its role in defence. **Current Opinion in Plant Biology**, Saint Louis,v. 7, n. 4, p. 400-407, 2004.

VORSTER, B.; SCHLÜTER, U.; DU-PLESSIS, M.; VAN WYK, S.; MAKGOPA, M.E.; NCUBE, I.; QUAIN, M.D.; KUNERT, K.; FOYER, C.H. The cysteine protease-cysteine protease inhibitor system explored in soybean nodule development. **Agronomy**, Basel, v. 3, n. 3, p. 550-570, 2013.

WALLER, F.; ACHATZ, B.; BALTRUSCHAT, H.; FODOR, J.; BECKER, K.; FISCHER, M.; HEIER, T.; HÜCKELHOVEN, R.; NEUMANN, C.; VON WETTSTEIN, D.; FRANKEN, P.; KOGEL, K.H. The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, v. 102, n. 38, p. 13386-13391, 2005.

WANG, W. A universal and rapid protocol for protein extraction from recalcitrant plant tissues for proteomic analysis. **Electrophoresis**, Weinheim, v. 27, n. 13, p. 2782-2786, 2006.

WANG, X.; BAUW, G.; VAN DAMME, E.J.; PEUMANS, W.J.; CHEN, Z.L.; MONTAGU, M.; ANGENON, G.; DILLEN, W. Gastrodianin-like mannose-binding proteins: a novel class of plant proteins with antifungal properties. **Plant Journal: for Cell and Molecular Biology**, Oxford, v. 25, n. 6, p. 651-661, 2001.

WANG, Z.; GERSTEIN, M.; SNYDER, M. RNA-Seq: a revolutionary tool for transcriptomics. **Nature Review Genetics**, New York, v. 10, n. 1, p. 57-63, 2009.

WILHELM, B.T.; LANDRY, J. RNA-Seq- quantitative measurement of expression through massively parallel. **Methods**, Manchester, v. 48, n. 3, p. 249-257, 2009.

WOJCECHOWSKYJ.J.A; LEE, J.Y.; SEEHOLZER, S H.; DOMS, R. W. Quantitative phosphoproteomics of CXCL12 (SDF-1) signaling. **PloS One**, London, v. 6, n. 9, e24918, 2011.

WOLTERING, E.J.; BENT, A.; VAN DER HOEBERICHTS, F.A. do plant caspases exist? **Plant Physiolog**y, Minneapolis, v. 130, n. December, p. 1764-1769, 2002.

WU, W.W.; WANG, G.; BAEK, S.J.; SHEN, R. Comparative study of three proteomic quantitative methods, DIGE , cICAT , and iTRAQ, using 2D Gel- or LC-MALDI TOF/TOF research articles. **Journal of Proteome Research,** Washington, v. 5, p. 651-658, 2006.

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 *Dendrobiumofficinale*. **PloS One**, London, v. 8, n. 8, e72705, 2013.

4 TRANSCRIPTOME ANALYSES SUGGEST ENHANCED NUTRIENT METABOLISM AND ALLEVIATION OF DEFENSE RESPONSES IN *Oeceoclades maculata* (ORCHIDACEAE) MYCORRHIZAL ROOTS

Abstract

In contrast to other types of endomycorrhiza, the molecular events taking place during orchid mycorrhiza development are mostly unknown. As in arbuscular mycorrhiza, it is likely that an intricate signaling process between the symbionts and alteration of host gene expression may take place in orchid mycorrhiza in response to cellular changes necessary to accommodate the fungus intracellularly, and allow nutrient transfer between symbionts. In order to complement previous proteomic studies, we have used large scale sequencing of the transcriptome (RNAseq) of *Oeceoclades maculata*mycorrhizal rootsto determine the molecular mechanisms regulating orchid mycorrhizal symbiosis. A total of 81,666 contigs were generated by *de novo* assembly of the transcriptome. Among 12,740 successfully annotated genes, 1,359 up- and 816 down-regulated genes were detected in mycorrhizal roots, as compared to non-mycorrhizal roots. The data revealed a strong increase in general stress responses, accompanied by changes in signaling pathways possibly related to fungal recognition and establishment of a compatible plant-microbe interaction. Some up-regulated genes may be involved in the reorganization of cell structure, likely related to accommodation of the fungal symbiont in the plant roots. We also observed up-regulation of genes involved in carbon metabolism, including glycolysis/gluconeogenesis and amino sugars metabolism, together with genes involved on enhanced nitrogen assimilation processes in mycorrhizal roots. The down-regulation of genes involved in the jasmonate and ABA pathways, together with key genes encoding anti-fungal proteins such as chitinase and a mannose-specific binding lectin, strongly suggests an alleviation of plant defense responses in *O. maculata* mycorrhizal roots. These results broadly expand the current knowledge on the mechanisms of regulation of orchid mycorrhiza and suggest more similarities than dissimilarities with arbuscular mycorrhiza.

Keywords: Orchid mycorrhiza; RNAseq; Transcriptomic; Defense responses

4.1 Introduction

Orchid mycorrhiza (OM) has peculiar features that makes it an interesting model to investigate plant-fungal interactions (DEARNALEY, 2007). All orchids are fully mycoheterotrophic during seed germination and embryo development (RASMUSSEN; RASMUSSEN, 2007). Most orchids associate with *Rhizoctonia*-like fungi, a polyphyletic group comprising teleomorphs in at least three unrelated basidiomycetous families (DERNALEY; MARTOS; SELOSSE, 2013). Fungi belonging to the anamorphic genus *Rhizoctonia* include species playing different roles in the ecosystems, such as pathogens, saprotrophs, besides forming ectomycorrhiza and OM (GONZÁLES-GARCÍA; ONCO; SUSAN, 2006).

It is acknowledged that, in nature, the nutrient balance in OM interactions tends to favor plant over fungal growth. Some authors categorize OM as a reverse parasitism, also called mycophagy (RASMUSSEN; RASMUSSEN, 2007, 2009; SELOSSE; ROY, 2009). This would suggest that the plants involved in this type of symbiosis are capable of controlling fungal virulence and infection. On the other hand, Cameron (2006) showed that a two-way nutrient transport of carbon (C) and nitrogen (N) takes place in adult *Goodyera repens,* and stated that the mycorrhizal association of a green-leaved terrestrial orchid can function mutualistically, with photosynthate translocated from the plant to the fungus in return for mineral nutrients (N) translocated from the fungus to the plant.

The molecular mechanisms that modulate OM symbiosis are largely unknown. Watkinson and Welbaum (2003) were the first to study gene expression in orchid mycorrhiza using differential display of reverse transcripts. They detected in *Cypripedium parviflorum* var. *pubescens* mycorrhizal roots an up-regulation of a nucleotide binding protein and a down-regulation of a trehalose-phosphate phosphatase. Only recently, Zhao *et al.* (2013) used suppression subtractive hybridization (SSH) to identify genes expressed in symbiotically germinated seeds of *Dendrobium officinale*. However, with the recent advances in sequencing techniques , it is now possible to yield a very large number of gene sequences, identify them and predict their roles in symbiosis using semi-automated biological function and cellular location prediction tools (ASHBURNER et al., 2000). The advantages of using a non-targeted approach is that there are no biases towards known genes that might have known functions in other plant-microbe interactions, and it may reveal novel genes potentially involved in the regulation of the symbiosis (WATKINSON; WELBAUM, 2003). The use of high-throughput sequencing techniques may also be useful for better understanding the co-evolution of orchids and their mycorrhizal fungi, and the functional significance of these associations. Coupling data from differential protein and gene expression studies should reveal whether OM is evolutively closer to compatible interactions, such as arbuscular mycorrhiza (AM), or to a unilateral relationship in favor of the plant, as in parasitism.

In chapter 3 we have explored the differential protein accumulation in mycorrhizal roots of *Oeceoclades maculata*, and detectedseveral proteins with roles in defense response, hormonal regulation and plant metabolism potentially important in the regulation of OM. However the number of proteins identified was very low, as compared to the potential output of RNAseq techniques. In this study, we used Illumina-based RNA sequencing to determine changes in transcript accumulation in *O. maculate* mycorrhizal roots as compared to non-

mycorrhizal roots, in order to determine possible mechanisms regulating OM development. After sequencing, bioinformatics steps were applied for *de novo assembly* of reads, identification, annotation and statistical evaluation of gene expression. Even though, the amount of data generated is very large, the analyses presented is limited to plant transcripts putatively involved in nutrient acquisition and control of the symbiosis.

4.2 Material and Methods

Biological material

Adult plants of *Oeceoclades maculata* were harvested at the campus of Piracicaba of the University of Sao Paulo, Brazil, during the dry season (August) of 2012. Plants were immediately brought to the laboratory, had their roots cut, their velamen removed and were washed with distilled water. Roots were then screened visually using a stereoscopic microscope for the presence or absence of mycorrhizal fungi structures (pelotons). Three biological replicates consisting of a pool of mycorrhizal (Myc) or non-mycorrhizal (Non-myc) roots from three plants were used for RNA extraction immediately after sample screening.

RNA extraction and enrichment of mRNA from total RNA

Total RNA from root samples was extracted using the TRIzol (Invitrogen) reagent according to Fracetto et al. (2013). RNA quantity and integrity were determined using Agilent Bioanalyzer 2100 (Agilent; Palo Alto, CA, USA), according to the manufacturer's instructions. Only samples with an RNA Integrity Score > 6.6 were used for cDNA synthesis.

cDNA library preparation and sequencing

Complementary DNA (cDNA) libraries were constructed, using 2 µg of total RNA and following the TruSeq RNA Sample preparation v.2 kit instructions (Low Throughput protocol, Illumina, Inc.). The quality of the isolated RNA was further determined using a Nanodrop 1000 spectrophotometer (Thermo Scientific, USA) and a Bioanalyzer 2100 (Agilent). Only samples with a ration OD 260 nm/ OD 280 nm between 1.8–2.1 and a 28S/18S ratio within 1.5–2 were further processed. The TruSeq RNA Sample Prep Kit v2 (Illumina, Inc., CA, USA) was used in the subsequent steps. Briefly, total RNA samples were polyA-enriched, reverse-transcribed and double-stranded cDNA synthesized. TruSeq adapters were attached to double-stranded cDNA. Enriched in fragments containing TruSeq adapters

on both ends were performed using PCR. The quantity and quality of the enriched libraries were validated using Nanodrop 1000 and Bioanalyzer 2100 respectively. The libraries were normalized to 10 nM of amplicons in Tris-HCl 10 mM, pH 8.5 containing 0.1% Tween 20.

Bar-coded amplicons (library) were spread over one Illumina HiScan 1000 lane. The TruSeq PE Cluster Kit v3-cBot-HS (Illumina, Inc., California, USA) was used for cluster generation using 2 pM of pooled normalized libraries on the cBOT. Sequencing was performed on the Illumina HiScan 1000 to yield paired-end reads of 2×101 bp using the TruSeq SBS Kit v3-HS (Illumina, Inc.). Illumina reads were quality-checked with Fastqc, which computes various quality metrics for the raw reads.

