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Comparative proteomic analysis of leaf tissue from tomato plants colonized with *Rhizophagus irregularis*

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Abstract A comparative proteomic approach was performed to analyze the differential accumulation of leaf proteins in response to the symbiosis between *Solanum lycopersicum* and the arbuscular mycorrhizal fungus (AMF) *Rhizophagus irregularis*. Protein profiling was examined in leaves from tomato plants colonized with AMF (*M*), as well as non-colonized plants fertilized with low phosphate (20 μM P; *NM-LP*) and non-colonized plants fertilized with regular phosphate Hoagland's solution (200 μM P; *NM-RP*). Comparisons were made between these groups, and 2D-SDS-PAGE revealed that 27 spots were differentially accumulated in *M* vs. *NM-LP*. Twenty-three out of the 27 spots were successfully identified by mass spectrometry. Two of these proteins, 2-methylene-furan-3-one reductase and auxin-binding protein ABP19a, were up-accumulated in *M* plants. The down-accumulated proteins in *M* plants were associated mainly with photosynthesis, redox, and other molecular functions. Superoxide dismutase, harpin binding protein, and thioredoxin peroxidase were down-accumulated in leaves of *M* tomato plants when compared to *NM-LP* and *NM-RP*, indicating that these proteins are responsive to AMF colonization independently of the phosphate regime under which they were grown. 14-3-3 protein was up-accumulated in *NM-RP* vs. *NM-LP* plants, whereas it was down-accumulated in *M* vs. *NM-LP* and *M* vs. *NM-RP*, regardless of their phosphate nutrition. This suggests a possible regulation by P nutrition and AMF colonization. Our results demonstrate AMF-induced systemic changes in the expression of tomato leaf proteins, including the down-accumulation of proteins related to photosynthesis and redox function.

Keywords Arbuscular mycorrhiza · Proteomics · SOLANUM lycopersicum · Leaf

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1 Introduction

Arbuscular mycorrhizal fungi (AMF) are soilborne microorganisms that establish a symbiotic association with most land plants (Smith and Read 1997). The AMF-plant interaction is a mutually beneficial event in which the plant supplies the fungus with carbon while the fungus assists the plant in the uptake of water, phosphate, and other mineral nutrients from the soil (Black et al. 2000; Peterson et al. 2004; Strack et al. 2003). In the AM symbiosis, the fungus invades the root cortical cells and forms differentiated hyphae, known as arbuscules, within the cells. As each arbuscule develops, the plant cell envelops it in a *de novo* synthesized membrane, and the resulting symbiotic interface is equipped with unique transporters that function mainly in nutrient exchange. In parallel to this intraradical colonization, the fungus develops an extensive network of hyphae in the soil surrounding the root. This extraradical mycelium explores and exploits the soil for nutrient acquisition and gives rise to new colonization events (Harrison 2005; Peterson et al. 2004; Strack et al. 2003).

The benefits of AM symbiosis on plant fitness are widely known and include improved mineral nutrition (mainly phosphorus), as well as the increased ability to overcome abiotic

and biotic stresses (Pozo and Azcón-Aguilar 2007). Importantly, the establishment of this symbiosis requires a developmental program finely regulated at the genetic level (Harrison 2005; Parniske 2004; Peterson et al. 2004). Root colonization by AM fungi also induces complex morphological, physiological, biochemical and molecular changes in both symbiosis partners (Pozo and Azcón-Aguilar 2007; Samra et al. 1997).

Although the physical interaction between the AM symbionts occurs in the root cortex, the physiology of the whole plant affects the development of the symbiosis, and in turn the AM symbiosis alters the physiology of the whole plant (Taylor and Harrier 2003). Indeed, alterations in photosynthetic rates, leaf hydration, leaf osmotic potential, stomatal conductance, reproduction, and transpiration have been well-documented within the leaf tissues of AMF-colonized plants, as compared to non-colonized plants (Boldt et al. 2011; Kapoor et al. 2008; Porcel and Ruiz-Lozano 2004; Wu and Xia 2006).

Various transcriptional profiling studies have provided insight into the effect of AMF colonization on gene expression. These studies were performed on the model plants *Medicago truncatula* (Gomez et al. 2009; Hohnjec et al. 2005; Liu et al. 2003, 2007), *Lotus japonicus* (Guether et al. 2009), *Oryza sativa* (Campos-Soriano et al. 2011; Guimil et al. 2005), *Populus alba* (Cicatelli et al. 2012), and *Solanum lycopersicum* (Cervantes-Gámez et al. 2015; Fiorilli et al. 2009; Taylor and Harrier 2003). While most of these studies describe differential gene expression in roots, only about half of them have analyzed the changes in gene expression in shoots of *M. truncatula* AMF-colonized plants (Aloui et al. 2011; Liu et al. 2007), *Oryza sativa* (Campos-Soriano et al. 2011), *Populus alba* (Cicatelli et al. 2012), and tomato (Fiorilli et al. 2009; Cervantes-Gámez et al. 2015).

Conversely, proteomics-based approaches combining two dimensional electrophoresis, mass spectrometry and bioinformatics have been useful in monitoring protein regulation in root-microbe interactions (Bestel-Corre et al. 2004), including proteins associated with AMF-colonized roots (Benabdellah et al. 2000; Bestel-Corre et al. 2002; Colditz et al. 2005; Couto et al. 2013; Samra et al. 1997; Valot et al. 2005, 2006). One study of the arsenic hyperaccumulating fern *Pteris vittata* investigated the effect of AMF colonization in the frond proteome when colonized by *Glomus mosseae* and *Gigaspora margarita*, revealing that leaf proteins were up- and down-accumulated during either colonization event (Bona et al. 2010). The authors found that the intensities of 19 spots decreased in both *G. mosseae* and *G. margarita* plants, eight of which belonged to the photosynthesis and carbon fixation group. In another proteomic study, a clone of *Populus alba* L., previously selected for its tolerance to copper and zinc, was used to investigate the effects of symbiosis with the AM fungus *G. intraradices* on leaf protein expression (Lingua et al. 2012). These authors found that fungal colonization promoted the up and down-regulation of several proteins.

No reports have yet addressed the differential accumulation of leaf proteins induced by AMF in colonized tomato plants, although there is experimental evidence for differential gene expression between the shoots of AMF-colonized and non-colonized tomato, rice, *Populus alba*, and *M. truncatula* plants (Aloui et al. 2011; Campos-Soriano et al. 2011; Cervantes-Gámez et al. 2015; Cicatelli et al. 2012; Fiorilli et al. 2009; Liu et al. 2007), as well as differential protein accumulation in fern and *Populus alba* L. (Bona et al. 2010; Lingua et al. 2012). The aim of the present study was to improve our understanding of the differential protein accumulation in tomato, using comparative proteomics to investigate the biochemical and physiological changes in leaves resulting from AM colonization. By comparing the protein profiles from leaves of tomato plants grown under three conditions (AMF-colonized plants fertilized with low-P, non-colonized plants fertilized with low-P, and non-colonized plants fertilized with regular-P) we could identify proteins that were differentially accumulated between the different conditions. The analysis of this differential protein expression provides a description of which proteins may be involved in the leaf response to AMF colonization. This approach will contribute to elucidating how plant physiology is altered by this widely present symbiosis.

