



Regulation of the leaf proteome by inoculation of *Populus × canescens* with two *Paxillus involutus* isolates differing in root colonization rates

Agnieszka Szuba¹ · Łukasz Marczak² · Leszek Karliński¹ · Joanna Mucha¹ · Dominik Tomaszewski¹

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Abstract

During ectomycorrhizal symbioses, up to 30% of the carbon produced in leaves may be translocated to the fungal partner. Given that the leaf response to root colonization is largely unknown, we performed a leaf proteome analysis of *Populus × canescens* inoculated in vitro with two isolates of *Paxillus involutus* significantly differing in root colonization rates ($65 \pm 7\%$ vs $14 \pm 7\%$), together with plant growth and leaf biochemistry analyses to determine the response of plant leaves to ectomycorrhizal root colonization. The isolate that more efficiently colonized roots (isolate H) affected 9.1% of the leaf proteome compared with control plants. Simultaneously, ectomycorrhiza in isolate H-inoculated plants led to improved plant growth and an increased abundance of leaf proteins involved in protein turnover, stress response, carbohydrate metabolism, and photosynthesis. The protein increment was also correlated with increases in chlorophyll, foliar carbon, and carbohydrate contents. Although inoculation of *P. × canescens* roots with the other *P. involutus* isolate (isolate L, characterized by a low root colonization ratio) affected 6.8% of the leaf proteome compared with control plants, most proteins were downregulated. The proteomic signals of increased carbohydrate biosynthesis were not detected, and carbohydrate, carbon, and leaf pigment levels and plant biomass did not differ from the noninoculated plants. Our results revealed that the upregulation of the photosynthetic protein abundance and levels of leaf carbohydrate are positively related to rates of root colonization. Upregulation of photosynthetic proteins, chlorophyll, and leaf carbohydrate levels in ectomycorrhizal plants was positively related to root colonization rates and resulted in increased carbon translocation and sequestration underground.

Keywords Ectomycorrhiza · Root colonization rate · Plant biometrics · Protein turnover · Stress response · Leaf carbohydrates

Abbreviations

2DE Two-dimensional gel electrophoresis
Chl *a* Chlorophyll *a*
Chl *b* Chlorophyll *b*
DW Dry weight
FW Fresh weight

ECM Ectomycorrhizal
Rca RuBisCo activase
SC Soluble carbohydrates
SEM Scanning electron microscopy
TNC Total non-structural carbohydrates

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✉ Agnieszka Szuba
agnieszkalapa@wp.pl

¹ Institute of Dendrology, Polish Academy of Sciences, Parkowa 5, 62-035 Kórnik, Poland

² Institute of Bioorganic Chemistry, Polish Academy of Sciences, Z. Noskowskiego 12/14, 61-704, Poznań, Poland

Introduction

The ectomycorrhizal (ECM) symbiosis is considered a mutualistic association between soil fungi and roots of woody plants. The interaction is usually highly beneficial to the host trees (Smith and Read 2008). The ECM symbiosis plays a significant role in carbon sequestration. An estimated 30% of the carbon in ECM interactions is transferred underground to fungal cells (Nehls et al. 2007; Smith and Read 2008). In exchange for carbohydrates delivered by the plant, ECM fungi improve host nutrition, particularly in terms of N (Willmann

et al. 2014) and water supply (Marjanović et al. 2005). Thus far, molecular studies, particularly those investigating N (Willmann et al. 2014; Szuba 2015) and carbohydrate flow (reviewed in: Nehls et al. 2010), have mainly focused on ECM plant roots. Although physical interactions only occur in roots, ECM fungi alter the physiologies of the entire plants (Smith and Read 2008), including the biochemistry of leaves (e.g., fatty acids (Luo et al. 2011) or flavonoids (Kaling et al. 2018) compositions). Leaves produce carbohydrates, and their transfer from plants to fungal partners is one of the most important factors in ECM symbiosis. The interaction between an ECM fungus and a plant is a dynamic process, especially during the early phases of root colonization. As fungal colonization of roots increases, the plant must continually match the symbiotic partner's requirements. This process rearranges the entire protein turnover machinery by increasing protein transcription and translation as well as protein degradation, as has been frequently reported previously for plants colonized by arbuscular mycorrhizal (AM) fungi (Aloui et al. 2011; Lingua et al. 2012; Song et al. 2015). However, how colonization by ECM fungi modifies the main proteomic pathways associated with carbohydrate production in leaves is less well understood.