RNA-Seq data processing

The CLCBio 6.5 software (Qiagen) was used for *de novo* assembly, blast searching, annotation and mapping of reads. All reads, from either Myc or Non-myc samples were used for the *de novo* assembly of transcriptome with a minimun contig lenght of 120 bp. The contigs were extracted and compared with the Swissprot plant protein database (http://www.uniprot.org, 11/2013) with an expect level =10, using BLASTx. Subsequently, we produced RNAseq mappings for all reads. In the RNAseq mapping pipeline we set the minimum read length fraction to 0.9, minimum similarity to 0.95, allowed up to 10 unspecific matches, and selected RPKM as expression value. Contigs were categorized functionally using Blast2GO PRO (ASHBURNER et al., 2000; CONESA et al., 2005) and the annotation table was further merged with the RNAseq experiment table. We have annotated both the whole transcriptome, and up-regulated genes for better presentation of the dataset.

4.3 Results

Sequencing, de novo assembly and mapping of sequencing reads

Six cDNA samples derived from three biological replicates of Myc and Non-Myc roots each were tagged sequenced using an Illumina HiScan 1000 sequencer. Sequencing produced approximately 83 millions of reads with a mean length of 101 base pairs (bp) and mean quality score of 38 (Phred Score). Paired-ended reads (2 x 101 bp) were merged, according to CLCbio default parameters, to produce reads of an average length of 150 bp. Trimming was performed in order to remove ambiguous nucleotides (maximum of 2 nucleotides allowed), terminal nucleotides (1 from the 5' end and 1 from the 3' end), adapter sequences, reads with

less than 15 and more than 1000 nucleotides and low quality reads (limit =0.02). Sequencing merging and trimming information is shown in Table 4.1

Table 4.1 -Estatistics of cDNA sequences derived from *Oeceoclades maculata* roots

Sample[1]	Total reads	Total reads after merging[2]	% trimmed	Number of reads completely removed	Avg. lenght before merging (bp)	Avg. lenght after merging (bp)	Avg. lenght after trimming (bp)
M1	13,258,869	18,843,992	0.9980	50,334 (0,26%)	101	150	147
M2	15,768,946	21,089,332	0.9985	56,111 (0,26%)	101	148	145
M3	14,149,071	18,921,006	0.9965	55,806 (0,29%)	101	148	144
NM1	14,526,535	19,241,370	0.9975	53,539 (0,27)	101	148	144
NM2	12,992,840	17,001,980	0.9995	48,969 (0,28)	101	147	144
NM3	12,631,275	16,334,516	0.9996	42,959 (0,26)	101	145	142
Total	83,354,536	111,432,196					

[1]M= Mycorrhizal root; NM= Non-Mycorrhizal root. [2]Sum of non-merged + merged reads

After the removal of reads containing low quality and/or ambiguous bases (>2% "N" per reads), we used the CLCbio assembler to cluster the reads in high quality contigs (*de novo* assembly). *De novo* assembly of cDNA reads yielded 81,666 contigs with more than 120 bp, with an average length of 717 bp and N50 length of 1066 bp (Table 4.2).

Table 4.2 -Length distributionof *de novo* assembled contigs

Parameter*	**Base pairs**
N75	471
N50	1066
N25	2069
Minimum length	120
Maximum length	16788
Average length	717

* N75 is the point where 75% of the entire assembly is contained contigs equal o larger than this value. Accordingly N50 is the point of half of the mass of the contig lenght distribution and N25 is the point where 25% of the assembly is contained in contigs equal to or larger than this value. A total of 81,666 contigs was obtained

Identification and functional categorization of O. maculata root transcripts

Contig sequences were compared to sequences in the the Swissprot plant database (November 2013), using BLASTx with an expect value =10 (default). 58 % of the contigs were successfully identified.

The Gene Ontology (GO) annotation was used to provide descriptions of gene products associated with molecular functions, cellular components and biological processes. To determine the possible functions of the genes, we used the GO classification system for plants developed by the GO consortium. A total of 36,000 contigs were mapped and 12,740 contigs (15.6 % of the dataset) were successfully annotated.They were categorized into 24 *Biological Processes* categories (Figure 4.1), 9 *Cellular Components* categories (Figure 4.2) and 9 *Molecular Functions* categories (Figure 4.3). We have filtered categories. Among the GO biological processes, "Response to Stress" was the most represented with 2,211 entries, followed by "Single-organism Developmental Process" with 1,735 entries and "Single-organism Transport" with 1,572 entries. The largest proportion of contigs in the *Molecular Function* categories was in "Hydrolase Activity" with 2,243 entries, "Protein Binding" with 1,755 entries and "Nucleic Acid Binding" with 1,411 entries. Concerning the Cellular component categories, most contigs (2,716 entries) encoded proteins putatively located in the "nucleus", followed by "intracellular organelle part" (2,421 entries) and "plasma membrane" (1,848 entries).

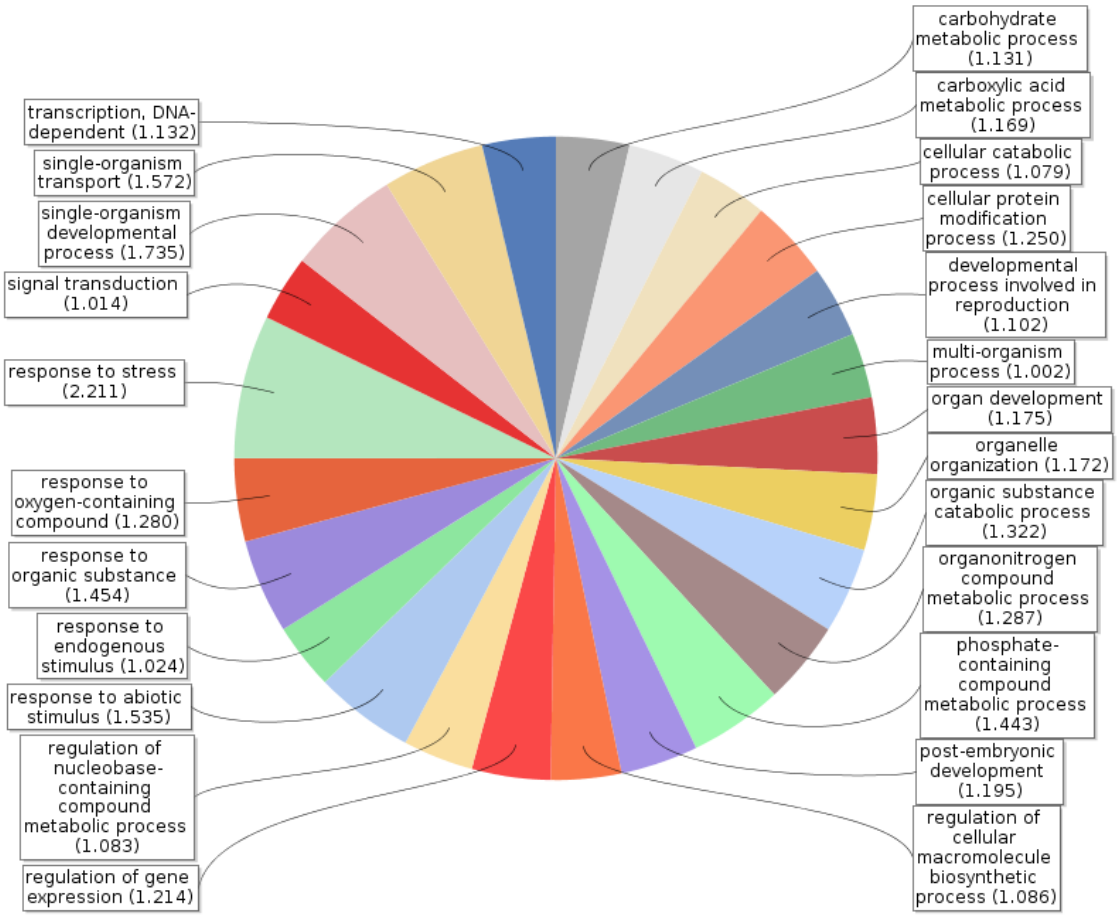


Figure 4.1 -Gene Ontology categorization of *Oeceoclades maculata* root transcripts according to their putative biological processes

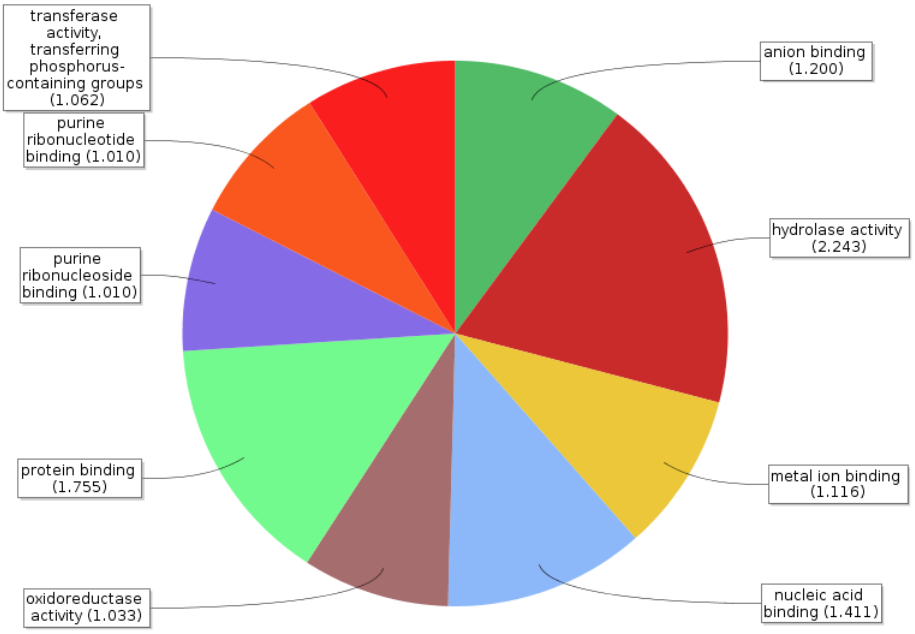


Figure 4.2 -Gene Ontology categorization of *Oeceoclades maculata* transcripts according the their putative molecular functions

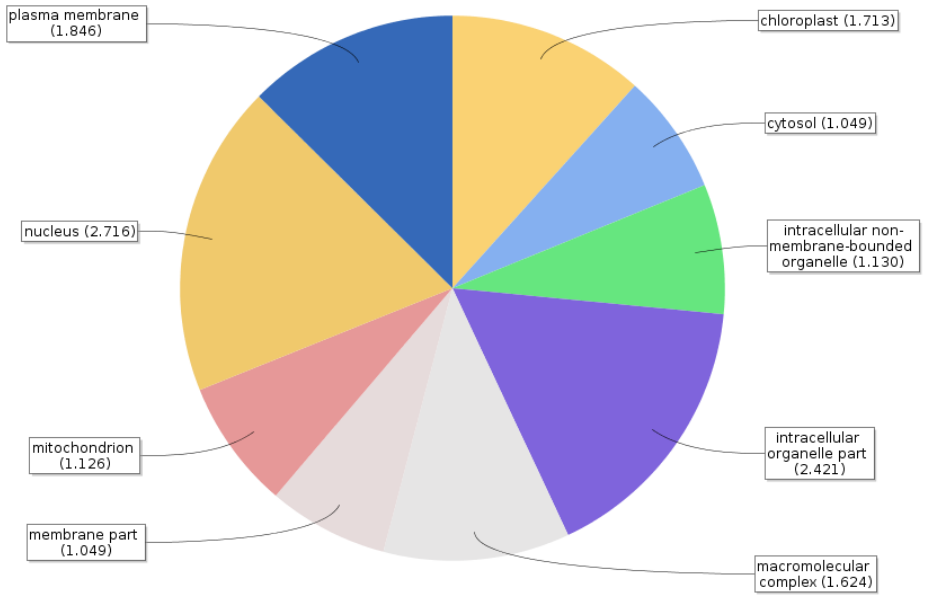


Figure 4.3 -Gene Ontology categorization of *Oeceoclades maculata* roots transcripts according to their sub-cellular component

Mapping

Subsequently, we produced RNA-Seq mappings for reads from the six samples. Both sets of reads were mapped to the 81,666 contigs from the *de novo* assembly. In the RNA-Seq mapping pipeline we set the minimum read length fraction to 0.9, minimum similarity to 0.95, allowed for up to 10 unspecific matches, and selected RPKM as expression value. An average of 89.6% of total number of reads were successfully mapped back to the contigs (89.1% of the single reads and 90.1% of paired reads) (Figure 4).