2 Materials and methods

2.1 Plant growth conditions and inoculation with *Rhizophagus intraradices*

Tomato seeds (*S. lycopersicum* Mill cv. Missouri) were surface-sterilized with a commercial bleach solution (10% v/v), rinsed with distilled water and germinated in vermiculite. Fifteen plantlets were transplanted when the first true leaf was expanded; plantlets were then grown individually in 250-mL pots containing a sterile mixture of vermiculite and fine sand (3:1 v/v) previously washed in running water and rinsed with distilled water.

R. irregularis (previously known as *Glomus intraradices* DAOM197198) was maintained on monoxenic culture associated with carrot roots in minimal medium (Chabot et al. 1992) followed by spore isolation (Doner and Bécard 1991). Roots were removed from the plate, and the solid medium was liquefied in an equal volume of 10 mM sodium citrate (using a blender on high setting for 5-10 seconds). Subsequently, the fungal material was sieved using 250- and 50-μm nylon meshes. The separated spores were then suspended in 10 mL of distilled water, counted under a stereoscope, and adjusted to 400 spores/mL in water.

Three different conditions, comprising five plants each, were investigated in this study: *M*, and the controls *NM-LP* and *NM-RP*. For *M*, plants were colonized with the AMF *R. irregularis* by applying approximately 400 spores to the

root zone of each plant. The plants were irrigated with water and fertilized twice weekly with a modified Hoagland's solution (Millner and Kitt 1992) containing low potassium phosphate (20 μM) in order to facilitate the establishment of AMF colonization. For *NM-LP*, plants were fertilized in low phosphate Hoagland's solution (20 μM P), whereas *NM-RP* plants were fertilized in regular phosphate Hoagland's solution (200 μM P) as a control for the phosphorus nutritional status. The latter control group was included to distinguish the responses of the plant due to improved phosphate nutrition, since some effects observed during the mycorrhiza association can be caused by improved phosphate uptake rather than the mycorrhiza itself. Plants were grown in a Binder KBW 400 growth chamber (Binder; Tuttlingen, Germany) for three months, using a 16 h light (25°C) and 8 h dark (20°C) photoperiod/temperature regime

2.2 Determination of mycorrhizal colonization

Leaves and roots were collected from *R. irregularis M* and *NM* plants. Leaves were immediately frozen in liquid nitrogen and stored at -70°C until further analysis, while roots were washed with distilled water to remove substrate particles and weighed. Roots were fixed in 50% ethanol (v/v) for at least one hour, washed twice in distilled water, and clarified with 20% KOH (w/v) for one day at room temperature. Next, roots were washed twice in distilled water and incubated for two hours in 1% (v/v) HCl, washed twice in distilled water and stained with a solution containing 0.05% trypan blue (w/v) (modified from Phillips and Hayman 1970) for two days. Roots were washed and maintained in lactoglycerol 1:1:1 (water:lactic acid:glycerol) at room temperature.

Colonization percentages were determined by the gridline method (Giovannetti and Mosse 1980). Fifty trypan blue-stained root pieces (2 cm in length) were placed on a glass slide and three lines were drawn along the slide. Any fungal element (vesicle, arbuscule, or hyphae) found at the intersection of a root with a line was counted as a hit. The percentage of colonization was calculated using the following formula: (number of hits/total number of root pieces) x 100.

2.3 Inorganic phosphate determination

The inorganic phosphorus concentration of the leaves from the *M*, *NM-LP*, and *NM-RP* tomato plants was determined by a modified molybdenum blue method (Chen et al. 1956). Absorbance was measured at 820 nm in a spectrophotometer using KH_2PO_4 as a phosphate standard. The ANOVA test was used to compare the means of treatments ($P < 0.05$). The Fisher test was used to determine significant differences between treatments.

2.4 Phenolic protein extraction

Three randomly selected biological replicates from each independent treatment (*M*, *NM-LP* and *NM-RP* leaf tissues) were submitted to protein extraction and separation by two-dimensional electrophoresis (2D-SDS-PAGE). Total leaf protein was extracted and precipitated according to a modified protocol (Hurkman and Tanaka 1986). Whole-leaf tissue from each plant stored at -70°C was ground briefly in liquid nitrogen. Approximately 0.5 g of leaf tissue was homogenized in 750 μL lysis buffer comprising 500 mM Tris-HCl pH 8.0, 50 mM EDTA, 700 mM sucrose, 100 mM KCl, 1% PVPP (w/v), 0.4% (v/v) β-mercaptoethanol and 1% protease inhibitor cocktail for plant cell and tissue extracts (Sigma-Aldrich; St Louis, MO, USA). A 0.1 M Tris-HCl pH 8.0-saturated phenol aliquot (750 μL) was added and the samples were homogenized extensively using a CP 50 ultrasonic processor (Cole-Parmer; Vernon Hills, IL, USA). Samples were kept on ice at all times. The phenolic phase was recovered by centrifugation (10,000 x g for 10 min at 4°C) and then mixed with an equal volume of lysis buffer. Proteins were precipitated from the phenolic phase by adding 5 volumes of 100 mM ammonium acetate in methanol and incubating at -20°C overnight. Total protein was pelleted by centrifugation (13,000 x g for 30 min at 4°C), solubilized in 200 μL of solution (7 M urea, 2 M thiourea), and quantified colorimetrically with Bradford's reagent (Sigma-Aldrich) using BSA (Sigma-Aldrich) as a standard in a DTX 880 multimode detector (Beckman Coulter; Fullerton, CA, USA). Protein contaminants were removed with the 2-D Clean-Up Kit (Amersham Biosciences; San Francisco, CA, USA). Samples were stored at -70°C until electrophoresis.

2.5 Two-dimensional electrophoresis

Isoelectric focusing (IEF) was performed with 500 μg of protein diluted in 300 μL destreak rehydration solution (Amersham Biosciences) containing 0.4% ampholyte (v/v) pH 3-10 (Bio-Rad, Inc.; Hercules, CA, USA). Protein was loaded onto 17-cm IEF strips (pH 4-7; Bio-Rad) using the in-gel rehydration method. IEF was performed using the Protean IEF Cell system (Bio-Rad) at 20°C for 15 min at 500 V, followed by a linear ramp until reaching 10 kV (which occurred in 3 h). The IEF was then maintained at 10 kV for 6 h. The focused strips were soaked for 15 min in equilibration solution I (20% glycerol v/v, 50 mM Tris-HCl pH 8.8, 6 M urea, SDS 2% w/v, a trace of bromophenol blue, 2% DTT w/v), followed by 15 min in equilibration solution II (20% glycerol v/v, 50 mM Tris-HCl pH 8.8, 6 M urea, 2% SDS w/v, a trace of bromophenol blue, 2.5% iodoacetamide w/v). For each biological replicate, at least two 2D-SDS-PAGE gels were run.

