Proteome changes in Oncidium sphacelatum (Orchidaceae) at different trophic stages of symbiotic germination

Proteome changes in *Oncidium sphacelatum* (Orchidaceae) at different trophic stages of symbiotic germination

R. B. S. Valadares, S. Perotto, E. C. Santos, M. R. Lambais Original Paper

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 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 photosynthetic stage. Part of these changes may be also related to the obligatory nature of the interaction with the endomycorrhizal fungus.

Keywords

Orchid mycorrhiza Proteomic iTRAQ Symbiotic germination

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 and Rasmussen 2009). Although some orchid species remain achlorophyllous, and therefore mycoheterotrophic, throughout their life cycle (Rasmussen and 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 and Roy 2009).

Photosynthetic orchids form endomycorrhiza mainly with heterobasidiomycetes 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 et al. 2006).

Orchid mycorrhiza (OM) is 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 and Rasmussen 2007; 2009), some studies indicate the occurrence of nutrient exchange through intact cell membranes (see Dearnaley et al. 2012). During the achlorophyllous stages of their life cycle, orchids receive mostly C from the mycobiont, even though P, N, and water may also be transferred from the fungus to the plant (Peterson et al. 1998; Rasmussen and Rasmussen 2007; Cameron et al. 2007). Despite the evidence of C transfer from plant to fungus in photosynthetic orchids (Cameron et al. 2006, 2008), the benefit of the symbiosis for the fungus is still a matter of discussion (Rasmussen and Rasmussen 2009).

Enhanced activity of antioxidant 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 et al. 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 and 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, 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; Shimura et al. 2007), and are likely involved in restraining fungal growth in the plant tissues.

More recently, Zhao et al. (2013) used suppression subtractive hybridization to identify genes expressed in symbiotically germinated seeds of *Dendrobium officinale*. By 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 to better understand 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.

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 2 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 min, rinsed (four times) in sterile distilled water and suspended 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 9-mm 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 III (Zettler and Hofer 1998; Otero et al. 2005) 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-h (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 s. Proteins were precipitated overnight in three volumes of acetone at −20 °C and solubilized in 0.1-mM Tris–HCl pH 7.6. Proteins were quantified using a Qubit® fluorometer (QIAGEN, Gaithersburg, MD, USA).

Protein extracts were subjected to reduction, cysteine blocking, and digestion with trypsin. Peptides were tagged with isobaric tags using iTRAQ™ 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 five 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 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 (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 5 min. The spectra were acquired in the MS/MS mode in the 50–2,000-m/z range. A green fluorescent protein peptide was used as a

standard to correct variations in equipment calibration. All parameters were defined using the Mass Lynx v 2.1 software (Waters® MassLynx, Waters Inc., Milford, MA, USA).

Database search was performed using the software MassMatrix v. 2.4.0 (Xu and Freitas 2009). Fixed modifications were: iTRAQ-N, iTRAQ-K, and methylthio. Variable modifications were: iTRAQ-Y, methionine 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/Swiss-Prot, 2012-01-27), and the non-identified peptides were compared to *Orchidaceae* (UniProt/Swiss-Prot, 2011-07-24) and Viridiiplantae databases (UniProt/Swiss-Prot, 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 Munich Information Center for Protein Sequences (MIPS) functional catalog database (Ruepp et al. 2004; Recorbet et al. 2009). Proteins uncategorized by MIPS were classified manually based on their putative functions. Metabolic pathways were drawn using the software yED 3.10.2 (www.yworks.com); enzyme abbreviations followed the International Union of Biochemistry and Molecular Biology (http://www.chem.qmul.ac.uk/iubmb/).

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 an 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.

Table 1

Proteins identified in the symbiotic protocorms of *Oncidium sphacelatum* in association with a *Ceratobasidium* sp. isolate

Most similar accession	Organism	Description	Score	Coverage (%)	Number of Peptides	Mass (monoisotopic)	117/116 Ratio
Cell cycle and DNA processing							
E5L4N8	*Oncidium* hybrid cultivar	Actin-like protein (Fragment)	56	21	3	24.555	**0.77**
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**

Most similar accession	Organism	Description	Score	Coverage (%)	Number of Peptides	Mass (monoisotopic)	117/116 Ratio
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**
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
Q0GKF3	*Gymnadenia conopsea*	Chloroplast FLU-like protein	16	14	5	41.576	0.88
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 adenylyl-transferase	22	19	12	61.401	**1.31**
C7E4Z8	*Phragmipedium schlimii*	Glyceraldehyde-3-phosphate dehydrogenase (Fragment)	71	39	10	24.923	1.15