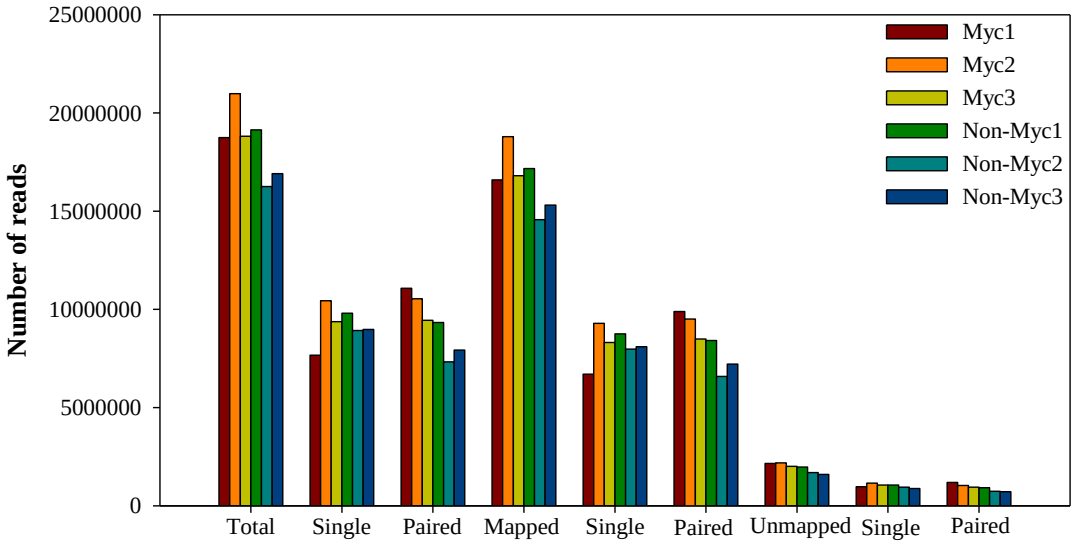


Figure 4.4 -Mapping distribution of single and paired reads. Myc1, Myc2, Myc3 refer to biological replicates of mycorrhizal roots. Non-Myc1, Non-Myc 2, Non-Myc 3 refer to biological replicates of Non-mycorrhizal roots

Differential expression analysis

After RNAseq mapping and counting, the two conditions (i.e. Myc and Non-Myc roots) were analyzed separately, each one represented by three biological replicates.

Data were normalized and a t-test was applied in order to evaluate differential expression between Myc and Non-myc roots of *O. maculata*. Sequences with fold-change between samples >1.5 (or < -1.5) and *p*-value < 0.05 were considered to be differentially expressed. Among the 81,666 contigs generated by the *de novo* assembly, 9,639 (11.8%) represented genes differentially expressed, 5854 being up-regulated and 3514 down-regulated. Out of these genes, 1359 up-regulated and 816 down-regulated genes were successfully identified and annotated.

The volcano plot in Figure 4.5 shows the changes in gene expression in terms of fold-change *versus* probability values. Dots above the red threshold represent genes with significant fold-change values at 95 % of confidence.

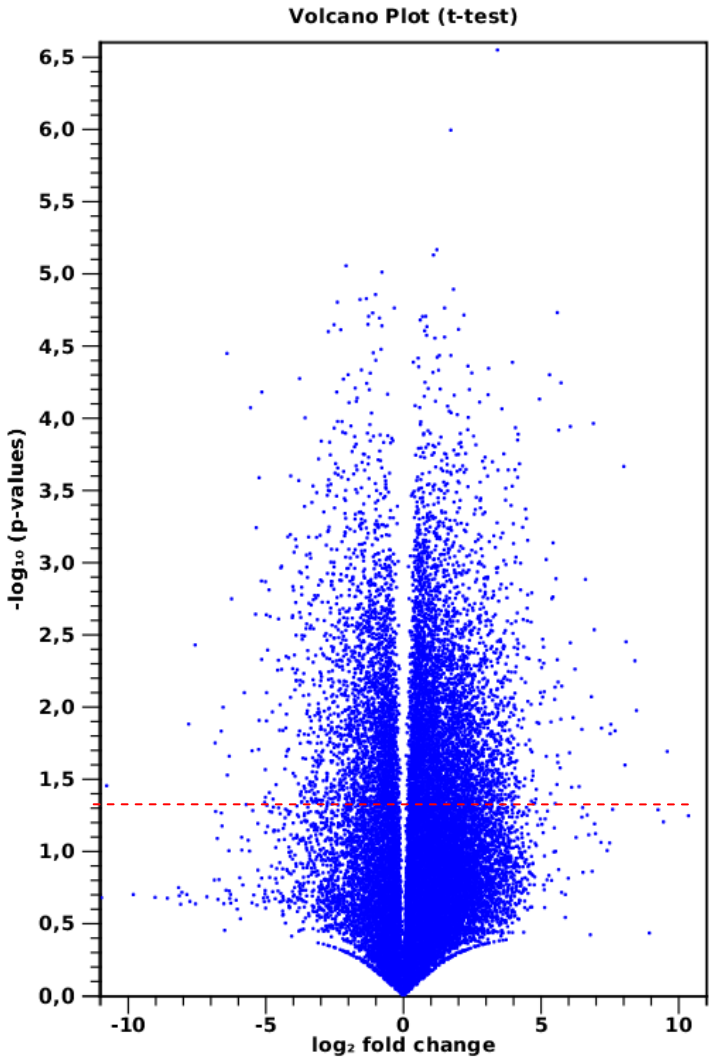


Figure 4.5-Volcano plot. The horizontal axis is the $\log_2$ fold change between the treatment means (myc vs non-myc). The $-\log_{10}$ (P-value) is plotted on the vertical axis. Each contig is represented by one dot on the graph. Dots above the red dashed line have *p* -values <0.05

Up-regulated genes in O. maculata mycorrhizal roots

GO annotation was also used to provide descriptions of genes that were up-regulated in *O. maculata* Myc roots (fold change >1.5; *p* <0.05). The annotations of sequences of *O. maculata*, for which GO categories could be assigned, were categorized in 24 *Biological Processes*, 9 *Cellular Components* and 9 *Molecular Functions*(Figure 4.6). Among the represented biological processes, "response to stress" was the most represented with 2,211 entries, followed by "single-organism developmental process" with 1,735 entries, and "single-organism transport" with 1,572 entries. Among the molecular functions, most expressed sequence tags (ESTs) were categorized as "hydrolase activity" with 2,243 entries, "protein

binding" with 1,755 entries and "nucleic acid binding", with 1,411 entries. Concerning the putative sub-cellular localization, most of the products were assigned to the "nucleus" (2,716 entries), followed by "intracellular organelle" (2,421 entries) and "plasma membrane" (1,848 entries).

The up-regulated genes were also classified using KEGG annotations, which provide an alternative functional annotation of genes according to their associated biochemical pathways, based on sequence similarity searches against the KEGG database (http://www.genome.jp/kegg/pathway.html). "Starch and Sucrose Metabolism" was the most represented pathway with 31 genes, representing 20 enzymes, followed by "Purine Metabolism" (26 genes; 15 enzymes) and "Glyoxylate and Dicaboxylate metabolism" (11 genes; 7 enzymes).

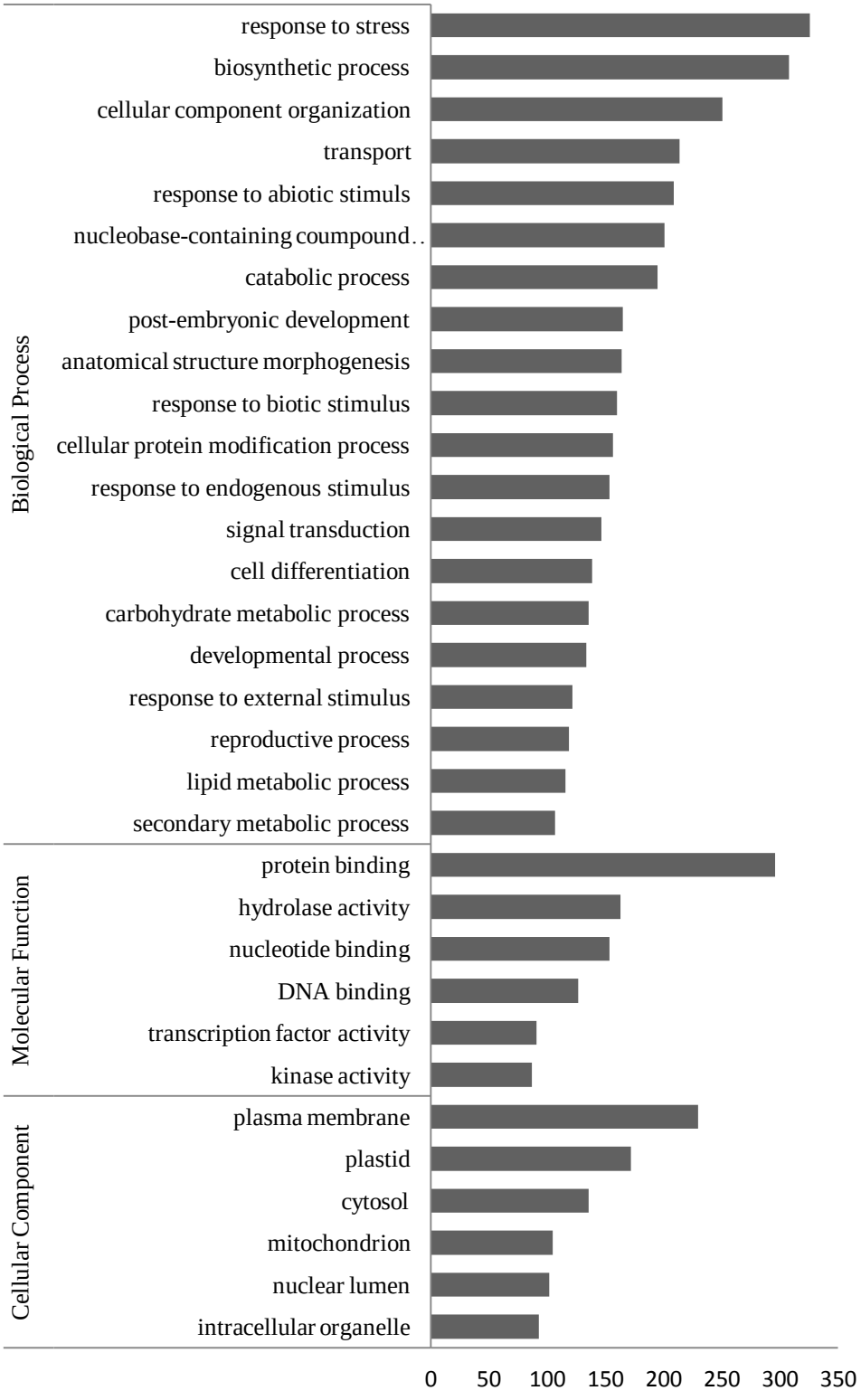


Figure 4.6 -Gene Ontology categorization of transcripts up-regulated in mycorrhizal roots of *Oeceoclades maculata*

Most abundant genes in mycorrhizal roots

In Table 4.3 are shown the 50 most abundant genes in mycorrhizal roots of *O. maculata*, among those with positive fold change. Syntaxin-24 was the most abundant gene, with 2,616.30 RPKM (Reads Per Kilobase of transcript per Million mapped reads) and it was 2.8-fold induced in myc roots. A syntaxin-24 homologue, syntaxin-121 (369.95 RPKM; 3.20 fold change) was assigned to the GO biological function "biotic stimulus, MAPK cascade and positive regulation of flavonoid biosynthetic process".