The resolved and equilibrated proteins were separated based on their molecular weight in 12% SDS-polyacrylamide gels (SDS-PAGE). Gel electrophoresis was run in a Protean II xi cell (Bio-Rad) at room temperature and 110 V for 10 h using 1X electrophoresis buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS w/v). The gels were stained with 0.1% Coomassie blue R-250 w/v and subsequently destained with a mixture of 10% methanol v/v and 10% acetic acid v/v, and were finally documented using the Chemi Doc system (Bio-Rad). Two-dimensional gel images were analyzed using the PD-Quest software (Bio-Rad) to detect differentially accumulated proteins according to the Student's t-test and the partial least squares test. The density of each differential protein (intensity x area) was also determined for the images.

2.6 Protein identification

Differential protein spots were manually excised from the gels and washed in distilled water, followed by 50% acetonitrile (v/v), acetonitrile/0.1 M ammonium bicarbonate (1:1 v/v), and 100% acetonitrile. Proteins were then reduced, carbamidomethylated, and digested with trypsin overnight at 37°C. The resulting peptides were then extracted from the gel as described by Shevchenko et al. (1996). The peptide mixture was analyzed using a Micromass Q-ToF spectrometer (Waters; Beverley, MA, USA) equipped with a nanospray ionization source, in which some of the peptides were sequenced from their collision-induced dissociation (CID) spectra using the MassLynx software (Waters) provided with the equipment. The sequences obtained were compared with those in databases using the MS Blast search tool (Shevchenko et al. 2001) and the MS/MS search tool provided by the Mascot program (Perkins et al. 1999). The peptide lists were searched against the NCBI non-redundant (nr) protein database (http://www.ncbi.nlm.nih.gov).

3 Results

3.1 Plant growth and fungal colonization

The fresh weight of leaves and roots was measured for *M*, *NM-LP*, and *NM-RP* plants. No significant differences were found among treatments (Table 1). The colonization percentage of *M* plants with *R. irregularis* was 84 ± 5%. No fungal structures were ever found in non-inoculated plants. Finally, inorganic phosphate (Pi) measurements from leaves of *M*, *NM-LP*, and *NM-RP* plants indicated that *NM-LP* plants displayed lower leaf Pi levels than *M* or *NM-RP* plants (Table 1).

3.2 Identification of differentially accumulated proteins

To compare the leaf protein profiles of *M* and *NM-LP* plants, we assembled a master gel using PD-Quest software (Fig. 1), in which over 236 proteins were reproducibly displayed within the range of pI 4-7, with molecular masses ranging from 10-160 kDa. The polypeptide pattern analysis revealed 25 proteins that were differentially accumulated according to the Student's t-test and/or the partial least squares test. Specifically, seventeen proteins were differentially accumulated according to the Student's t-test (Fig. 1, indicated in red) and eighteen proteins were differentially accumulated according to the partial least squares test (Fig. 1, indicated in yellow). Ten proteins were differentially expressed according to both tests. Two proteins were additionally selected as quantitatively differential by PD-Quest, but were not statistically significant (spots 1101 and 8101, indicated in Fig. 1 as circles). Twenty-three out of the 27 analyzed differentially accumulated proteins were successfully identified by mass spectrometry (Table 2). Two of these proteins, 2-methylene-furan-3-one reductase (spot 7402) and the auxin-binding protein ABP19a (spot 8102), were up-accumulated in *M* plants, whereas the other twenty-one proteins were down-accumulated. These proteins were grouped based on their function according to UniProt (http://www.uniprot.org). Twelve of the down-accumulated proteins are associated with photosynthesis and represent ATP synthase delta chain (chloroplastic; spot 1101), thylakoid lumenal 16.5 kDa protein (spot 2002), photosystem II stability/assembly factor HCF136 (spot 3301), chlorophyll a-b binding protein 6A (spot 4102), oxygen-evolving enhancer protein 1 (spot 4202), ribulose bisphosphate carboxylase small chain (spot 5004), type I (26 kDa) CP29 polypeptide (spot 5103), transketolase (spot 7901), chlorophyll a-b binding protein 8 (spot 8101), and three ferredoxin-NADP reductase proteins (spots 6301, 7303 and 8302). Four other proteins were related to redox function: thioredoxin peroxidase 1 (spot 5101), superoxide dismutase (Cu-Zn) 2 (spot 7002), alcohol dehydrogenase homolog (spot 8203), and 2-methylene-furan-3-one reductase (spot 8401). The five last proteins were identified as plastid lipid-associated protein CHRC (spot 1203), 14-3-3 protein (spot 1204), harpin binding protein 1 (spot 2103), annexin p34 (spot 6302), and ATP-dependent Clp protease (spot 6903). Since AM symbiosis improves phosphate levels in leaf tissue with respect to *NM-LP* plants (as reported in Table 1), it cannot be ruled out that some differential proteins detected by this comparison could be regulated by phosphate nutrition, rather than by mycorrhiza colonization.

The leaf protein profiles of *M* and *NM-RP* plants were analyzed in a second comparative proteomic analysis, as described in the previous section. Twenty proteins were differentially accumulated according to the Student's t-test and/or the partial least squares test. All proteins were down-accumulated in *M* plants. Only nine proteins could be identified by mass spectrometry, and are listed in Table 3.

Table 1 Fresh weight and inorganic phosphate (Pi) content in *M*, *NM-LP*, and *NM-RP* tomato plants

Treatment	Leaf fresh weight (g)	Root fresh weight (g)	Pi in leaves (μg Pi/ g fresh weight)
M	19.22g ± 1.87[a]	5.02g ± 0.52[a]	644.24 ± 197.61[a]
NM-LP	19.38g ± 2.19[a]	5.08g ± 0.98[a]	357.67 ± 153.49[b]
NM-RP	18.82 ± 2.46[a]	5.28g ± 0.49[a]	899.52 ± 267.45[a]

Data are expressed as mean ± standard deviation

Different letters in the same column mean significant differences based on ANOVA and Fisher test at $p < 0.05$

In order to identify proteins regulated by phosphate, but not by AMF colonization, a third protein profile comparison was performed between *NM-LP* and *NM-RP* leaf tissues. Twelve proteins were differentially accumulated according to the Student's t-test and/or the partial least squares test. Only five of these proteins could be identified by mass spectrometry. Four proteins were up-accumulated in the *NM-RP* condition as compared to *NM-LP* leaf extracts, including a 14-3-3 protein (spot 1202), a plastid lipid-associated protein CHRC (spot 1303), and two isoforms of photosystem I reaction center subunit II: spot 6703 (pI 5.8/79.6 kDa), and spot 9101 (pI 6.9/20.9 kDa). The only down-accumulated protein was a chloroplast precursor of the elongation factor TuB (spot 5607; Table 4).

Proteins exhibiting differential accumulation in more than one comparison are reported in Table 5. Superoxide dismutase, harpin binding protein 1, and thioredoxin peroxidase 1 were down-accumulated in leaves of *M* vs. *NM* tomato plants (under either *LP* or *RP* conditions), whereas no differential accumulation was detected when comparing *NM* plants maintained at *RP* and *LP*. This indicates that the expression of these proteins changes in response to AMF colonization, independently of the phosphate regime under which they were grown. Furthermore, 14-3-3 protein was up-accumulated in *NM-RP* vs. *NM-LP* plants, whereas it was down-accumulated in *M* vs. *NM-RP* and *M* vs. *NM-LP*, regardless of their phosphate nutrition (Table 5).