ECM symbiosis can lead to altered leaf anatomies or larger leaf sizes (Martins et al. 1997) that could increase a plant's photosynthetic abilities. ECM fungi can influence, for example, the N status of leaves, which results in an increase (Reid et al. 1983; Hobbie and Colpaert 2003) or decrease (Hobbie and Colpaert 2003; Koele et al. 2012; Näsholm et al. 2013; Szuba et al. 2017) in foliar N concentrations. Thus, these changes may be consequences of leaf proteome modifications by ECM symbiosis. In contrast to the increased abundances of proteins associated with photosynthesis, carbohydrate metabolism, protein turnover, and the stress response in leaves of AM plants (Aloui et al. 2011; Lingua et al. 2012), ECM colonization does not lead to any significant changes in oak leaves (Sebastiana et al. 2017). However, the latter studies were mainly focusing on proteins expressed in roots. Consequently, the response of the leaf proteome to ECM colonization is still unknown. The relationship between C and nutrient exchange is still not fully understood but is probably the most important aspect of the symbiosis, particularly because the C/N balance determines the outcome of the ECM interaction (Corrêa and Martins-Loução 2011). In mutualistic relationships, resource exchanges are very complex (Veneault-Fourrey and Martin 2011) and depend on many factors. The root colonization ratio has been found to significantly affect carbohydrate-nutrient exchange (Corrêa and Martins-Loução 2011) via modulation of a plant's N uptake and probably via the so-called fungal-induced “carbon sink”—the carbon demand of the fungal partner (Dosskey et al. 1990). Although N uptake can occur via a mantle (Garcia et al. 2016), it is commonly believed that the resource

exchange mainly occurs via the Hartig net—a network of fungal cells located between epidermal and cortical plant cells (Smith and Read 2008). As the formation of the ectomycorrhizae proceeds, the architecture of root tips and thus their functioning changes, e.g., due to altered nutrient uptake in root tips, devoid of root hairs but still without well-developed Hartig nets (Smith and Read 2008; Szuba et al. 2017).

To elucidate the response of plant leaves to colonization by ECM fungi, we have to consider the duration of the experiments to ensure fully functioning ectomycorrhizae (Koele et al. 2012; Lingua et al. 2012), which affects the levels of root colonization and the extent of the biochemical changes in both partners (Tschaplinski et al. 2014). The dynamics of *in vitro* root colonization with *Paxillus involutus* are similar (Gafur et al. 2004; Müller et al. 2013; Szuba unpublished data) to those in the *Laccaria bicolor* interaction with poplars, in which the largest molecular shifts occur 6 weeks after inoculation (Tschaplinski et al. 2014). Thus, we harvested poplar samples 6 weeks after inoculation with two *P. involutus* isolates that significantly differed in their root colonization ratios to answer the following questions: (i) How does the leaf proteome response vary between plants inoculated with the two analyzed isolates; (ii) how does root colonization affect photosynthetic proteins; (iii) how do these proteomic changes relate to leaf biochemistry and root colonization ratio.

During non-axenic pot or field studies, even noninoculated controls have been frequently found to possess low levels of root tip colonization, which may influence biochemical traits (Karst et al. 2008; Koele et al. 2012). Thus, to answer the above questions, we chose a *Populus × canescens*–*P. involutus* *in vitro* fully controlled model. This simplified model has frequently been used to understand the basics of the molecular changes associated with the ECM symbiosis (Müller et al. 2013; Szuba 2015) and allowed us to understand the extent to which ECM colonization can modify the leaf proteome.

Material and methods

In vitro cultures and experimental design

Populus × canescens microcuttings (clones) provided by Prof. Krystyna Bojarczuk of the Institute of Dendrology, Polish Academy of Sciences, were propagated in a modified Murashige and Skoog (MS) agar medium containing 1.5% sucrose, as described previously (Szuba et al. 2017). *Paxillus involutus* isolates were obtained from fruit bodies growing in poplar monocultures and maintained *in vitro* on agar in a modified Melin-Norkrans medium (MMN; Kottke et al. 1987) containing 1% glucose and 0.5% maltose.