Most similar accession	Organism	Description	Score	Coverage (%)	Number of Peptides	Mass (monoisotopic)	117/116 Ratio
AQ9SE26	*Dendrobium crumenatum*	Isocitrate lyase	24	17	10	67.975	**0.59**
D5HQ13	*Oncidium* Gower Ramsey	NAD(P)H-quinone oxidoreductase subunit 4 L	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**
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
Metabolism							
Q52QS5	*Oncidium* Gower Ramsey	9-*cis*-epoxycarotenoid dioxygenase	26	22	6	29.190	**1.82**
Q96413	*Dendrobium crumenatum*	Aminocyclopropane-1-carboxylic acid (ACC) synthase	20	5	3	52.684	**4.48**
C3VEQ1	*Oncidium* Gower Ramsey	Beta-carotene hydroxylase	24	18	3	36.016	**0.79**

Most similar accession	Organism	Description	Score	Coverage (%)	Number of Peptides	Mass (monoisotopic)	117/116 Ratio
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**
B2LU34	*Oncidium* Gower Ramsey	Chromoplast specific carotenoid associated protein	31	5	2	37.413	**0.64**
B4F6G1	*Oncidium* Gower Ramsey	Fibrillin-like protein	31	9	3	37.604	**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**
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

Most similar accession	Organism	Description	Score	Coverage (%)	Number of Peptides	Mass (monoisotopic)	117/116 Ratio
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

Most similar accession	Organism	Description	Score	Coverage (%)	Number of Peptides	Mass (monoisotopic)	117/116 Ratio
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
D5HQ50	*Oncidium* Gower Ramsey	30S ribosomal protein S12, chloroplastic	18	20	4	15.596	**0.32**
Signaling							
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. Proteins were identified using MassMatrix software ($p < 0.05$). Score represents the protein identification score from MassMatrix. Coverage represents the protein sequence coverage by the peptides identified. Mass represents the monoisotopic mass of each protein hit. 117/116. Ratio represents the relative abundance of the two iTRAQ tags used to label proteins extracted from green (iTRAQ 117) and achlorophyllous (iTRAQ 116) *O. sphacelatum* protocorms

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.

Changes in orchid carbon metabolism during protocorm development

Given the lack of storage reserves typical of the minute orchid "dust-seeds" (Arditti 1992), 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 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 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 and Nose 2012). In addition, these changes were accompanied by the activation of the phenylpropanoid pathway (Danson et al. 2000).

The enhanced accumulation of a glucose-1-phosphate adenylyl-transferase (Table 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 Fig. 1.