Two different isoforms of the proteins annotated as plastid lipid-associated protein CHRC and photosystem II stability/assembly factor HCF136 displayed differential expression during the comparisons (Tables 2, 3 and 4). Whereas plastid lipid-associated protein CHRC (spot 1203, pI 4.7/33.4 kDa) was down-accumulated in leaves of *M* plants as compared to *NM-LP*, a second isoform (spot 1303, pI 4.7/32.8 kDa) was down-accumulated in *M* plants as compared to *NM-RP* plants, although it was up-accumulated in *NM-RP* plants as compared to *NM-LP* plants (Table 5). One isoform of photosystem II stability/assembly factor HCF136 (spot 3301, pI 5.1/35.9 kDa) was down-accumulated in leaves of *M* plants as compared to *NM-LP* plants, whereas another isoform (spot 3301b, pI 5.2/36.2 kDa) was down-accumulated in *M* plants vs. *NM-RP* plants. This isoform did not display any altered accumulation in leaves when comparing *NM-RP* and *NM-LP* plants.

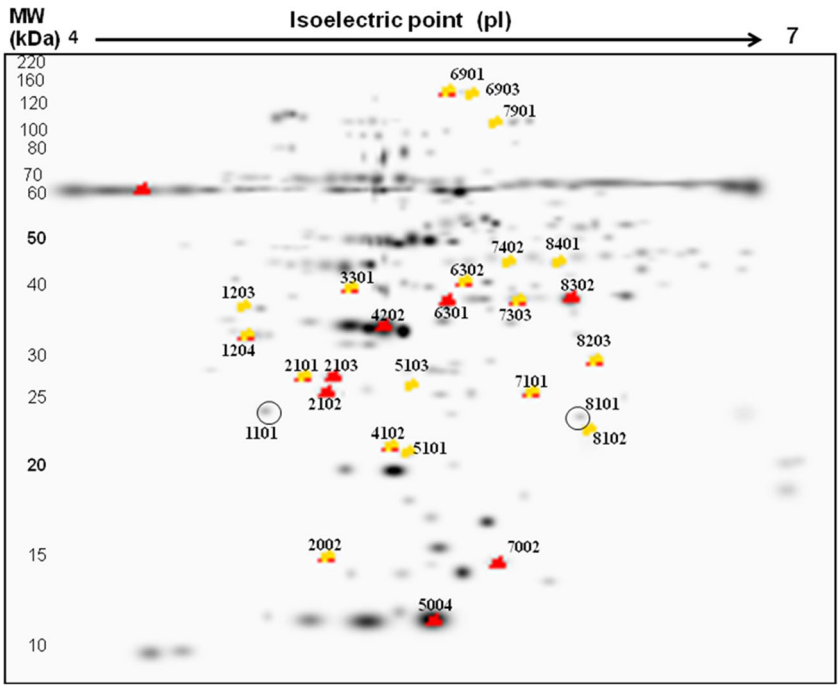

Fig. 1 Master gel assembled from 2D-SDS-PAGE gels of leaf proteins extracted from *M* (colonized with *R. irregularis*) and *NM-LP* (fertilized with Hoagland's solution containing 20 μM potassium phosphate) tomato plants (*S. lycopersicum*). *Red*: proteins that were differentially accumulated based on the Student's t-test. *Yellow*: proteins that were differentially accumulated based on the partial least squares test. *Circles* indicate proteins with differences at the quantitative level that are not statistically significant. MW: molecular weight in kDa. The linear range of isoelectric points is presented with the corresponding proteins

Table 2 Mass spectrometric identification of leaf proteins differentially accumulated in *M* vs. *NM-LP* tomato plants

Spot number	GenBank accession number	Protein	pI/MW (kDa)	Score/identities	Peptide sequences matched	Density ratio: *M*/*NM-LP*
UP-REGULATED						
7402	NP_001296292.1	2-methylene-furan-3-one reductase (*S. lycopersicum*)	5.8/40.4	103 (14/14 100%) 89 (12/12 100%) 63 (9/9 100%)	QFGSLAEYTAVEEK VVDAFSYLETGR IVAAALNPVFDK	1.570
8102	XP_004235542.1	Auxin-binding protein ABP19a (*S. lycopersicum*)	6.1/22.9	83 (11/11 100%) 100 (14/14 100%) 44 (8/11 73%)	AVQDFCVADLK LVAATTFLDEATIK APESPSPLPCK	2.043
DOWN-REGULATED PHOTOSYNTHESIS						
1101	XP_004252554.1	ATP synthase delta chain, chloroplastic (*S. lycopersicum*)	4.8/23.7	101 (15/15 100%) 102 (14/14 100%)	WSAAGSYANALADVAK SNETLEQTTADLEK	0.138
2002	XP_004251724.1	Thylakoid lumenal 16.5 kDa protein, chloroplastic (*S. lycopersicum*)	5.0/14.9	83 (12/12 100%)	TAFVASASAFEK	0.188
3301	XP_004231481.1	Photosystem II stability/assembly factor HCF136, chloroplastic isoform X1 (*S. lycopersicum*)	5.1/35.9	93 (13/13 100%) 75 (10/10 100%)	AADNIAANLYSVK GFGILDVGYR	0.065
4102	XP_004239673.1	Chlorophyll a-b binding protein 6A, chloroplastic (*S. lycopersicum)*	5.3/22.0	71 (11/13 85%)	YPGQFDPLGYSK	0.277
4202	NP_001296294.1	Oxygen-evolving enhancer protein 1, chloroplastic (*S. lycopersicum*)	5.3/29.8	117 (17/17 100%) 124 (17/17 100%) 85 (11/11 100%)	GGSTGYDNAVALPAGGR DGIDYAAVTVQLPGGER FCLEPTSFTVK	0.441
5004	P07180	Ribulose bisphosphate carboxylase small chain 3A/3C, chloroplastic (*S. lycopersicum*)	5.5/11.6	109 (13/13 100%)	GLWVPCLEFETEHGFYVR	0.560
5103	CAA43590.1	Type I (26 kD) CP29 polypeptide (*S. lycopersicum*)	5.4/25.4	97 (13/13 100%) 76 (10/10 100%)	TQLLLDGNTLNYFGK IFLPEGLLDR	0.214
6301	XP_004232495.1	Ferredoxin-NADP reductase, leaf-type isozyme, chloroplastic (*S. lycopersicum*)	5.5/34.5	112 (16/16 100%)	LYSIASSALGDFGDSK	0.329
7303	XP_004232495.1	Ferredoxin-NADP reductase, leaf-type isozyme, chloroplastic (*S. lycopersicum*)	5.8/34.5	112 (16/16 100%) 113 (14/14 100%)	LYSIASSALGDFGDSK MAQYAEELWTLLQK	0.568
7901	XP_004248560.1	Transketolase, chloroplastic (*S. lycopersicum*)	5.7/97.5	89 (13/15 87%) 110 (15/15 100%) 98 (13/13 100%) 86 (12/12 100%) 86 (12/12 100%)	AIGVDGFGASAPGEK ALPTYTPESPADATR SIITGELPAGWEK NLSQQNLNALAK VTTTIGFGSPNK	0.463
8101	XP_004248217.1	Chlorophyll a-b binding protein 8, chloroplastic-like (*S. lycopersicum*)	6.1/23.8	121 (17/17 100%) 81 (10/11 91%)	FAMLGAAGAIAPEILGK WLAYGEVIDGR	0.311
8302	O04977	Ferredoxin--NADP reductase, leaf-type isozyme, chloroplastic (*N. tabacum*)	6.1/35.2	80 (10/10 100%) 49 (5/7 71%) 40 (5/6 83%)	DGIVWADYKK NDTFIYMESNK QTTFIYFCLGK	0.422
OXIDO-REDUCTASE						
5101	NP_001234171.1	Thioredoxin peroxidase 1 (*S. lycopersicum*)	5.4/21.2	82 (11/11 100%)	YALLVDDLEVK	0.313
7002	Q43779.3	Superoxide dismutase (Cu-Zn) 2 (*S. lycopersicum*)	5.8/14.8	96 (13/13 100%)	QIPLTGPQSIIGR	0.411
8203	AAB00109.1	Alcohol dehydrogenase homolog, partial (*S. lycopersicum*)	6.2/27.7	114 (17/17 100%) 95 (13/13 100%)	VAIITGAASGIGEASAR VVVADIQDELGQK	0.317
8401	NP_001296292.1	2-methylene-furan-3-one reductase (*S. lycopersicum*)	6.0/40.4	102 (13/14 93%)	AWSYTDYGSVNVLK	0.624
OTHER MOLECULAR FUNCTIONS						
1203	NP_001234183.1	Plastid lipid-associated protein CHRC (*S. lycopersicum*)	4.7/33.4	109 (16/16 100%) 77 (11/11 100%) 83 (11/11 100%)	KGLITSVQDTASSVAK GDAGSVFVLIK QLADSFYGTNR	0.392
1204	NP_001234097.1	14-3-3 protein (*S. lycopersicum*)	4.7/29.6	113 (16/16 100%)	VVAAADGAEELTVEER	0.280
2103	NP_001234460.1	Harpin binding protein 1 (*S. lycopersicum*)	5.1/26.2	121 (17/17 100%) 90 (12/12 100%) 77 (11/11 100%)	ELESCAGAVDLAADLDK LLPITLGQVFQR EAEAELIGSLK	0.465
6302	NP_001234104.1	Annexin p34 (*S. lycopersicum*)	5.6/37.6	73 (10/10 100%)	LLVPLVSSYR	0.432
6903	XP_004252280.1		5.7/120.7	95 (13/13 100%)	VLENLGADPSNIR	0.361