It is also noteworthy the high accumulation of two ethylene-responsive transcription factors (492.44 and 689.69 RPKM; 1.8 and 1.57 fold change). Indeed, several transcription factors were detected among the most induced transcripts, including MYB-related, WRKY transcription factors and transcripts containg MADS-box, homeobox, and u-box. Proteinases and proteins related to redox homeostasis are also found in Table 4.3.

Several of these genes have functions (inferred by GO biological process annotation) related to response to biotic stimulus, signal transduction, response to fungus, and response to chitin. In particular, a bel1-like homeodomain protein was categorized in the category "response to symbiotic fungus" (compatible interaction). In general, these genes are most likely to be related to fungal recognition, signal transduction and negative regulation of defense responses.

Table 4.3 -The 50 most abundant genes in mycorrhizal roots, among those with positive fold change within mycorrhizal and non-mycorrhizal roots of *Oeceoclades maculata*

(continue)

Swissprot ID	Swissprot - Acession	Myc - Means	Non-Myc - Means	Fold change	*p*-value	Annotations - GO biological process
Q9C615	syntaxin-24	2616.30	917.79	2.85	0.01	0051641 // cellular localization /// 0016043 // cellular component organization
O49169	elongation factor 1-alpha	779.21	447.43	1.74	0.04	0006184 // GTP catabolic process /// 0006414 // translational elongation
Q9SFE4	ethylene-responsive transcription factor erf012	710.69	342.90	2.07	0.01	0009741 // response to brassinosteroid stimulus /// 0009753 // response to jasmonic acid stimulus dependent
Q9FDW1	myb-related protein 44 short	689.69	439.41	1.57	0.01	0010200 // response to chitin response to ethylene stimulus /// 0050832 // defense response to fungus
P53492	actin-7	540.40	340.05	1.59	0.01	0006094 // gluconeogenesis /// 0009733 response to wounding
Q40477	ethylene-responsive transcription factor 4	492.44	273.78	1.80	0.03	0006952 // defense response /// 0009873 // ethylene mediated signaling pathway
Q8H273	dehydration-responsive element-binding protein 1e	453.50	114.66	3.96	0.03	0006355 // regulation of transcription, DNA-dependent /// 0009414 // response to water deprivation
Q9AST3	sphinganine c -monooxygenase	450.98	66.40	6.79	0.04	0016126 // sterol biosynthetic process /// 0009640 // photomorphogenesis /// 0046520 // sphingoid biosynthetic process
P83332	thaumatin-like protein	434.19	154.82	2.80	0.04	0006952 // defense response
P59169	histone	374.10	235.35	1.59	0.04	0006605 // protein targeting /// 0006334 // nucleosome assembly
Q8H273	dehydration-responsive element-binding protein 1e	370.05	118.85	3.11	0.02	0006355 // regulation of transcription, DNA-dependent /// 0009414 // response to water deprivation

Table 4.3 -The 50 most abundant genes in mycorrhizal roots, among those with positive fold change within mycorrhizal and non-mycorrhizal roots of *Oeceoclades maculata.*

(continuation)

Swissprot ID	Swissprot - Acession	Myc - Means	Non-Myc - Means	Fold change	*p*-value	Annotations - GO biological process
Q9SJ56	bel1-like homeodomain protein 1	355.68	180.46	1.97	0.01	1901576 // organic substance biosynthetic process /// 0009610 // response to symbiotic fungus /// 0045087 // innate immune response
P48534	l-ascorbate cytosolic	290.90	149.44	1.95	0.01	0055114 // oxidation-reduction process /// 0042744 // hydrogen peroxide catabolic process
P19950	40s ribosomal protein	245.32	154.34	1.59	0.00	0006412 // translation
Q9ZWA6	zinc finger protein magpie	221.87	140.58	1.58	0.01	0006355 // regulation of transcription, DNA-dependent
Q9FH57	gata transcription factor 5	218.48	92.25	2.37	0.02	
P49043	vacuolar-processing enzyme	208.55	4.48	46.56	0.00	0006508 // proteolysis
Q6K5Q0	germin-like protein	197.93	2.77	71.57	0.01	0009651 // response to salt stress
Q9SIA4	mate efflux family protein 5	190.20	47.31	4.02	0.00	0042991 // transcription factor import into nucleus
O54922	exocyst complex component	187.10	94.74	1.97	0.03	
Q5ZJX4	rRNA-binding protein 38	181.61	100.00	1.82	0.01	0030330 // DNA damage response, signal transduction by p53 class mediator
Q6H754	zinc finger a20	180.45	83.03	2.17	0.00	

Table 4.3 -The 50 most abundant genes in mycorrhizal roots, among those with positive fold change within mycorrhizal and non-mycorrhizal roots of *Oeceoclades maculata.*

(continuation)

Swissprot ID	Swissprot - Acession	Myc - Means	Non-Myc - Means	Fold change	*p*-value	Annotations - GO biological process
A5BUU4	40s ribosomal protein	178.72	106.68	1.68	0.00	0006412 // translation /// 0000028 // ribosomal small subunit assembly
Q944S9	mads-box transcription factor 16	161.90	64.32	2.52	0.01	0009860 // pollen tube growth /// 0010093 // specification of floral organ identity
P36181	heat shock cognate protein 80	159.86	88.77	1.80	0.01	0006457 // protein folding **/// 0006950 // response to stress**
Q9FEF8	RNA polymerase II transcription subunit 36b	151.10	98.76	1.53	0.01	0016571 // histone methylation /// 0000478 // endonucleolytic cleavage involved in rRNA processing
Q9LW00	methyl-binding-domain-containing protein 11	142.42	77.97	1.83	0.01	
P94549	probable enoyl- hydratase	139.68	49.76	2.81	0.00	0008152 // metabolic process
P52409	glucan endo- -beta-glucosidase ame	132.33	55.06	2.40	0.04	0008152 // metabolic process
Q0D3J9	zinc finger ccch domain-containing protein	125.60	75.72	1.66	0.00	
Q42569	cytochrome p450	125.09	54.38	2.30	0.00	0048767 // root hair elongation /// 0010268 // brassinosteroid homeostasis /// 0071555 // cell wall organization
P27484	glycine-rich protein 2	121.94	70.82	1.72	0.00	0006355 // regulation of transcription, DNA-dependent

Table 4.3 - The 50 most abundant genes in mycorrhizal roots, among those with positive fold change within mycorrhizal and non-mycorrhizal roots of *Oeceoclades maculata*.

(continuation)

Swissprot ID	Swissprot - Acession	Myc - Means	Non-Myc - Means	Fold change	*p*-value	Annotations - GO biological process
Q8GRL4	homeobox-leucine zipper protein	120.41	47.98	2.51	0.00	0006351 // transcription, DNA-dependent
Q05001	NADPH-cytochrome p450 reductase short	119.00	57.76	2.06	0.02	0055114 // oxidation-reduction process
Q9ZWA6	zinc finger protein magpie	116.38	75.49	1.54	0.00	0006355 // regulation of transcription, DNA-dependent
Q9C8D1	u-box domain-containing protein	115.22	20.35	5.66	0.04	0010200 // response to chitin
Q9SMT7	4-coumarate-- ligase-like 10	113.50	37.78	3.00	0.04	0033611 // oxalate catabolic process /// 0010167 // response to nitrate /// 0015706 // nitrate transport /// 0050832 // defense response to fungus
Q9LT67	transcription factor bhlh113	112.47	40.24	2.79	0.00	0006355 // regulation of transcription, DNA-dependent
Q93YS6	transcription factor gte9	111.12	66.65	1.67	0.05	
Q9UKV3	apoptotic chromatin condensation inducer in the nucleus	107.31	69.09	1.55	0.05	0030263 // apoptotic chromosome condensation /// 0030218 // erythrocyte differentiation
B8AM21	u2 small nuclear ribonucleoprotein b short	106.44	64.56	1.65	0.01	

Table 4.3 -The 50 most abundant genes in mycorrhizal roots, among those with positive fold change within mycorrhizal and non-mycorrhizal roots of *Oeceoclades maculata.*

(conclusion)

Swissprot ID	Swissprot - Acession	Myc - Means	Non-Myc - Means	Fold change	*p*-value	Annotations - GO biological process
F4KFC7	probable mediator of RNA polymerase II transcription subunit 26c	104.66	52.08	2.01	0.00	
Q39433	ras-related protein rab1bv	103.59	66.97	1.55	0.01	0015031 // protein transport /// 0007264 // small GTPase mediated signal transduction
Q10716	cysteine proteinase	103.30	57.92	1.78	0.02	
P93257	probable mannitol dehydrogenase	101.93	48.01	2.12	0.00	0055114 // oxidation-reduction process
Q9FM19	hypersensitive-induced response	100.38	57.08	1.76	0.00	0010075 // regulation of meristem growth /// 0019344 // cysteine biosynthetic process
Q948P6	ferritin- chloroplastic	98.55	27.31	3.61	0.03	0055114 // oxidation-reduction process /// 0006826 // iron ion transport /// 0006879 // cellular iron ion homeostasis
Q9C7A2	ankyrin repeat-containing protein	97.94	61.34	1.60	0.00	0009651 // response to salt stress /// 0034613 // cellular protein localization
Q9SUP6	WRKY transcription factor 53	95.33	22.36	4.26	0.01	0006612 // protein targeting to membrane /// 0031348 // negative regulation of defense response //// 0050832 // defense response to fungus

Most abundant genes inO. maculata non-mycorrhizal roots

In table 4.4 we list the 50 most abundant genes in non-mycorrhizal roots of *Oeceoclades maculata*, among those negative fold change (Table 4.4). The most abundant gene in non-mycorrhizal roots was a mannose-specific binding lectin, with striking 43,808.67 RPKMand -37.92 fold change (Myc *versus* Non-Myc). This gene has been previously characterized to be involved in anti-fungal responses in *Gastrodia elata* (WANG, 2001). Also whithin the most expressed genes in non-mycorrhizal roots was a chitinase (181.17 RPKM; -11.64 fold change), which is a well-known hydrolytic enzyme activated in response to fungal infections and show to be suppressed in arbuscular mycorrhizal roots (VOLPIN et al., 1994)

Also, more abundant in non-mycorrhizal roots was an allene oxide synthase (658.41 RPKM; -4.33 fold change)and a 9-lipoxygenase (758,38 RPKM; -5.71 fold change), both involved in jasmonate biosynthesis, and an abscisic acid stress-ripening protein (1009.19 RPKM; -6.14 fold change).