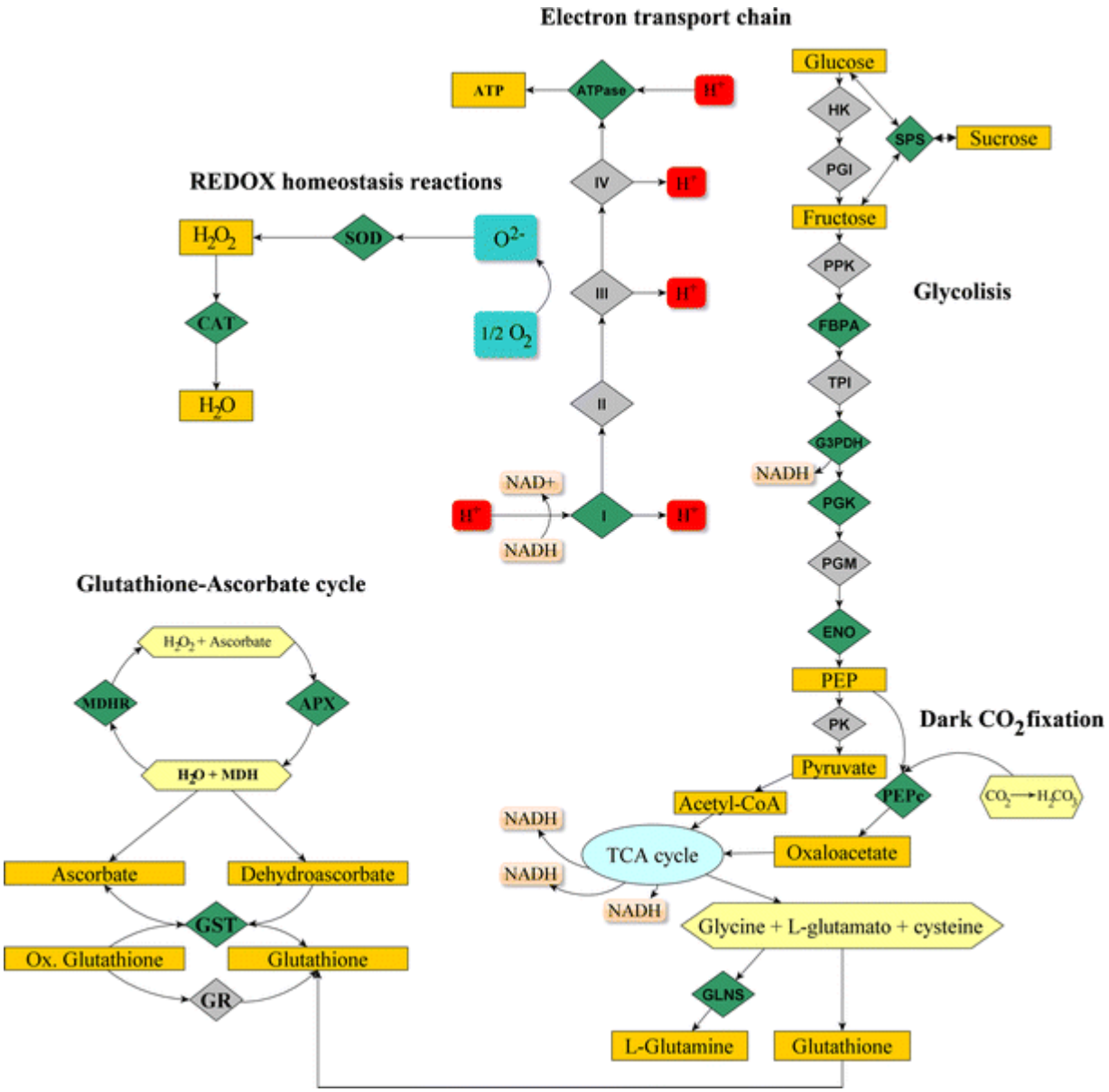

Fig. 1

Core reactions of the energetic metabolism and associated redox homeostasis mechanisms involving proteins identified in the symbiotic protocorms of *Oncidium sphacelatum* in association with a *Ceratobasidium* sp. isolate. Proteins represented inside green diamonds (*dark gray* in the printed version) were identified in this study. Abbreviations used: *SPS* sucrose phosphate synthase; *HK* hexokinase, *PGI* phosphoglucoisomerase, *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*, and *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 (Volpin et al. 1994; Volpin et al. 1995; Larose et al. 2002; Aloui et al. 2011) and nodulating bacteria as well (Hassan and Mathesius 2012). The higher PAL activity in symbiotic protocorms of *Anacamptis* (*Orchis*) *morio*, as compared to non-mycorrhizal protocorms (Beyrle et 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 *Oncidium 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 chalcone isomerase (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 *Medicago truncatula* roots colonized by *Glomus versiforme* when compared to non-mycorrhizal roots (Harrison and 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 the 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 and 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 et al. 2000). Indeed, specific carotenoids (C_{14} mycorradicin and C_{13} cyclohenenone) accumulate in roots during AM development (Klingner et al. 1995; Fester et al. 2002; Walter et al. 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 1, Fig. 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 1, Fig. 2).