Table 2 (continued)

Spot number	GenBank accession number	Protein	pI/MW (kDa)	Score/identities	Peptide sequences matched	Density ratio: *M*/*NM-LP*
		ATP-dependent Clp protease ATP-binding subunit ClpA homolog CD4B, chloroplastic (*S. lycopersicum*)		41 (7/10 70%)	NAVAVEITLMPR	

Spot number corresponds to numbers given in Fig. 1

Leucine = Isoleucine

M designates AMF-colonized plants

NM-LP designates plants not colonized by AMF, fertilized with low phosphate Hoagland's solution (20 μM P)

4 Discussion

We focused our comparative proteomic analysis primarily on the symbiotic interaction between *S. lycopersicum* and *R. irregularis*, in order to identify systemic changes in the proteome of the aerial part of the plant in response to AMF colonization. Several proteins in leaves were detected as differentially accumulated in *M* vs. *NM* plants. *M* plants were maintained in a low-P (20 μM) fertilizer regime in order to favor colonization, since it is known that high phosphate inhibits the establishment of symbiosis (Smith and Read 1997). Initially, the leaf proteome of *M* plants was compared to the corresponding *NM* plants, which were also maintained in low-P (20 μM). This comparative analysis resulted in 23 differentially accumulated proteins (Table 2). Two of these proteins displayed a higher accumulation under *M* conditions, whereas the other 21 proteins were down-accumulated.

The protein 2-methylene-furan-3-one reductase, also known as enone oxidoreductase or *SlEO* (spot 7402, GenBank NP_001296292.1), was one of two up-accumulated proteins in *M* vs. *NM-LP* plants (Table 2). This protein is related to the fruit ripening process, and is a

Table 3 Mass spectrometric identification of leaf proteins differentially accumulated in *M* vs. *NM-RP* tomato plants

Spot number	GenBank accession number	Protein	pI/MW (kDa)	Score/identities	Peptide sequences matched	Density ratio: *M*/*NM-RP*
DOWN- REGULATED						
1202	NP_001234097.1	14-3-3 protein (*S. lycopersicum*)	4.7/29.6	113 (16/16 100%)	VVAAADGAEELTVEER	0.206
1303	NP_001234183.1	Plastid lipid-associated protein CHRC (*S. lycopersicum*)	4.7/32.8	110 (15/16 94%) 77 (11/12 92%) 70 (9/9 100%) 102 (15/15 100%)	NPNPAPTEALTLLDGK PLATTSISTDAK TTYLDDELR GLITSVQDTASSVAK	0.596
1502	XP_004238411.1	Phosphoprotein ECPP44 (*S. lycopersicum*)	4.7/41.9	81 (11/12 92%) 66 (9/9 100%) 59 (8/8 100%)	ETVGTDVEATDR EDTSVPVEK VSEEVEPK	0.165
2201	NP_001234460.1	Harpin binding protein 1 (*S. lycopersicum*)	5.0/26.2	121 (17/17 100%) 90 (12/12 100%) 77 (11/11 100%)	ELESCAGAVDLAADLDK LLPITLGQVFQR EAEAELIGSLK	0.465
3103	NP_001234171.1	Thioredoxin peroxidase 1 (*S. lycopersicum*)	5.4/21.2	82 (11/11 100%)	YALLVDDLEVK	0.316
3204	XP_004230885.1	Triosephosphate isomerase, chloroplastic (*S. lycopersicum*)	5.3/26.4	109 (15/15 100%) 65 (8/8 100%)	VASPEQAQEVHVAVR FFVGGNWK	0.213
3301b	XP_004231481.1	Photosystem II stability/assembly factor HCF136, chloroplastic isoform X1 (*S. lycopersicum*)	5.2/36.2	93 (13/13 100%)	AADNIAANLYSVK	0.580
6001	Q43779.3	Superoxide dismutase (Cu-Zn) 2 (*S. lycopersicum*)	5.8/14.8	96 (13/13 100%)	QIPLTGPQSIIGR	0.366
6604	XP_010324204.1	Phosphoglycerate kinase, cytosolic (PGK) (*S. lycopersicum*)	6.0/47.3	131 (18/18 100%) 68 (9/9 100%)	LASLADLYVNDAFGTAHR YSLKPLVPR	0.497

Leucine = Isoleucine

M designates AMF- colonized plants

NM-RP designates plants not colonized by AMF, fertilized with regular phosphate Hoagland's solution (200 μM P)