Two fungal *P. involutus* isolates were selected for the proteomic experiments: an isolate characterized by a high mycorrhization level (hereinafter referred to as “isolate H”), and an isolate characterized by a low colonization level (hereinafter referred to as “isolate L”; for details about isolate origins, see Szuba et al. 2017).

A two-layered agar medium (full MS medium (40 ml) covered with an approximately 3-mm layer of full MMN medium (10 ml)) was used for both the inoculated and control treatments. The freshly transferred poplars were inoculated by placing a 5-mm² fragment of fresh mycelium on the growth medium near a 3-cm-high poplar microcutting (Szuba et al. 2017). The poplars (18 plants per treatment) were cultivated in 300-ml glass jars in a growth chamber at 21 °C under a cool white fluorescent light with 150 μM photons m⁻² s⁻¹, 60% humidity, and a 16/8-h day/night photoperiod.

Biometrical and root colonization analysis

The plant heights and root, stem and leaf fresh weights were measured. Leaves from randomly selected plants were scanned with a high-resolution Epson Perfection V700 photo scanner. Leaf images were processed with WinFolia software (*Régent Instruments Inc.*). The number of leaves per plant, average leaf area, and total leaf area (sum of leaf area per plant) was calculated. Three representative leaves per treatment were used for scanning electron microscopy (SEM). The middle parts of the leaf fragments were mounted on aluminum stubs with conductive carbon adhesive tabs. Samples were subsequently air-dried under ambient conditions and coated with gold. The surface of the abaxial side of leaves was viewed with a Hitachi S3000N SEM equipped with a secondary electron detector (SE) with an accelerating voltage of 15 kV. The cell wall thickness was measured from the SEM images using ImageJ v1.48 software (Wayne Rasband, Bethesda, MD, USA).

Root colonization was assessed as described previously (Szuba et al. 2017). Briefly, the roots were classified into two stages of symbiosis: “fully mycorrhizal” (with a well-developed mantle and Hartig net); “changed” root tips, which were covered with fungi but lacked a fully developed Hartig net; and “nonmycorrhizal” root tips (Fig. 1a and b). Representative root tips for each class were selected, fixed, and analyzed under a microscope (Szuba et al. 2017).

The roots, stems, and leaves were dried at 60 °C for 48 h to determine the dry weight (DW). Next, dry weight percentages were estimated as follows:

$$DW (\%) = \frac{\text{dry weight (mg)}}{\text{fresh weight (mg)}} \times 100$$

Biochemical analysis

The total N and C percentages in dried leaf, stem and root samples were measured using a CHNS analyzer (2400 CHNS/O Series II System, PerkinElmer, Waltham, MA), and the C/N ratio was calculated according to the following formula:

$$C/N \text{ ratio} = \frac{C \text{ concentration in DW}/12.011}{N \text{ concentration in DW}/14.007}$$