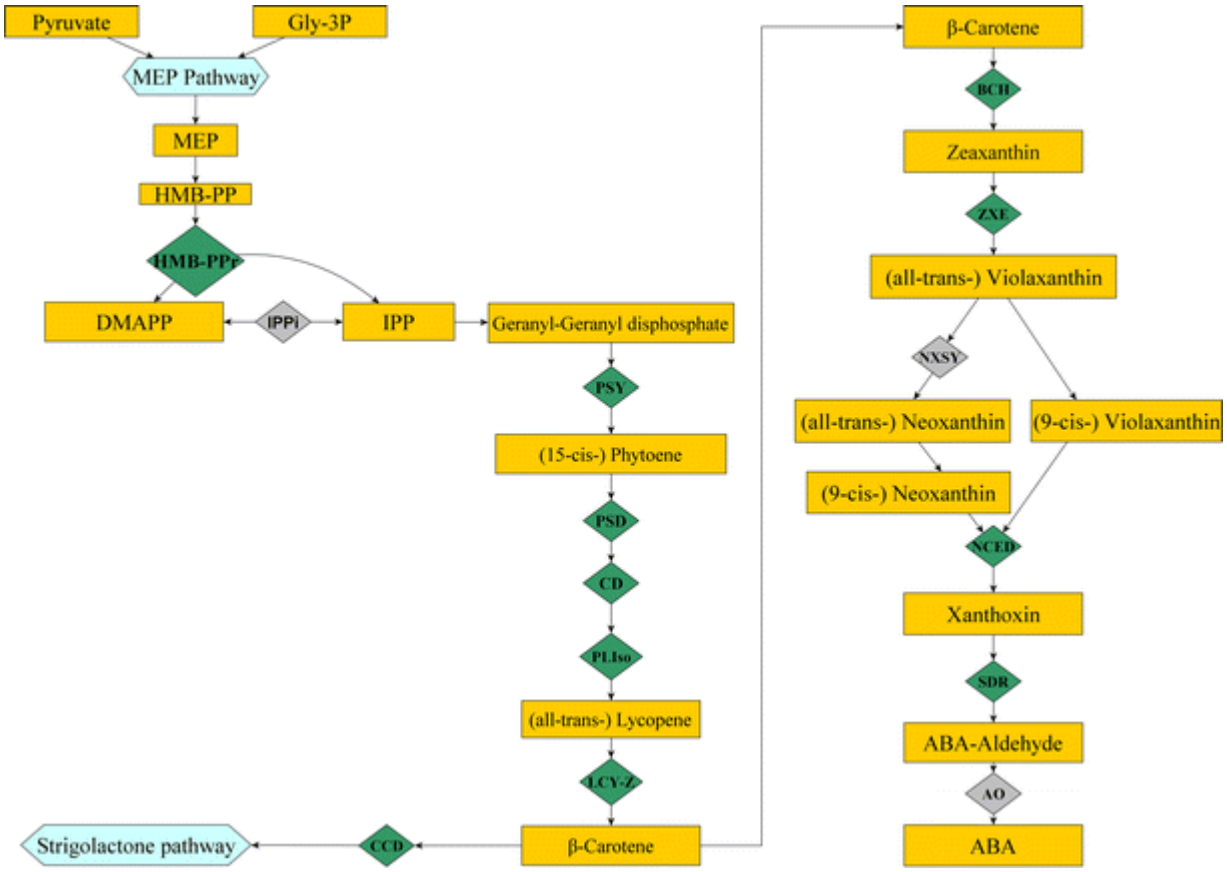

Fig. 2

Proteins involved in the biosynthesis of carotenoids, ABA and strigolactone identified in the symbiotic protocorms of *Oncidium sphacelatum* in association with a *Ceratobasidium* sp. isolate. Proteins represented inside *green diamonds* (*dark gray* in the printed version) 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

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 (Matusova et al. 2005; Bouvier et al. 2005; Akiyama et al. 2005; Martín-Rodríguez et al. 2011). In our study, NCED and 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 1, Fig. 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 et al. 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 (Zsögön et al.

2008; Penmetsa et al. 2008; Riedel et al. 2008; Fracetto et al. 2013), 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 and Zeevaart 1997), and an ethylene receptor showed enhanced accumulation in green protocorms, as compared to achlorophyllous protocorms (Table 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 (Zsögön et al. 2008; Martín-Rodríguez et al. 2011; Fracetto et al. 2013). 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 et 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 ET-signaling often operate synergistically to activate the expression of defense-related genes after pathogen infection (Penninckx et al. 1998; Thomma et al. 2001; Bari and Jones 2009). 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 the enhanced biosynthesis of JA at this stage.

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 and 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 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 and 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 and Manchanda 2009; Torres 2010). Differential expression of antioxidant enzymes has been observed in AM (Blilou et al. 2000; Lambais et al. 2003; Fracetto et al. 2013) and rhizobium-legume interactions (Pauly et al. 2006; Torres 2010). Staining techniques showed that intracellular accumulation of H_2O_2 in 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 and 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 (Salzer et al. 1999; Lanfranco et al. 2005), 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 *M. 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 (Sagi et al. 1999; Hesberg et al. 2004; Yesbergenova et al. 2005; Zarepour et al. 2010), and a bibenzyl synthase, known to catalyze the biosynthesis of phytoalexins in orchids (Reinecke and Kindl 1994), was also observed in green protocorms (Table 1), suggesting that the regulation of OM may occur at the cellular 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 and 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 1), although their significance in the regulation of OM interactions is currently unknown. The presence of a calmodulin in this dataset is noteworthy, due to its central role in the transduction of calcium signaling (Yang and 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 and 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 *Arabidopsis thaliana* (Burnette et al. 2003), points to a role of calcium in the regulation of OM.

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 an 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.

Acknowledgments

This research was funded by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). RBSV and ECS were supported by FAPESP.

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