Table 4 Mass spectrometric identification of leaf proteins differentially accumulated in *NM-RP* vs. *NM-LP* tomato plants

Spot number	GenBank Accession Number	Protein	pI/MW (kDa)	Score/identities	Number or sequence of peptides matched	Density ratio: *NM-RP*/ *NM-LP*
UP-REGULATED						
1202	NP_001234097.1	14-3-3 protein (*S. lycopersicum*)	4.7/29.6	113 (16/16 100%)	VVAAADGAEELTVEER	1.351
1303	NP_001234183.1	Plastid lipid-associated protein CHRC (*S. lycopersicum*)	4.7/32.8	110 (15/16 94%) 77 (11/12 92%) 70 (9/9 100%) 102 (15/15 100%)	NPNPAPTEALTLLDGK PLATTSISTDAK TTYLDDELR GLITSVQDTASSVAK	1.307
6703	NP_001295880.1	Photosystem I reaction center subunit II, chloroplastic (*S. lycopersicum*)	5.8/79.6	53 (7/8 88%)	QGVGQNFR	2.033
9101	NP_001295880.1	Photosystem I reaction center subunit II, chloroplastic (*S. lycopersicum*)	6.9/20.9	118 (15/15 100%) 61 (7/7 100%)	EQIFEMPTGGAAIMR INYQFYR	1.955
DOWN-REGULATED						
5607	Q43364	Elongation factor TuB, chloroplast precursor (EF-TuB) (*Nicotiana sylvestris*)	5.7/48.1		9[(a)]	0.726

Leucine = Isoleucine

NM-RP designates plants not colonized by AMF, fertilized with regular phosphate Hoagland's solution (200 μM P)

NM-LP designates plants not colonized by AMF, fertilized with low phosphate Hoagland's solution (20 μM P)

[a] Obtained by PMF: Peptide mass fingerprinting (MALDI-TOF)

negatively auxin-regulated enzyme that catalyzes the last step in the formation of the important strawberry flavor compound 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF, or furaneol). Indeed, biochemical studies have confirmed the involvement of *SlEO* in the formation of the volatile furaneol in tomato fruit (Buttery et al. 1995; Klein et al. 2007; Raab et al. 2006). Volatile organic compounds induce defense priming and enhance the resistance against pathogens (Quintana-Rodríguez et al. 2015). Additionally, several reports indicate that mycorrhiza colonization triggers a defense priming mechanism in colonized plants (Mora-Romero et al. 2015a, 2015b; Whipps 2004). While these observations could promote speculation that the production of furaneol by 2-methylene-furan-3-one reductase might have a role in defense signaling due to furaneol's volatile nature, additional studies are required to confirm this hypothesis.

The other accumulated protein in leaves of *M* tomato plants was auxin-binding protein ABP19a (spot 8102, GenBank XP_004235542.1), which exhibited a two-fold change (Table 2). Auxin-binding proteins (ABPs) are a class of low-abundance proteins in plants that bind active auxins with high specificity and affinity. It is possible that ABP could initiate the auxin signal pathways leading to various cellular responses through ABP-auxin binding, which would thus accord it with a plant hormone receptor function (Kim et al. 2001; Ohmiya et al. 1998). Although the role of auxins in

Table 5 Densitometric quantification of selected leaf proteins differentially accumulated in *M*, *NM-LP* and *NM-RP* tomato plants

No.	Protein	Density ratio (*M*/*NM-LP*)	Density ratio (*M*/*NM-RP*)	Density ratio (*NM-RP*/ *NM-LP*)
1	Superoxide dismutase (Cu-Zn)	0.411	0.366	Ndp
2	Harpin binding protein 1	0.465	0.565	Ndp
3	Thioredoxin peroxidase 1	0.313	0.316	Ndp
4	14-3-3 protein	0.28	0.206	1.351
5	a) Plastid lipid-associated protein CHRC (Spot 1203, pI 4.7/33.4 kDa)	0.392	Ndp	Ndp
	b) Plastid lipid-associated protein CHRC (Spot 1303, pI 4.7/32.8 kDa)	Ndp	0.596	1.307
6	a) Photosystem II stability/assembly factor (Spot 3301, pI 5.1/35.9 kDa)	0.065	Ndp	Ndp
	b) Photosystem II stability/assembly factor (Spot 3301b, pI 5.2/36.2 kDa)	Ndp	0.58	Ndp

Ndp no densitometric differential protein

AMF colonization is not completely understood, it has been reported in several systems that auxins increase in the roots after inoculation by AMF (Fitze et al. 2005; Hause et al. 2007; Kaldorf and Ludwig-Muller 2000; Ludwig-Muller and Guther 2007). In AMF-colonized maize, indol-3-butyric acid content has been shown to increase in leaves, probably through *de novo* synthesis or transport from the root (Fitze et al. 2005). It is also possible that a polypeptide similar to an ABP may play a role in an auxin-signaling pathway in the upper part of the plant, in response to AMF colonization. However, the exact role of this protein remains unknown.

4.1 Photosynthesis-related proteins

Twenty-one proteins were down-accumulated in leaves of *M* plants in comparison to leaves of *NM-LP* plants. Twelve of these proteins are related to photosynthesis function (Photosystems I and II and the Calvin cycle) or structural components (Table 2).

A photosystem II stability/assembly factor HCF136 protein (spot 3301, pI 5.1/35.9 kDa) decreased its accumulation significantly in *M* vs. *NM-LP* plants (density ratio: 0.065; Tables 2 and 5). Similarly, an isoform of this protein (spot 3301b, pI 5.2/36.2) was down-accumulated in the *M* condition as compared to *NM-RP* (density ratio: 0.58; Tables 3 and 5), which might indicate that this isoform decreases its expression through AMF colonization, and not based on the phosphate nutritional status.

Our results do not support the findings from previous reports that showed an increase in net photosynthesis in AMF-colonized plants in different associations such as *Glycine max* and *Cucumis sativus* colonized by *F. mosseae* (Brown and Bethlenfalvay 1988; Black et al. 2000), *Plantago lanceolata* and tomato colonized by *G. fasciculatum* (Parádi et al. 2003; Sánchez-Rocha et al. 2005), *Citrus aurantium* associated with *G. intraradices* (Johnson 1984), and *C. tangerine* colonized by *Endogone versiformis* (Wu and Xia 2006). However, our results are in agreement with other proteomic (Bona et al. 2010; Lingua et al. 2012) and transcriptomic reports (Wipf et al. 2014) that indicate that mycorrhiza colonization induces the down-regulation of photosynthesis-related proteins in shoots. Some of these proteins were also down-regulated in shoots of tomato mycorrhiza plants reported in this work, such as four oxygen-evolving enhancer proteins (OEE1), a probable oxygen-evolving enhancer protein 2 (OEE2), an ATP synthase CF1 α-subunit, three ATP synthase beta-subunits, an enolase, a thylakoid lumenal protein, and three ferredoxin NADP reductases (Table 2).