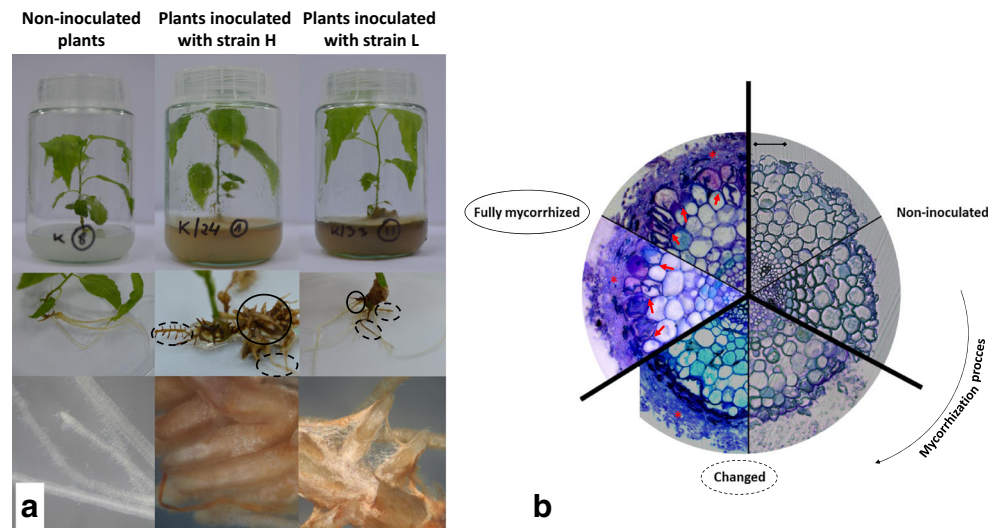
Nonstructural soluble carbohydrates (SC) and starch were extracted from dried leaf samples (20 mg) and determined using a standard colorimetric assay as described previously (Szuba et al. 2013). Leaf pigments were analyzed in the fresh leaf samples (10 mg) according to the photometric acetone method (Lichtenthaler and Wellburn 1983). C, N, SC, and starch contents (g per plant) were calculated according to the formula DW × Concentration of the analyzed feature (mg g of DW⁻¹). Leaf pigment contents (g per plant) were calculated according to the formula fresh weight (FW) × Concentration of the analyzed feature (mg g of FW⁻¹). C, N, and foliar carbohydrates and pigment concentrations were normalized to DW and FW and are presented in Online Resource 1.

Proteomic analysis

Leaves from eight randomly selected plants per treatment were used for the proteomic analysis. Three to five biological replicates per treatment were used for protein extraction. For each replicate, proteins were isolated from 500 mg of fresh leaves (each sample consisted of a pool of leaves from three plants randomly selected from the eight plants chosen for the proteomic analysis) according to the modified phenol extraction method (Hurkman and Tanaka 1987).

Plant materials were ground in liquid N₂ and extracted after 45 min on ice in a (1:1) mixture of extraction buffer (0.7 M sucrose; 0.5 M Tris, pH 7.5; 0.1 M KCl; 50 mM EDTA; 5% 2-mercaptoethanol; 1% SDS and 2 mM PMSF) and phenol solution (pH 8.0 from Sigma Aldrich) with continuous shaking. Fresh buffer was added to the phenol phase for the next 30 min of the extraction. After centrifugation, the phenol phase was collected and mixed with 5 vol. of ice-cold 0.1 M ammonium acetate in methanol. After overnight precipitation, the proteins were centrifuged and then air-dried after two washes with ammonium acetate solution. The proteins were resuspended in rehydration buffer (5 M urea, 2 M thiourea, 2% CHAPS, 2% carrier ampholyte (Pharmalyte 4–7, GE Healthcare LS, Uppsala, Sweden)), and the protein concentrations were estimated using a 2-D Quant Kit according to the manufacturer’s procedure (GE Healthcare LS). Protein (300 μg, with one or two technical replicates for each protein extract) was loaded in 11-cm pH 4–7 linear IPG strips (GE Healthcare LS) and

Fig. 1 Representative images of the analyzed plants. Images of the analyzed plants, representative root images, and root tip close-ups are given for all experimental treatments (a). Circles with solid lines: examples of fully mycorrhizal root tips; circles with dotted lines: examples of changed root tips (b). Microscopic images of cross-sections of fixed noninoculated, changed, and fully mycorrhizal root tips. Asterisk represents fungal mantle; arrows represent Hartig net (c)



isofocused in a Manifold II (GE Healthcare LS) apparatus using the following parameters: 300 V for 1 h, followed by 3500 V for 1:30 h, and 3500 V for 3:30 h. The strips were equilibrated for 15 min in buffer (6 M urea; 0.75 M Tris, pH 8.8; 2% SDS; 30% glycerol; 1% DTT) and then for 15 min in 2.5% iodoacetamide. The equilibrated proteins were electrophoresed on a 12% sodium dodecyl sulfate–polyacrylamide gels. Finally, the proteins were visualized with Coomassie Brilliant Blue G-250, and the protein profiles were captured using the LabScan 2.20 system (GE Healthcare LS). The best protein profiles ($n = 3$ selected from a minimum of 6 profiles) were analyzed with Image Master 2D Platinum 7.0 Software (IMP 7.0; GE Healthcare LS). The protein profiles of leaves of inoculated treatments were compared to those of noninoculated poplars in two independent analyses (comparison of noninoculated treatment vs isolate H and noninoculated treatment vs isolate L). Spot-normalized volumes (%Vol) were compared, and proteins with altered abundances were selected according to the following parameters: ANOVA < 0.05 and ratio ≥ 1.2 , with manual verification. Selected spots were removed from the gel with SpotPicker (GE Healthcare LS) and subjected to MS/MS identification. All spots selected in one experiment were compared using IMP 7.0 software to corresponding spots in the rest of the analysis.