Genes related to energy metabolism, likely involved in sucrose and starch biosynthesis, were also shown to be more expressed in non-mycorrhizal roots (Table 4.4).

Table 4.4 - The 50 most abundant genes in non-mycorrhizal roots, among those with negative fold change within mycorrhizal and non-mycorrhizal roots of *Oeceoclades maculata*

(continue)

SwissProt ID	Swiss prot - acession	Myc - Means	Non-Myc Means	Fold change	*p* - value	Annotations - GO biological process
B2KNH9	mannose-specific lectin	1155,31	43808,67	-37,92	0,01	0050832 // defense response to fungus
O49169	elongation factor 1-alpha	2193,34	3897,52	-1,78	0,01	0006184 // GTP catabolic process /// 0006414 // translational elongation
Q41607	sucrose synthase	684,52	1973,04	-2,88	0,00	0005985 // sucrose metabolic process /// 0009058 // biosynthetic process
Q7FAH2	glyceraldehyde-3-phosphate dehydrogenase	864,21	1305,94	-1,51	0,01	/// 0042542 // response to hydrogen peroxide /// 0006096 // glycolysis
Q9ATM4	aquaporin pip2	622,81	1032,22	-1,66	0,04	0006810 // transport
P37220	abscisic stress-ripening protein 3	164,23	1009,19	-6,14	0,00	0006950 // response to stress
P33627	tubulin alpha-6 chain	500,17	937,00	-1,87	0,01	0051258 // protein polymerization /// 0006184 // GTP catabolic process /// 0007017 // microtubule-based process
Q9SYT0	annexin d1	276,13	759,04	-2,75	0,00	0009409 // response to cold ///
Q43191	linoleate 9s-lipoxygenase 5	132,21	755,38	-5,71	0,02	0006633 // fatty acid biosynthetic process
Q39613	peptidyl-prolyl cis-trans isomerase	418,42	693,36	-1,66	0,01	0000413 // protein peptidyl-prolyl isomerization /// 0006457 // protein folding
P48494	triosephosphate cytosolic	437,06	693,27	-1,59	0,01	0006094 // gluconeogenesis /// 0006096 // glycolysis /// 0044262 // cellular carbohydrate metabolic process
P48417	allene oxide synthase	151,98	658,41	-4,33	0,03	0006633 // fatty acid biosynthetic process
P10978	retrovirus-related pol polyprotein	388,36	632,65	-1,63	0,01	0043170 // macromolecule metabolic process /// 0044238 // primary metabolic process
Q9SJY5	mitochondrial uncoupling protein	104,26	509,63	-4,89	0,03	0010200 // response to chitin /// 0015992 // proton transport /// 0006839 // mitochondrial transport
Q9ZWA8	fasciclin-like	203,33	406,20	-2,00	0,02	

Table 4.4 -The 50 most abundant genes in non-mycorrhizal roots, among those with negative fold change within mycorrhizal and non-mycorrhizal roots of *Oeceoclades maculata*

(continuation)

SwissProt ID	Swiss prot - acession	Myc - Means	Non-Myc Means	Fold change	*p* - value	Annotations - GO biological process
O82627	granule-bound starch synthase	153,24	390,43	-2,55	0,04	0009250 // glucan biosynthetic process
Q6Z844	coatomer subunit zeta-2	239,39	376,71	-1,57	0,01	0016192 // vesicle-mediated transport /// 0015031 // protein transport
P21616	pyrophosphate-energized-vacuolar membrane proton pump	205,39	347,54	-1,69	0,01	0015992 // proton transport
P48724	eukaryotic translation initiation factor 5	177,06	311,64	-1,76	0,00	0006412 // translation
B3H4F1	protein spiral1-like 1	191,57	304,51	-1,59	0,01	
O24381	plastidic atp adp-transporter	173,63	273,93	-1,58	0,05	
Q94G86	glucan endo- -beta-d-glucosidase	135,64	265,64	-1,96	0,00	
P46225	triosephosphate chloroplastic	148,50	244,83	-1,65	0,02	0006096 // glycolysis
Q42850	protochlorophyllide reductase	43,71	238,88	-5,46	0,01	/// 0015995 // chlorophyll biosynthetic process /// 0015979 // photosynthesis
Q96262	plasma membrane-associated cation-binding protein	137,67	228,63	-1,66	0,02	/// 0031115 // negative regulation of microtubule polymerization
P46265	tubulin beta-5 chain	138,29	225,60	-1,63	0,03	0051258 // protein polymerization /// 0006184 // GTP catabolic process /// 0007018 // microtubule-based movement
Q5W7C1	upf0014 membrane protein star2 ame	45,53	217,04	-4,77	0,00	0010044 // response to aluminum ion /// 0015786 // UDP-glucose transport
P46302	40s ribosomal protein s28	138,32	215,46	-1,56	0,02	0006412 // translation
P52416	glucose-1-phosphate adenylyltransferase	72,86	209,99	-2,88	0,01	0005978 // glycogen biosynthetic process /// 0019252 // starch biosynthetic process
O22319	metallothionein-like protein type 2	112,23	201,41	-1,79	0,01	
O48528	outer envelope pore protein	116,85	196,88	-1,68	0,01	0006511 // ubiquitin-dependent protein catabolic process
P55233	glucose-1-phosphate adenylyltransferase	88,82	190,29	-2,14	0,03	0005978 // glycogen biosynthetic process /// 0019252 // starch biosynthetic process

Table 4.4 -The 50 most abundant genes in non-mycorrhizal roots, among those with negative fold change within mycorrhizal and non-mycorrhizal roots of *Oeceoclades maculata*

(conclusion)

SwissProt ID	Swiss prot - acession	Myc - Means	Non-Myc Means	Fold change	*p* - value	Annotations - GO biological process
Q8GYN5	rinrpm1-interacting protein 4	85,92	190,03	-2,21	0,00	0009816 // defense response to bacterium, incompatible interaction
O82199	zinc finger ccch domain-containing protein 20	110,13	181,21	-1,65	0,04	0006661 // phosphatidylinositol biosynthetic proces/// 0006979 response to oxidative stress
Q9MA41	chitinase	15,57	181,18	-11,64	0,00	0009611 // response to wounding
O22126	asciclin-like arabinogalactan protein 8	27,14	180,31	-6,64	0,00	0009664 // plant-type cell wall organization /// 0009832 // plant-type cell wall biogenesis
Q9M040	pyruvate decarboxylase	115,67	179,63	-1,55	0,04	
Q7XLR1	probable aquaporin pip2	79,20	165,18	-2,09	0,01	0006810 // transport
Q9LZS3	alpha-glucan-branching enzyme 2	91,65	165,09	-1,80	0,03	/// 0010021 // amylopectin biosynthetic process /// 0019252 // starch biosynthetic process
P93092	acyl carrier protein	106,94	162,11	-1,52	0,02	0006631 // fatty acid metabolic process
P46265	tubulin beta-5 chain	89,78	159,34	-1,77	0,01	0051258 // protein polymerization /// 0006184 // GTP catabolic process
P34913	bifunctional epoxide hydrolase 2 includes	92,60	148,47	-1,60	0,02	0019222 // regulation of metabolic process 0016311 // dephosphorylation
Q7X996	cbl-interacting protein kinase	58,62	136,24	-2,32	0,01	0006468 // protein phosphorylation /// 0007165 // signal transduction
Q6L440	late blight resistance protein	21,14	134,93	-6,38	0,02	
Q8RZQ8	bidirectional sugar transporter	6,11	134,38	-21,99	0,00	0008643 // carbohydrate transport
Q9FNE2	glutaredoxin	48,29	128,56	-2,66	0,00	
Q6AT26	probable cellulose synthase	78,05	127,53	-1,63	0,04	0030244 // cellulose biosynthetic process /// 0009833 // primary cell wall biogenesis
Q9LM02	cycloartenol-c-24-methyltransferase	75,54	120,52	-1,60	0,00	0016126 // sterol biosynthetic process /// 0009793 /// 0009805 // coumarin biosynthetic process
Q9SJY5	mitochondrial uncoupling protein 5	15,59	119,12	-7,64	0,04	0010200 // response to chitin /// 0015992 // proton transport /// 0006839 // mitochondrial transport

4.4 Discussion

Although the diversity of orchid mycorrhiza (BRUNDRETT, 2007; OTERO; BAYMAN; ACKERMAN, 2005; RASMUSSEN, 2002) and the characterization of fungal symbionts and symbiotic germination (VALADARES et al., 2012; ZETTLER; HOFER, 1998) have been the subjects of several studies, studies on the molecular mechanisms regulating orchid mycorrhiza are lacking (DEARNALEY, 2007). Here we provide the first deep sequencing of an OM transcriptome, mostly focusing on plant genes with differential expression. We have constructed six independent cDNA libraries by using enriched mRNA isolated from three mycorrhizal and three non-mycorrhizal root samples. A variety of plant genes have been identified and classified according to the Gene Ontology (GO) database. Their putative roles in OM symbiosis are discussed below.

OM and the energetic metabolism of the host plant

The role of orchid mycorrhizal fungi in providing organic carbon to the host plant during its germination is probably one of the key features and first observed phenomena in OM. Indeed, orchids were amongst the first group of plants in which benefits of mycorrhizal associations were recognized (BERNARD, 1899).

Orchids develop from extremely small endosperm-lacking seeds into mycoheterotrophic structures called protocorms (RASMUSSEN, 2002). Whilst this completely fungus-dependent trophic strategy is retained throughout the life of non-photosynthetic orchids, most of the orchid species produce green leaves and are therefore assumed to be at least partially autotrophic (SELOSSE; ROY, 2009). Conversely, very little is known about nutrient dynamics of these plants when growing in nature, especially concerning the return of carbon from the plant to the fungus (CAMERON; LEAKE; READ, 2006). It is known that OM fungi can utilize many carbon sources including complex polysaccharides, cellulose, lignin, fructose and dextrose (Harley, 1968), but the carbon form that is transferred to the host plant is currently unknown.

From the up-regulated genes, 31 (corresponding to 20 proteins) were assigned to the starch and sucrose metabolism pathway (APPENDIX A) and 10 genes (corresponding to 10 proteins) were assigned to the glycolysis/gluconeogenesis pathway (APPENDIX B). With the current approach, we are not able to predict if these enzymes are working in the direction of energy generation or storage, neither to confirm whether any source of carbon is being

redirected back to the fungus. It is also important to highlight that, as discussed in the chapter 1, a possible role of enzymes from the glycolytic pathway in general plant responses to fungal infection may also be considered (MUTUKU; NOSE, 2012; VALADARES et al., 2013).