Gene expression in the photosynthesis-related proteins OEE and the small subunit of Rubisco was down-regulated in a previous transcriptome study of tomato plant shoots colonized by the AMF *F. mosseae* (Fiorilli et al. 2009). These two proteins were also down-regulated in the present study. Liu et al. (2007) reported that the small subunit of Rubisco was the most highly down-regulated gene in the shoots of *G. intraradices*-colonized *M. truncatula* plants, which is in agreement with the down-accumulation of this protein in shoots of mycorrhiza-colonized tomato plants reported here. Furthermore, down-regulation of photosynthesis-related genes has been reported in *Arabidopsis* leaves that display symbiotic associations with plant growth promoting rhizobacteria (PGPR), and inoculated plants were more resistant to subsequent infections by the virulent pathogen *Pseudomonas syringae* pv. tomato than control plants (Cartieaux et al. 2003; Wang et al. 2005).

Similarly, there is a reduced susceptibility to the leaf pathogen *X. campestris* in leaves of mycorrhiza tomato plants (Mora-Romero et al. 2015b); we observed that several photosynthesis-related genes are significantly down-regulated as well (Table 2). Whether the decrease in the accumulation of some photosynthesis proteins is associated with the protection response induced by mycorrhiza is unknown. Interestingly, no effect from the reduced accumulation of photosynthesis proteins was observed on biomass production (Table 1). This observation suggests the possibility for the existence of an alternative regulation of photosynthesis in mycorrhizal plants. Clearly, further studies are necessary to understand how the decreased accumulation of several proteins involved in photosynthesis may be associated with leaf responses in *M* plants. Although in this work only two proteins were over-accumulated in mycorrhizal plants in comparison to the controls, we cannot rule out the existence of other differentially accumulated proteins that were not extracted or separated using the present method. A more exhaustive proteomic approach would certainly provide more answers.

4.1.1 Redox-related proteins

Several enzymes that modulate redox systems in the cell displayed specific responses to AMF colonization in this work (Table 2). The control of cellular oxidative levels is achieved by antioxidative systems (Asada 1999). These defense systems are composed of different metabolites (*i.e.* ascorbate, glutathione, or tocopherol), as well as scavengers of activated oxygen such as superoxide dismutases, peroxidases, and catalases (Noctor and Foyer 1998; Schutzendubel and Polle 2002). Plants have developed defense systems against reactive oxygen species (ROS), both to limit their formation and to promote their removal. Under unstressed conditions, the formation and removal of O_2 are in balance; however, ROS formation can be very abundant during a defense response.

Superoxide dismutase (SOD) constitutes the first line of defense against ROS in a cell, and is considered to be a hallmark of plant defense responses against pathogens (Alscher et al. 2002). Different environmental stresses can lead to the

enhanced production of O_2^- radicals within plant tissues, which plants must subsequently detoxify (Liochev and Fridovich 1994; Fridovich 1995). The main function of SOD is to scavenge O_2^- radicals generated in various physiological processes to yield molecular oxygen and H_2O_2, thus preventing the oxidation of biological molecules. Plants possess three types of SODs with different prosthetic metal groups: CuZnSOD, MnSOD, and FeSOD (Alscher et al. 2002). In the present work, superoxide dismutase Cu-Zn (Cu-Zn SOD) and thioredoxin peroxidase 1 significantly decreased their accumulation in the *M* condition, in comparison to *NM-LP* (spots 5101 and 7002; density ratios: 0.313 and 0.411, respectively) and *NM-RP* (spots 3103 and 6001; density ratios: 0.316 and 0.366, respectively) (Table 5).

Interestingly, contradictory results have been reported regarding the regulation of redox-modulating proteins by mycorrhiza in leaves. Whereas some studies have found that SOD activity increased in leaves of *Poncirus trifoliate* (Zou et al. 2015) and *Cajanus cajan* (Garg and Chandel 2015) plants colonized with *Funneliformis mossea*, others have shown that SOD and peroxidase activities significantly decreased in leaves of soybean plants colonized with *G. intraradices* (Porcel and Ruiz-Lozano 2004), and in leaves of *Phillyrea angustifolia* plants inoculated with a mixture of three AM fungi (Caravaca et al. 2005). Consistent with the latter reports, one proteomic study has documented a decrease in protein concentration of two thioredoxin peroxidases in shoots of *Pteris vittata* plants colonized with the AMF *G. mosseae* or *G. margarita* (Bona et al. 2010).

In the present work, SOD and thioperoxidase were down-accumulated in leaves of mycorrhizal plants (Table 2), in line with other reports that have demonstrated a decrease in SOD activities in parallel with an increase in ROS concentration (Porcel and Ruiz-Lozano 2004; Caravaca et al. 2005). Recently, it was shown that leaves of AMF-colonized tomato plants improved their defense response to foliar pathogen attack in comparison to non-mycorrhizal plants (Mora-Romero et al. 2015a). Together, these reports suggest that ROS concentration increases in mycorrhiza tomato leaves could make plants more resistant to pathogen attack. Nevertheless, not every genotype of a given plant species is able to trigger mycorrhiza-induced defense (Mora-Romero et al. 2015b). Instead, it is probably those genotypes displaying an increase in ROS concentration in leaves as a consequence of the decrease in antioxidative enzymes (*i.e.* SOD and peroxidases) that can trigger mycorrhiza-induced defenses. This may explain the down-regulation of SOD and thioredoxin peroxidase in leaves of mycorrhizal tomato plants, as well as the contradicting reports on the regulation of redox-modulating enzymes. Further experiments are required to fully test this hypothesis.

4.1.2 Proteins related to other molecular functions

Two isoforms of plastid lipid-associated proteins were differentially accumulated in this work. The accumulation of one isoform (spot 1203, pI 4.7/33.4 kDa) decreased in the leaves of *M* plants as compared to *NM-LP* plants (density ratio: 0.392) (Table 2), whereas the other isoform (spot 1303, pI 4.7/32.8 kDa) was down-accumulated in leaves of *M* plants as compared to *NM-RP* plants (density ratio: 0.596) (Table 3). The plastid lipid-associated protein CHRC is a chromoplast-specific carotenoid-associated protein that has roles in sequestration and accumulation of carotenoid in plastid membranes during flower and fruit development (Chiou et al. 2008). Numerous CHRC/fibrillin homologs have been identified in plastids besides chromoplasts, and are collectively referred to as plastid lipid-associated proteins (PAPs). Previous research has suggested that these PAPs are not only involved in the storage of carotenoids but also in the general sequestration of hydrophobic compounds such as lipids, in a process that may be essential for plant survival under stress (Langenkamper et al. 2001; Leitner-Dagan et al. 2006). CHRC is activated in vegetative tissues through various biotic and abiotic stresses, as observed with other PAPs (Langenkamper et al. 2001; Leitner-Dagan et al. 2006). Interestingly, the accumulation of the second identified isoform (spot 1303) was higher in *NM-RP* plants than in *NM-LP* plants (density ratio: 1.307) (Table 4). This particular plastid lipid-associated protein isoform (pI 4.7/32.8 kDa) appears to be up-accumulated in leaves by high-P rather than through mycorrhizal colonization (Table 5), similar to what we observed for other proteins that are up-accumulated in leaves under stress conditions and down-accumulated in leaves under AMF colonization. The differential accumulation of these proteins suggests possible roles for isoform 1303 in response to P and isoform 1203 in AMF colonization. However, more studies are needed to understand the role these proteins play in the shoots of mycorrhiza plants.