Selected proteins were digested using a standard “in gel” digestion procedure (Szuba and Lorenc-Plucińska 2015). Peptide solutions (0.5 μ l) were deposited on a PAC plate (Bruker Daltonics GmbH) prespotted with α -cyano-4-hydroxycinnamic acid. When the spots were dried, spectra were collected with a minimum of 1500 laser shots in a MALDI TOF/TOF UltrafleXtreme mass spectrometer (Bruker Daltonics GmbH). All spectra were externally calibrated using a standard peptide solution (Bruker Daltonics GmbH). The mass spectra (combined peptide mass fingerprints and MS/MS fragments) were used for database searches

using the Viridiplantae taxonomy filter against the Swiss-Prot database (550,740 sequences), the NCBI nonredundant database (84,704,028 sequences), and the *Populus trichocarpa* Genome DB (47,351 sequences) with Mascot software (Matrix Science, London, UK, locally installed at the European Centre for Bioinformatics and Genomics, Poznań, Poland). The parameters for the mass tolerance of precursor and product ions were ± 30 ppm and ± 0.8 Da, respectively. Carbamidomethylation as a fixed modification and methionine oxidation as a variable modification were selected; one missed cleavage was allowed. For predicted or unknown proteins, identifications were performed by a maximum similarity search using Blastp software (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

The identified proteins were assigned to one of seven functional groups: protein metabolism (including, among others, proteins related to biosynthesis, refolding, transport, and proteolysis, P); proteins involved in the light-dependent phase of photosynthesis (L); carbon fixation (containing, among others, proteins that catalyze reversible and irreversible reactions in the Calvin-Benson cycle, and all proteins related to ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo), even those involved in protein refolding, F); carbohydrate metabolism (excluding proteins directly involved in the Calvin-Benson cycle pathway, C); proteins involved in the stress response (S) but with a distinction for proteins involved in ROS scavenging (R); and other processes (including mainly those involved in secondary metabolism and uncharacterized proteins, O). Functional classification of the identified proteins was based on the protein function according to data obtained from the gene ontology (GO) platform of the protein knowledge base, UniProtKB (<http://www.uniprot.org/uniprot>). For comparison, an additional analysis of COG (Clusters of Orthologous Groups) was performed, and the results are shown in Online Resource 2.

As many proteins had multiple functions, an additional identification of enriched cellular pathways was performed using the DAVID (Database for Annotation, Visualization and Integrated Discovery; <http://david.abcc.ncifcrf.gov/>) tool (Huang et al. 2009). For pathway annotations, the KEGG pathway database was searched. An analysis in DAVID was performed based on the *P. trichocarpa* gene list alone, and the fold enrichment and *p* value for each significantly altered metabolic pathway were calculated. The protein abundances (%Vol normalized using a Z-score algorithm) of differently abundant proteins are presented in the form of heatmaps that were prepared using the software PERSEUS (Max Planck, Germany).

Statistics

Statistical analyses were performed using STATISTICA 5 (StatSoft Inc., Tulsa, OK, USA) or JMP Pro 13.0.0 (SAS Institute Inc.), as well as Image Master Platinum 7.0 software (for protein profile analysis). The percentage data were transformed when necessary to achieve normality, according to the Bliss formula (Snedecor and Cochran 1976). All figures show nontransformed data. Variation among samples was assessed using ANOVA and compared with Tukey's honest significant difference test ($p < 0.05$).