Nine up-regulated genes (corresponding to 8 proteins) were assigned to the amino sugar metabolism pathway (APPENDIX C). Cameron et al. (2006) have used double-labeled [^{13}C - ^{15}N]-glycine to track carbon transfer from the fungus to the plant, showing that amino-sugars are a suitable carbon source for OM fungi. Our data corroborate their data, suggesting that amino sugars can be used as carbon sources by the fungus and then transferred to the plant. In the same pathway, the up-regulation of six genes involved in the conversion of L-arabinose to α-galactose, suggesting that this is an important carbon source in mycorrhizal roots.

Enhanced mineral nutrient assimilation in OM roots

Although the role of arbuscular mycorrhizal fungi (AMF) in plant nutrition is well described (SMITH; SMITH, 2011), the involvement of orchid mycorrhizal fungi in mineral nutrition of host plants is mostly unknown. Even though, direct evidence for uptake and transfer of N in OM is limited (SMITH; READ, 1997), there is increasing evidence that terrestrial orchids are particularly dependent on their fungal partners for N nutrition. The concentrations of N in terrestrial orchid shoots can be surprisingly high, even in achlorophyllous species which lack effective photosynthetic machinery, and would therefore have a very low N demand (GEBAUER; MEYER, 2003).

Cameron et al. (2006) were the first to demonstrate that N can be transported from fungal mycelia to *Goodyera repens.* They have shown that both C and N, when supplied as double-labeled glycine, were transferred from the hyphae to the roots, rhizomes and to green shoots of the plant. In our dataset, enhanced accumulation of transcripts encoding nitrate and nitrite reductases, which are involved in the assimilatory nitrate reduction, ammonia-ligase and L-glutamate synthase, involved in ammonia assimilation into amino acids, has been detected in mycorrhizal roots and assigned to the *Nitrogen Metabolism Pathway* (Appendix D). Additionally, six other enzymes involved in the alanine, aspartate and glutamate metabolism were also up-regulated in Myc roots. Our findings corroborate Cameron et al. (2006) results, and suggest that N assimilation and amino acid metabolism are also highly enhanced in *Oeceoclades maculata* roots.

Symbiosis control - stress responses, hormonal regulation and defense

Plant responses to different stresses are highly complex and involve changes in cell structure and physiology. In AM, upon recognition of the fungal partner, the plant actively reprograms the host cell in order to accommodate the fungus within the cortical tissue and controls its proliferation (BONFANTE; REQUENA, 2011). In our dataset, the most represented biological process, comprising 325 up-regulated genes from *O. maculata* Myc roots, was "Stress Responses", which includes genes involved in the regulation of reactive oxygen species homeostasis, membrane polarization, signaling recognition and transduction and reorganization of cell architecture. In chapter 2, using proteomic techniques, we have observed changes in proteins related to the regulation of reactive oxygen species homeostasis, proteases, transport, metabolism and cell wall modifications. Together with the proteomic data, our transcriptomic data suggest that stress responses in OM are likely related to fungal recognition and cellular modification required for intracellular accommodation of the fungal symbiont and functionality of the symbiosis.

Host changes in response to a fungal symbionts are also related to modifications in the relative abundance of phytohormones, most of which are thought to play a central role in the regulation of symbioses (FRACETTO et al., 2013; HAUSE; FESTER, 2005). Among plant hormones, ethylene (ET), salicylic acid (SA), abscisic acid (ABA), and jasmonic acid (JA) are known to be key elements in fine-tuning the plant defense responses during interactions with microorganisms (LÓPEZ-RÁEZ et al., 2010).

Jasmonates are important regulators of plant responses to biotic and abiotic stresses, as well as of plant development, and are considered essential for the defense responses against various types of plant pathogens (WASTERNACK; HAUSE, 2013). In the chapter 2, we have shown that a Jasmonate-ZIM domain protein (JAZ), which acts to repress transcription of jasmonate-responsive genes, was up-regulated in *O. maculata* mycorrhizal roots. On the other hand, an allene oxide synthase, which catalyze the first step in the biosynthesis of jasmonic acid, and a lipoxygenase, catalyzes the biosynthesis of oxylipins, which will further generate an extensive family of metabolites (including JA) were down-regulated under the same conditions. The current transcriptomic dataset corroborate the proteomic data. Transcripts coding a JAZ protein were 4.3 fold more abundant in Myc roots than in non-myc roots, whilst transcripts related to an allene oxide synthase and 9-lipoxygenase were 4.3 and 5.7 fold down-regulated in myc roots, respectively. Altogether, our results point to a repression of crucial steps of JA biosynthesis and perception in OM roots.

ABA is involved in the regulation of many aspects of plant development and defense responses (MAUCH-MANI; MAUCH 2005,MOHR; CAHILL 2007; TORRES-ZABALAet al., 2007; ADIE et al.,2007). However, the role of ABA in plant defense appears to be more complex, and vary among different types of plant-microbe interactions. In general, ABA is shown to be involved in the negative regulation of plant defense against various biotrophic and necrotrophic pathogens (BARI; JONES, 2009). Mutants deficient in ABA are more sensitive to infection by the fungal pathogens *A. brassicicola, Pythium irregulare* (ADIEet al., 2007) and *Leptosphaeria maculans* (KALIFFet al., 2007).In our study, transcripts encoding an ABA 8-hidroxylase have been detected exclusively in mycorrhizal roots. This enzyme catalyzes the first step in the oxidative degradation of ABA. On the other hand, an ABA receptor encoding transcript was shown to be suppressed 4.6 fold in mycorrhizal roots. Like JA, ABA transduction pathways are likely to be highly regulated in OM roots allowing fungal colonization and hyphal spread into root cortical cells.

Ethylene is another key phytohormone involved in the regulation of plant development (SCHALLER, 2012) and plant-microbe interactions (FRACETTO et al., 2013; PENMETSA et al., 2008; RIEDEL; GROTEN; BALDWIN, 2008; ZSÖGÖN et al., 2008). The inhibitory effect of exogenous ethylene in AM development has been observed in different AMF-host combinations (GEIL et al., 2001; GEIL; GUINEL, 2002 apud FRACETTO et al., 2013). However, it has been shown that exogenous ethylene may either inhibit or stimulate AM formation, depending on the concentration (ISHII et al., 1996). Whilst specific roles for ethylene are largely unknown, ethylene-responsive transcription factors are usually found among highly up-regulated genes in AM roots, suggesting regulatory roles (FRACETTO et al., 2013). Similar to AM, we have detected 15 ethylene responsive transcriptions factors and 3 ethylene-induced calmodulin with enhanced accumulation in *O. maculata* Myc roots, suggesting that ethylene is also involved in OM regulation.

Chitinases are hydrolytic enzymes that are able to cleave β-1,4-glycosidic bonds between N-acetyl glucosamine residues of chitin. This polysaccharide is a primary structural component of the cell wall of several fungi, including AMF, ectomycorrhizal fungi, and plant pathogens (SALZER et al., 2000). Chitinase is also one of the known down-stream ethylene-regulated genes highly regulated in AM roots (LAMBAIS; MEHDY, 1993). In AM, an early induction of chitinase transcript accumulation and activity followed by suppression, compared to non-infected controls, has been observed (SPANU; BONFANTE, 1988; LAMBAIS; MEHDY, 1993). Moreover, an anti-fungal protein has been identified in the orchid *Gastrodia*

elata, and it was named Gastrodianin (WANG et al., 2001). Similar proteins were further found in *Epipactis helleborine* and *Listera ovata*, also Orchidaceae, suggesting that they belong to a novel class of orchid proteins involved in controlling fungal growth. Their observed fungistatic effect is thought to be due to their chitin- and mannose-binding activities, affecting hyphal wall and membrane integrity, respectively (WANG et al., 2007).

The strong suppression of the levels of transcripts encoding a mannose-specific bind lectin (41 fold) and a chitinase (11 fold), both well known for their anti-fungal activities, in Myc roots suggests that both are key components of the defense responses in OM. Their down-regulation in Myc roots adds to the previous discussed evidences of the alleviation of the defense responses in *O. maculata* mycorrhizal roots. However, with the current approach their relationship with hormones or other signaling molecules cannot be inferred.

It is also important to note that the mannose-specific binding lectin encoding transcripts was the most abundant in the whole dataset with more than 40,000 RPKM. Comparatively the second most abundant transcript was a Late embryogenesis protein, with approximately 12,000 RPKM in Non-Myc and 10,000 RPKM in Myc roots. This could be an indication that, in the presence of a fungal elicitor, the expression of this gene is enhanced in other parts of the plant in order to avoid infection in non-desired tissues.

Final considerations

The current dataset broadly expands our results on *O. maculata* responses to mycorrhizal fungi, obtained by using proteomic techniques. In general, the proteomic an transcriptomic data point to the same directions. We observed strong differences in carbon and mineral nutrient metabolism, stress responses, structural cellular modifications, defense-related responses and phytohormones, when comparing myc and non-myc roots of O. maculata. Further studies should address specific roles of each component of the symbiotic machinery. For example it would be interesting to understand whether fungal derived N and C are transferred to the host plant when fungal structures are digested or when they are still intact.

The expression of key anti-fungal proteins should also be investigated in a time-course of symbiosis development and their localization in specific cell-types would reveal their roles in OM regulation and functioning. As suggested by Wang et al. (2007), studying orchid defense mechanisms may contribute to the identification of new phytopathogenic fungi controlling technologies. Finally OM can also be used as a model for understanding the role

of plant phytohormones in endomycorrhiza regulation and help to understand the mechanisms controlling AM, as well.

4.5 Conclusion

Mycorrhizal colonization in orchids is accompanied by dramatic changes in host plant metabolism, especially concerning the metabolism of carbohydrates. The up-regulation of transcripts encoding proteins involved in N assimilation is a strong evidence of the contribution of orchid mycorrhizal fungi to mineral nutrient uptake. We have also observed that colonization with mycorrhizal fungi triggers strong induction of general stress responses, which may lead to physiological and structural adaptations of host cells to accommodate the fungal symbiont and develop a functional symbiosis.

The down-regulation of transcripts involved in JA and ABA transduction pathways, together with the up-regulation of genes encoding ethylene responsive transcription factors seems to act synergically to suppress anti-fungal responses in mycorrhizal roots. Even though specific roles of these phytohormones in OM remain unclear, it is conceivable that a fine-tuning of hormone balance takes place in OM and it should be directed related to fungal control.

Our results suggest that there are well-conserved shared regulatory mechanisms between OM and AM, especially concerning the alleviation of defense responses in mycorrhizal roots. It seems that OM symbiosis is much closer to a compatible interaction than to a unilateral relationship in favor to the plant. Overall orchid mycorrhiza has proved to be a promising model for investigating plant-fungal interactions and further studies should now address specific roles of each of the discussed components of orchid symbiotic machinery.

References

ADIE, B.A.; PEREZ-PEREZ, J.; PÉREZ-PÉREZ, M.M.; GODOY, M.; SÁNCHEZ-SERRANO, J.J.; SCHMELZ, E.A.; SOLANO, R. ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defences in *Arabidopsis*. **Plant Cell**, Rockville, v. 19, p. 165-1681, 2007.