We identified a harpin binding protein that was down-accumulated in leaves of *M* plants in comparison to *NM-LP* (spot 2103; density ratio: 0.465) and *NM-RP* (spot 2201; density ratio: 0.565) plants (Tables 2 and 3). Harpins are effector proteins believed to be secreted by the type III secretion system of bacterial phytopathogens. Harpins were originally defined as elicitors of the hypersensitive response (HR), but recent evidence indicates that they can act as signaling molecules with multiple functions, inducing systematic acquired resistance (SAR) and associated defense responses in many plants that may or may not be accompanied by HR (Peng et al. 2003; Wang et al. 2007, 2008; Yang et al. 2005). For example, one harpin-induced protein has been reported to be down-accumulated at the transcriptional level in *M* tomato shoots (Fiorilli et al. 2009). Here, we document a harpin binding protein specifically down-accumulated in the AMF condition

and independent of the P status of the plant (Tables 2, 3 and 5). Although the role of these proteins is currently unknown, their expression patterns reveal a role in the response of the plant leaf to AMF colonization.

14-3-3 proteins regulate a wide range of target proteins in eukaryotes by acting as phosphoserine/phosphothreonine-binding proteins. Specifically, these proteins function in the regulation of signal transduction pathways, and generally act as adapters, chaperones, activators, or repressors. They also regulate the activities of a wide array of targets *via* direct protein-protein interactions. The 14-3-3 protein family plays a central role in stress resistance, disease, and growth control during the cell cycle (Bunney et al. 2002; Fulgosi et al. 2002; Porcel et al. 2006; Roberts and Bowles 1999; Roberts et al. 2002). Evidence suggests that 14-3-3 proteins help to regulate levels of jasmonic acid and ethylene, both of which are important signaling molecules in the activation of defense genes (Robb et al. 2007). There is further evidence that jasmonic acid and/or some of its derivatives may play a role in the signaling of defense priming in the leaves of mycorrhiza-colonized tomato (Mora-Romero et al. 2015a). Here, we demonstrated that a 14-3-3 protein was significantly down-accumulated in leaves of *M* plants in comparison to *NM-LP* (spot 1204, density ratio: 0.28) and *NM-RP* plants (spot 1202, density ratio: 0.206) (Tables 2 and 3). This pattern of differential accumulation could correspond to a protein potentially responding to AMF colonization. However, 14-3-3 protein accumulation (spot 1202) was 1.35 times higher in leaves of *NM-RP* vs. *NM-LP* plants, indicating that its accumulation is also regulated by the P status even in the absence of colonization (Table 4). The differential concentration of this protein in different conditions suggests a possible role for this protein in the cross-talk mechanism between P nutrition and AMF colonization. Nevertheless, the exact role of 14-3-3 protein in AMF colonization and P nutrition in leaves of tomato plants is still unknown.

We observed that the protein annexin (spot 6302) was differentially expressed in leaves of *M* tomato plants in comparison to *NM-LP* plant leaves (density ratio: 0.432; Table 2). Annexins are capable of both Ca^{2+}-dependent and Ca^{2+}-independent binding of phospholipids from the endomembrane and the plasma membrane (Talukdar et al. 2009). These proteins are ubiquitous throughout the plant and animal kingdoms, and have been associated with multiple processes including responses to stress stimuli such as drought, cold, high-salinity and pathogens (Jami et al. 2008; Talukdar et al. 2009). Several previous studies have shown that the annexin genes MtAnn1 and MtAnn2 are transcriptionally activated in the roots of *M. truncatula* in response to arbuscular mycorrhizal and rhizobial symbioses (Amiour et al. 2006; de Carvalho-Niebel et al. 1998, 2002; Manthey et al. 2004; Oldroyd and Downie 2004; Talukdar et al. 2009). In addition, an annexin protein has been reported to be down-accumulated at the transcriptional level in shoots of AMF-colonized tomato plants (Fiorilli et al. 2009). This is in agreement with our findings here, in which the accumulation of an annexin protein decreased in leaves of *M* plants in comparison to *NM-LP* plants. These reports clearly demonstrate that the up-regulation of annexin genes is involved in establishing mycorrhiza symbiosis in roots, although evidence of annexin regulation in shoots is still emerging.

In the present work we determined the profile of proteins extracted from leaves of *M*, *NM-LP*, and *NM-RP* tomato plants. Proteins were identified as differentially accumulated in the *M* vs. *NM-LP*, *M* vs. *NM-RP*, and *NM-RP* vs. *NM-LP* comparisons. The level of each differentially accumulated protein was quantified and expressed as a density value. Analysis of the differential accumulation of proteins in these three comparisons was used to infer their possible involvement in leaf responses to AMF colonization. This permitted us to determine that superoxide dismutase (Cu-Zn), harpin binding protein 1, and thioredoxin peroxidase 1 display the same expression pattern, since all of these genes were down-accumulated in *M* vs. *NM* tomato leaves (independently of the plant fertilizer condition). No differential accumulation was detected for these proteins in *NM-RP* vs. *NM-LP* (Table 5). Together, our results suggest that these proteins are regulated only by AMF colonization, and not by phosphate nutrition. Conversely, a 14-3-3 protein was down-accumulated in *M* leaf extracts in comparison to either *NM-LP* or *NM-RP* plants, although it was up-accumulated in *NM-RP* vs. *NM-LP* plants (Table 5). Thus, our results for the 14-3-3 protein suggest that it is regulated by both AMF colonization and phosphate nutrition.

5 Concluding remarks

Our results demonstrate that comparative proteome analysis is a powerful approach for identifying proteins with a potential role in the leaf response to AMF colonization. Most proteins identified as differentially accumulated in this work were primarily related to photosynthesis and, importantly, were down-accumulated under AMF-colonization. Interestingly, the expression of genes such as those encoding SOD, thioredoxin peroxidase, harpin binding protein and annexin are reported to be induced at the transcript level in leaves by biotic and abiotic stresses, while in this work they were detected as down-accumulated at the protein level in leaves of *M* vs. *NM* plants. We showed in a previous work that *M* tomato plants increased their bioprotection against the leaf pathogen *X. campestris* pv. *vessicatoria* in comparison to *NM* plants, independently of their phosphorous nutritional status (Mora-Romero et al. 2015b). We conclude that the down-regulation of some of these proteins may be part of a priming mechanism that prepares the plant leaves to respond to subsequent stresses, such

as pathogen attack. Further studies will help elucidate the specific roles of these proteins, and how their down-accumulation may be associated with different leaf responses in *M* plants, such as defense.

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Author contributions LIPG participated in the experimental design, conducted most of the experimental work (including 2D-electrophoresis and peptide sequence analyses) and drafted the manuscript. JALV participated in image analysis of the two-dimensional gel using the PD-Quest software, and in critical revision of the manuscript. IEMM, HGF and SCL participated in the experimental work and in critical revision of the manuscript. MLM and SMG conceived the study and participated in its design and coordination, as well as drafting of the manuscript. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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