Results

Biometrical and root colonization analysis

Three stages of root colonization by isolates of *P. involutus* were distinguished: nonmycorrhizal, changed root, and fully mycorrhizal tips (Fig. 1, Table 1). The total percentage of root tip colonization (percentage of fully mycorrhizal and changed root tips) was significantly higher for the H isolate compared with the L isolate ($65 \pm 7\%$ vs $14 \pm 7\%$; Table 1).

Although inoculation with either of the two *P. involutus* isolates resulted in increases in host plant height, only the H isolate increased the host dry biomass compared with the control (Table 1). Leaf DW did not differ between plants inoculated with isolate L and the controls, but it was higher in those inoculated with isolate H. The increase in DW of plants inoculated with isolate H was driven by a significantly higher DW (%) in those plants (Table 1)—the FW of both leaves and whole plants did not differ between the treatments (Table 1).

Plants inoculated with isolate H had fewer leaves but showed a tendency towards larger leaf blades, which resulted in a lack of a significant difference in leaf area per plant among the analyzed treatments (Table 1). Additionally, according to the SEM analysis, leaves of plants inoculated with isolate H had thicker cell walls compared with leaves in the other treatments (Fig. 2).

Biochemical traits of leaves inoculated with *P. involutus* isolates

In plants inoculated with isolate L, the foliar carbon contents did not differ from the noninoculated controls (Fig. 3a) but were significantly higher in the leaves and stems of plants inoculated with isolate H, as well as in the roots of poplars inoculated with either *P. involutus* isolate, all compared with noninoculated plants (Fig. 3a). The poplars inoculated with isolate H, but not isolate L, had higher C contents in whole plants compared with the controls (Fig. 3a).

The nitrogen contents in the leaves of inoculated plants did not differ compared with the controls, whereas in stems and roots, higher nitrogen concentrations were found for both inoculated treatments (Fig. 3b). There were no significant differences in N contents in whole plants between treatments (Fig. 3b).

C and N contents were strongly correlated for all treatments (Fig. 3c), but the slope coefficient of the regression line differed and increased in the following order: leaves of plants inoculated with isolate H → leaves of plants inoculated with isolate L → leaves of noninoculated plants.

The C/N ratio was significantly different in all analyzed treatments ($p < 0.0001$) and increased in the following order: noninoculated plants (7.35 ± 0.10) → plants inoculated with isolate L (8.96 ± 0.09) → plants inoculated with isolate H (10.91 ± 0.18).

Leaf soluble carbohydrates and starch contents did not differ between controls and plants inoculated with isolate L, but they were almost twice as high in plants inoculated with isolate H (Table 1). Increases in the contents of foliar total non-structural carbohydrates (TNC; sum of starch and soluble carbohydrates) were accompanied by higher root colonization levels (%) (Fig. 4a).

Chlorophyll (*a*) contents did not differ between the analyzed treatments, but a trend towards an increase in Chl (*a*) content in leaves of inoculated plants was possible (Table 1). Chlorophyll (*b*) contents were higher in the leaves of plants inoculated with both *P. involutus* isolates compared with those of control, noninoculated plants (Table 1). The contents of the sum of the analyzed leaf pigments (Chl (*a* + *b*)) were higher in plants inoculated with isolate H and did not differ in plants inoculated with isolate L, both compared with the controls (Fig. 4b; $p = 0.049$). The average Chl (*a* + *b*) contents (Fig. 4b), especially calculated per leaf blade area (reflecting the surface available for photosystems antennas; Fig. 4c), were positively related to the average root colonization (%) values.

Proteomic results

Concentrations of extracted leaf proteins were higher in plants inoculated with isolate H and did not differ in plants inoculated with isolate L, both compared with the controls (Table 1).

Table 1 Biometrical and biochemical characteristics of non-mycorrhizal and mycorrhizal *Populus × canescens* microcuttings. Mean \pm SE values are presented; different letters within rows indicate significant differences between the values (one-way ANOVA, Tukey's test, $\alpha = 0.05$)