ASHBURNER, M.; BALL, C.A.; BLAKE, J.A.; BOTSTEIN, D.; BUTLER, H.; CHERRY, J.M.; DAVIS, A.P.; DOLINSKI, K.; DWIGHT, S.S.; EPPIG, J.T.; HARRIS, M.A.; HILL, D.P.; ISSEL-TARVER, L.; KASARSKIS, A.; LEWIS, S.; MATESE, J.C.; RICHARDSON, J.E.; RUBIN, G.M.; SHERLOCK, G. Gene ontology: tool for the unification of biology. **Nature Genetics**, New York, v. 25, n. 1, p. 25-29, 2000.

BARI, R.; JONES, J.D.G. Role of plant hormones in plant defence responses. **Plant MolecularBiology**, Dordrecht, v. 69, n. 4, p. 473-488, 2009.

BERNARD, N. Sur la germination du *Neottia nidus-avis*. **Comptes Rendus Hebdomadaires des Séances de l'Académie des Sciences**, Paris, v. 128, p. 1253–1255, 1899.

BONFANTE, P.; REQUENA, N. Dating in the dark: how roots respond to fungal signals to establish arbuscular mycorrhizal symbiosis. **Current Opinion in Plant Biology**, Saint Louis,v. 14, n. 4, p. 451-457, 2011.

BRUNDRETT, M.C. Scientific approaches to Australian temperate terrestrial orchid conservation. **Australian Journal of Botany**, Melbourne, v. 55, n. 3, p. 293-307, 2007.

CAMERON, D.D.; LEAKE, J.R.; READ, D.J. Mutualistic mycorrhiza in orchids: evidence from plant-fungus carbon and nitrogen transfers in the green-leaved terrestrial orchid *Goodyera repens*. **New Phytologist**, Cambridge, v. 171, n. 2, p. 405-416, 2006.

CONESA, A.; GÖTZ, S.; GARCÍA-GÓMEZ, J. M.; TEROL, J.; TALÓN, M.; ROBLES, M. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. **Bioinformatics**, London, v. 21, n. 18, p. 3674-6, 2005.

DE TORRES-ZABALA, M.; TRUMAN, W.; BENNETT M.H.; LAFFORGUE, G.; MANSFIELD, J.W.; RODRIGUEZ, E.P, BÖGRE, L.; GRANT, M.*Pseudomonas syringae* pv. tomato hijacks the *Arabidopsis* abscisic acid signaling pathway to cause disease. **EMBO Journal**, Oxford, v. 26, p. 1434-1443, 2007.

DEARNALEY, J.D.W. Further advances in orchid mycorrhizal research. **Mycorrhiza**, Berlin, v. 17, p. 475–486, 2007.

DEARNALEY J.D.W.; MARTOS, F.; SELOSSE, M.A.; Orchid mycorrhizas: molecular ecology, physiology, evolution and conservation aspects. In: HOCK, B. (Ed.). **The mycota IX: Fungal associations**. New York: Springer-Verlag, 2012. p. 207-230.

FRACETTO, G.G.M.; PERES, L.E.P.; MEHDY, M.C.; LAMBAIS, M.R. Tomato ethylene mutants exhibit differences in arbuscular mycorrhiza development and levels of plant defense-related transcripts. **Symbiosis**, Rehovot, v. 60, n. 3, p. 155–167, 2013.

GEBAUER, G.; MEYER, M. ^{15}N and ^{13}C natural abundance of autotrophic and myco-heterotrophic orchids provides insight into nitrogen and carbon gain from fungal association. **New Phytologist**, Cambridge, v. 160, p. 209-223, 2003.

GEIL, R.D.; PETERSON, R.L.; GUINEL, F.C. Morphological alterations of pea (*Pisum sativum* cv. Sparkle) arbuscular mycorrhizas as a result of exogenous ethylene treatment. **Mycorrhiza**, Berlin, v. 11, p. 137-143, 2001.

GONZÁLES-GARCÍA, V.; ONCO, M.A.P.; SUSAN, V.R. Review. Biology and systematics of the form genus Rhizoctonia. **Spanish Journal of Agricultural Research**, Madrid, v. 4, n. 1, p. 55–79, 2006.

HAUSE, B.; FESTER, T. Molecular and cell biology of arbuscular mycorrhizal symbiosis. **Planta**, Berlin, v. 221, n. 2, p. 184-196, 2005.

ISHII, T.; SHRESTHA, Y.H.; MATSUMOTO, I.; KADOYA, K. Effect of ethylene on the growth of vesicular-arbuscular mycorrhizal fungi and on the mycorrhizal formation of trifoliate orange roots. **Journal of the Japanese Society for Horticultural Science**, Tokyo, v. 65, p. 525-529, 1996.

KALIFF, M.; STAAL, J.; MYRENAS, M.; DIXELIUS, C. ABA is required for *Leptosphaeria maculans* resistance via ABI1- and ABI4-dependent signaling. **Molecular Plant Microbe Interactions**, Saint Paul, v. 20, p. 335-345, 2007.

LÓPEZ-RÁEZ, J.A; VERHAGE, A.; FERNÁNDEZ, I.; GARCÍA, J.M.; AZCÓN-AGUILAR, C.; FLORS, V.; POZO, M.J. Hormonal and transcriptional profiles highlight common and differential host responses to arbuscular mycorrhizal fungi and the regulation of the oxylipin pathway. **Journal of Experimental Botany**, Oxford, v. 61, n. 10, p. 2589-2601, 2010.

MAUCH-MANI, B.; MAUCH, F. The role of abscisic acid in plant-pathogen interactions. **Current Opinion in Plant Biology**, Saint Louis,v. 8, p. 409-414, 2005.

MOHR, P.G.; CAHILL, D.M. Suppression by ABA of salicylic acid and lignin accumulation and the expression of multiple genes, in *Arabidopsis* infected with *Pseudomonas syringae* pv. tomato. **Functional & Integrative Genomics**, Berlin, v. 7, p. 181-191, 2007.

MORANDI, D. Effect of xenobiotics on endomycorrhizal infection and isoflavonoid accumulation in soybean roots. **Plant Physiology and Biochemistry**, New Delhi, v. 27, p.697-701, 1989.

MUTUKU, J.M.; NOSE, A. Changes in the contents of metabolites and enzyme activities in rice plants responding to *Rhizoctonia solani* Kuhn infection: activation of glycolysis and connection to phenylpropanoid pathway. **Plant & Cell Physiology**, Kyoto, v. 53, n. 6, p. 1017-1032, 2012.

OTERO, J.T.; BAYMAN, P.; ACKERMAN, J.D. Variation in mycorrhizal performance in the epiphytic orchid *Tolumnia variegata in vitro*: the potential for natural selection. **Evolutionary Ecology**, London,v. 19, n. 1,p. 29–43, 2005.

PENMETSA, R.V.; URIBE, P.; ANDERSON, J.; LICHTENZVEIG, J.; GISH, J.; NAM, Y.; ENGSTROM, E.; XU, K.; SCKISEL, G.; PEREIRA, M.; BAEK, J.M.; LOPEZ-MEYER, M.; LONG, S.R.; HARRISON, M.J.; SINGH, K.B.; KISS,G.B.; COOK, D.R. The *Medicago truncatula* ortholog of *Arabidopsis* EIN2, sickle, is a negative regulator of symbiotic and pathogenic microbial associations. **Plant Journal**, Oxford, v. 55, n. 4, p. 580–595, 2008.

RASMUSSEN, H.N. Recent developments in the study of orchid mycorrhiza. **Plant and Soil**, The Hague, v. 244, p. 149–163, 2002.

RASMUSSEN, H.N.; RASMUSSEN, F.N. Trophic relationship in orchid mycorrhiza - diversity and implications for conservation. **Lankesteriana**, Cartago, v. 7, n. 1, p. 334–341, 2007.

_____. Orchid mycorrhiza: implications of a mycophagous life style. **Oikos**, Copenhagen,v. 118, n. 3, p. 334-345, 2009.

RIEDEL, T.; GROTEN, K.; BALDWIN, I.T. Symbiosis between *Nicotiana attenuata* and *Glomus intraradices*: ethylene plays a role, jasmonic acid does not. **Plant, Cell & Environment**, Oxford, v. 31, n. 9, p. 1203–1213, 2008.

SALZER, P.; BONANOMI, A; BEYER, K.; VÖGELI-LANGE, R.; AESCHBACHER, R.A.; LANGE, J.; WIEMKEN, A.; KIM, D.; COOK, D.R.; BOLLER, T. Differential expression of eight chitinase genes in *Medicago truncatula* roots during mycorrhiza formation, nodulation, and pathogen infection. **Molecular Plant-Microbe Interactions**, Saint Paul, v. 13, n. 7, p. 763-777, 2000.

SCHALLER, G.E. Ethylene and the regulation of plant development. **BMC Biology**, Leiden, v. 10, p. 9, 2012.

SELOSSE, M.A.; ROY, M. Green plants that feed on fungi: facts and questions about mixotrophy. **Trends in Plant Science**, Oxford, v. 14, n. 2, p. 64–70. 2009.

SMITH, S.E.; SMITH, F.A. Roles of arbuscular mycorrhizas in plant nutrition and growth: new paradigms from cellular to ecosystem scales. **Annual Review of Plant Biology**, Palo Alto, v. 62, p. 227-250, 2011.

SPANU, P.; BONFANTE, P. Cell-wall-bound peroxidase activity in roots of mycorrhizal *Allium porrum*. **New Phytologist**, Cambridge, v. 109, p. 119-124, 1988.

VALADARES, R.B.S.; PEREIRA M.C.; OTERO, J.T. CARDOSO, E.J.B.N. Narrow fungal mycorrhizal diversity in a population of the orchid *Coppensia doniana*. **Biotropica**, Lawrence, v. 44, n. 1, p. 114–122, 2012.

VOLPIN, H.; PHILLIPS, D.A.; OKON, Y.; KAPULNIK, Y. Suppression of an isoflavonoid phytoalexin defense response. **Plant Physiology**, Minneapolis, v. 108, n. 1995, p. 1449–1454, 1995.

WANG, H.-X.; YANG, T.; ZENG, Y.; HU, Z. Expression analysis of the gastrodianin gene ga4B in an achlorophyllous plant *Gastrodia elata* Bl. **Plant Cell Reports**, Berlin, v. 26, n. 3, p. 253-259, 2007.

WANG, X.; WANG, X.; BAUW, G.; VAN DAMME, E.J.; PEUMANS, W.J.; CHEN, Z.L.; VAN MONTAGU, M.; ANGENON, G.; DILLEN, W. Gastrodianin-like mannose-binding proteins: a novel class of plant proteins with antifungal properties. **Plant Journal: for Cell and Molecular Biology**, Oxford, v. 25, n. 6, p. 651–661, 2001.

WASTERNACK, C.; HAUSE, B. Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development: an update to the 2007 review in Annals of Botany. **Annals of Botany**, London, v. 111, n. 6, p. 1021-1058, 2013.