	Non-inoculated	Plant inoculated with:		<i>p</i>	<i>n</i>
		Strain L	Strain H		
Root colonization characteristics					
Non-colonized root tips (%)	–	82.7 \pm 1.07b	44.5 \pm 12.1a	0.001	18
Changed root tips (%)	–	8.64 \pm 5.22a	40.3 \pm 8.0b	0.001	18
Fully mycorrhized root tips (%)	–	5.93 \pm 3.65a	24.5 \pm 7.6b	<0.0001	18
Whole plant biometrics					
Plant height (mm)	47.7 \pm 3.0a	65.9 \pm 2.5b	60.7 \pm 2.5b	<0.0001	18
Whole plant DW (g)	0.032 \pm 0.004a	0.038 \pm 0.002a	0.053 \pm 0.004b	0.008	10
Whole plant FW (g)	0.347 \pm 0.037	0.410 \pm 0.035	0.371 \pm 0.032	0.339	10
Leaf biometrics					
Leaf FW (g)	0.247 \pm 0.030	0.298 \pm 0.019	0.242 \pm 0.019	0.248	5 $\leq n \leq$ 7
Leaf DW (g)	0.025 \pm 0.003a	0.030 \pm 0.002ab	0.038 \pm 0.0001b	<0.0001	5 $\leq n \leq$ 7
Leaf DW (%)	10.3 \pm 0.03a	10.1 \pm 0.2a	16.2 \pm 1.0b	<0.0001	5 $\leq n \leq$ 7
Leaf number per plant	9.5 \pm 0.9b	7.8 \pm 0.8ab	6.2 \pm 0.3a	0.024	5 $\leq n \leq$ 7
Leaf average area (cm ²)	1.9 \pm 1.2	2.3 \pm 1.8	2.5 \pm 1.9	0.240	5 $\leq n \leq$ 7
Leaf area per plant (cm ²)	18.8 \pm 2.1	18.2 \pm 1.7	15.8 \pm 1.5	0.537	5 $\leq n \leq$ 7
Leaf biochemistry					
Foliar SC (mg per plant)	13.4 \pm 1.47a	17.8 \pm 1.13a	22.8 \pm 1.53b	0.002	9
Foliar starch (mg per plant)	0.141 \pm 0.015	0.172 \pm 0.011	0.225 \pm 0.015	0.004	9
Chlorophyll a (mg per plant)	0.515 \pm 0.064a	0.651 \pm 0.043ab	0.737 \pm 0.057b	0.065	10
Chlorophyll b (mg per plant)	0.129 \pm 0.016a	0.216 \pm 0.014b	0.187 \pm 0.015b	0.003	10
Leaf proteome characteristics					
Concentrations of extracted proteins (mg g ⁻¹ FW)	8.37 \pm 0.14a	8.51 \pm 0.25a	12.35 \pm 0.79b	0.009	6
Number of selected proteins	–	56	62	–	–
Percentage of selected spots (%) ^a	–	6.83%	9.13%	–	–
Proteins more abundant in inoculated variant ^b	–	9	57	–	–
Proteins less abundant in inoculated variant ^b	–	47	5	–	–

DW dry weight, FW fresh weight, SC soluble carbohydrates

^a Percentage of selected spots (100% = number of matches between the controls and inoculated plants). This is regarded as the final volatility level

^b Compared to control, non-inoculated plants

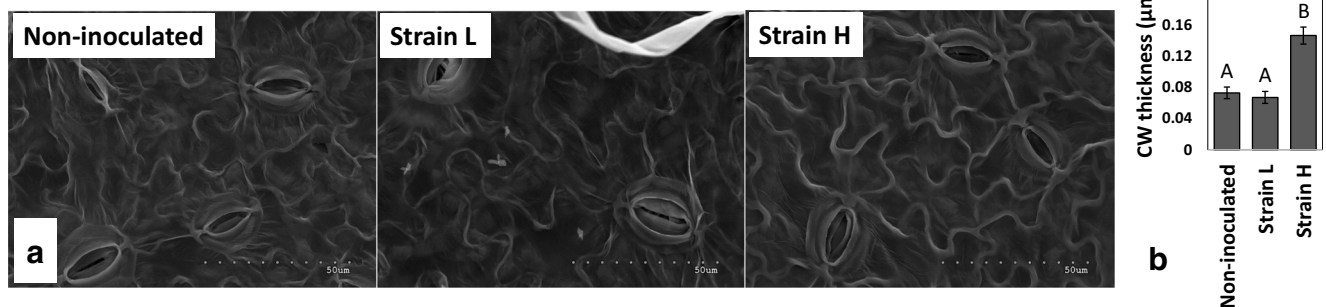


Fig. 2 Scanning electron microscopy (SEM) results for the analyzed plants showing the surface of the abaxial side of leaves (**a**). Thickness of the cell walls (CW) calculated based on the presented images (**b**).