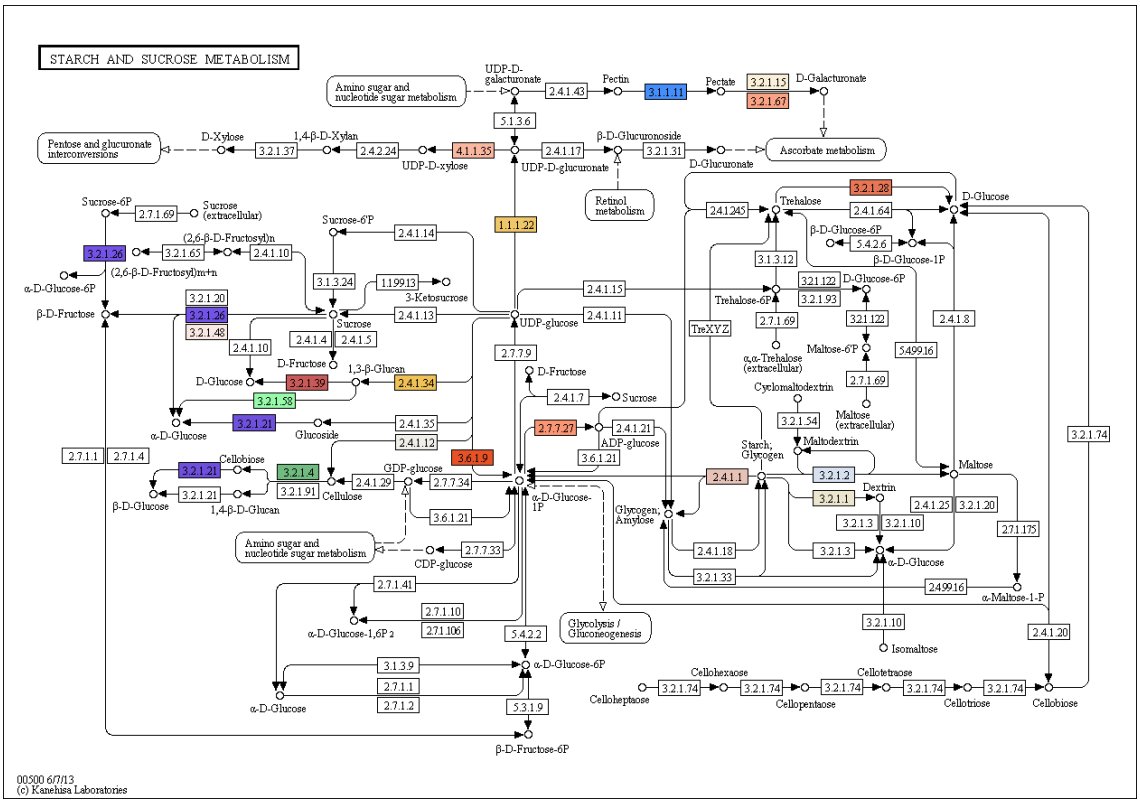
ZETTLER, L.W.; HOFER, C.J. Propagation of the little club-spur orchid (*Platanthera clavellata*) by symbiotic seed germination and its ecological implications. **Environmental and Experimental Botany**, Elmsford, v. 39, p. 189-195, 1998.

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 *Dendrobiumofficinale*. **PloS One**, London, v. 8, n. 8, e72705, 2013.

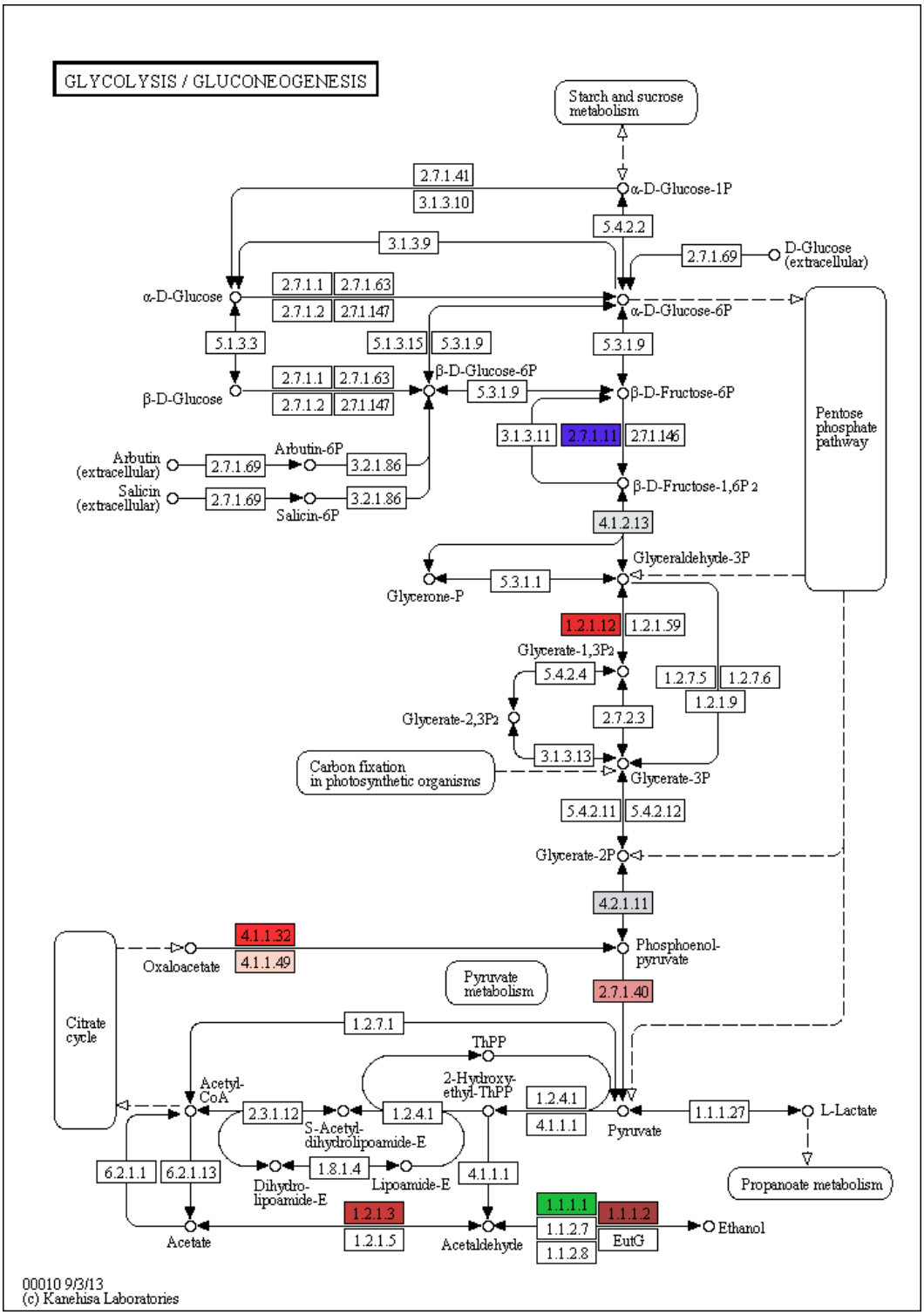
ZSÖGÖN, A.; LAMBAIS, M.R.; BENEDITO, V.A.; FIGUEIRA, A.V.O.; PERES, L.E.P. Reduced arbuscular mycorrhizal colonization in tomato ethylene mutants. **Scientia Agricola**, Piracicaba, v. 65, n. 3, p. 259–267, 2008.

APPPENDIX

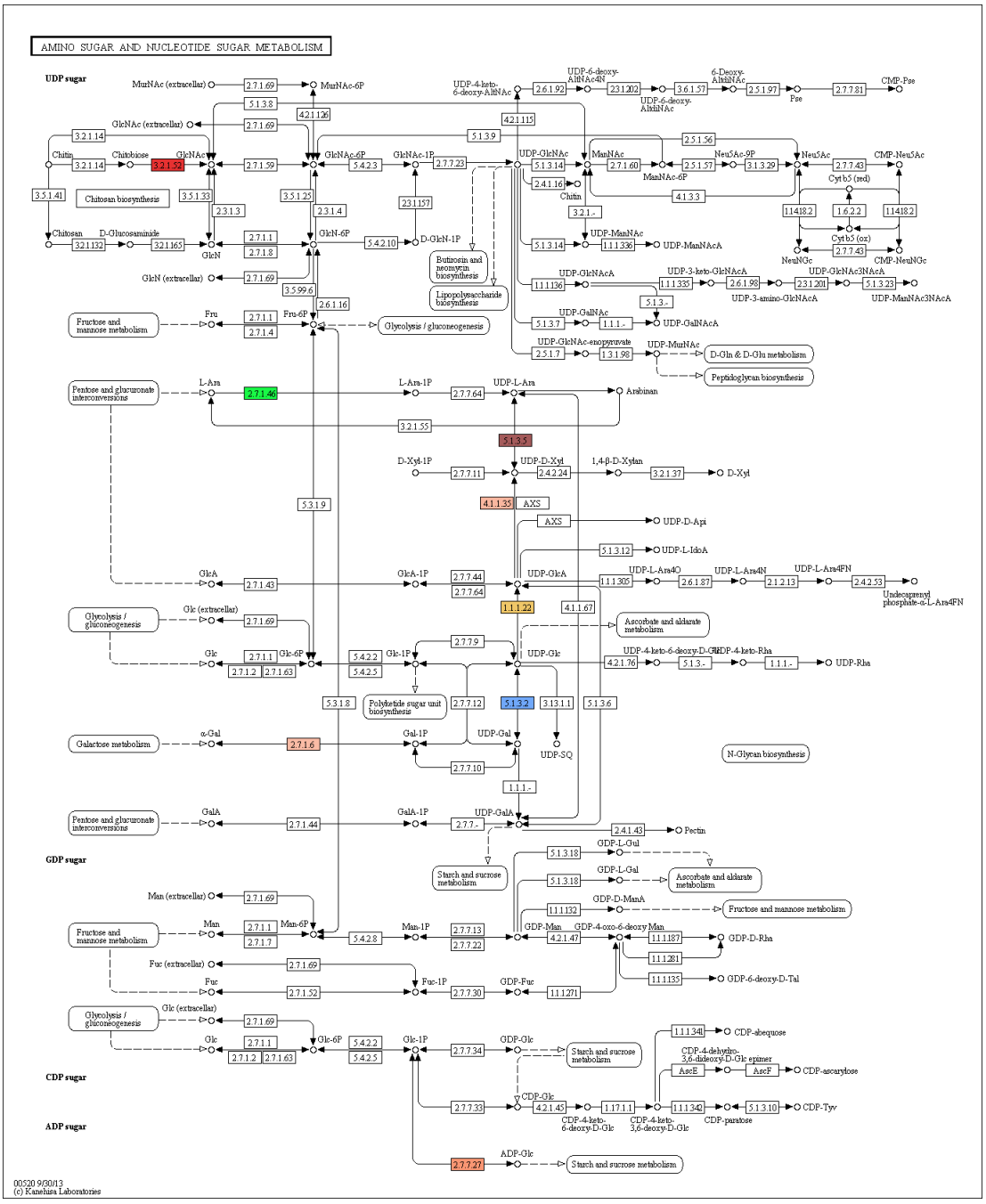
Appendix A - KEGG pathway representing amino starch and sucrose metabolism. Colored boxes represents enzymes related to genes up-regulatedin *Oeceoclades maculata* roots



Appendix B -KEGG pathway representing glycolysis/gluconeogenesis. Colored boxes represents enzymes related to genes up-regulatedin *Oeceoclades maculata* roots



Appendix C -KEGG pathway representing amino sugar and nucleotide metabolism. Colored boxes represents enzymes related to genes up-regulatedin *Oeceoclades maculata* roots



Appendix D -KEGG pathway of **nitrogen metabolism**. Colored boxes represents enzymes related to genes up-regulatedin *Oeceoclades maculata* roots