Mean \pm SE values are presented, and different letters indicate significant differences between treatments within each plant organ (one-way ANOVA, Tukey's test, $\alpha = 0.05$, $n = 10$)

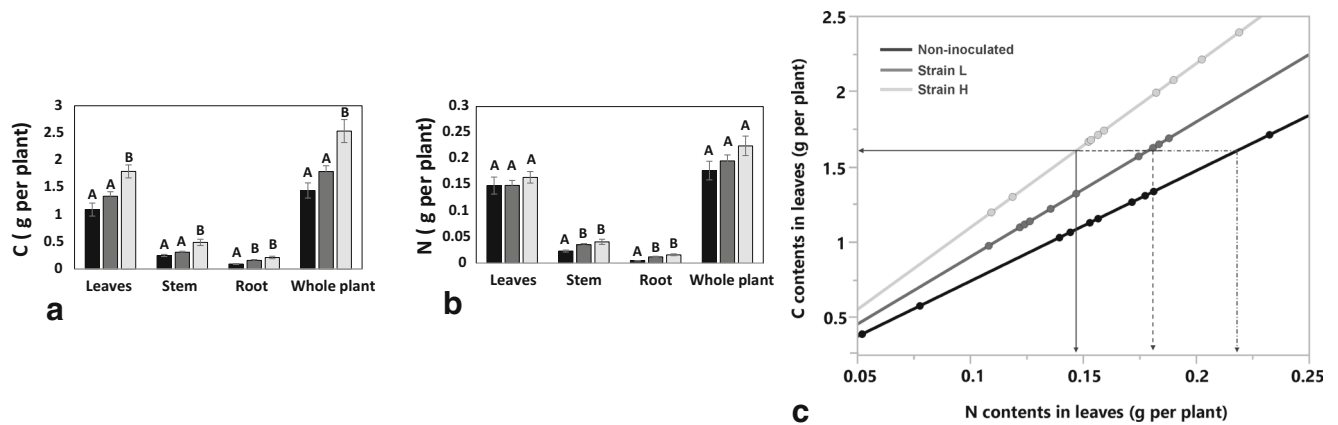


Fig. 3 Carbon and nitrogen relations in the analyzed plants. Calculated C (a) and N (b) contents (g per plant; $n = 10$). Correlation of foliar C and N contents (c). Mean \pm SE values are presented; the different letters within

the plant organs indicate significant differences between the values (one-way ANOVA, Tukey's test, $\alpha = 0.05$)

Two-dimensional gel electrophoresis (2DE) protein profile analysis

According to the protein 2DE profiles, within two independent comparisons (control vs isolate H and control vs isolate L), isolate H had 62 spots and isolate L had 56 spots, revealing a significant difference in abundance in inoculated plants compared with the leaf proteomes extracted from noninoculated poplars (Table 1). Inoculation with isolate H influenced approximately 9% of the leaf proteome, whereas inoculation with isolate L resulted in significant changes in abundance in almost 7% of the leaf proteins (Table 1).

There were significant differences in the abundances of the selected spots between the two isolates. More than 90% of the spots in the plant leaves inoculated with isolate H were more abundant in the leaves of inoculated plants; by contrast, leaves

of plants inoculated with isolate L exhibited spots with abundances lower than in the controls (Table 1, Fig. 6).

A comparison of spots from all 2DE profiles revealed a relatively small number of common selected spots between the fungal isolates (12 spots). The IMP 7.0 and MS/MS identification data are presented in Online Resource 2.

Among the selected spots, 21 proteins were not identified due to an insufficient spectrum quality or lack of information in the databases (Online Resource 2). All identified proteins are presented in the Supplemental Table S2 (located in the Online Resource 2) and Fig. 6.

Proteins involved in protein metabolism and turnover

Many of the identified proteins were associated with protein metabolism (Supplemental Table S2). Proteins involved in the

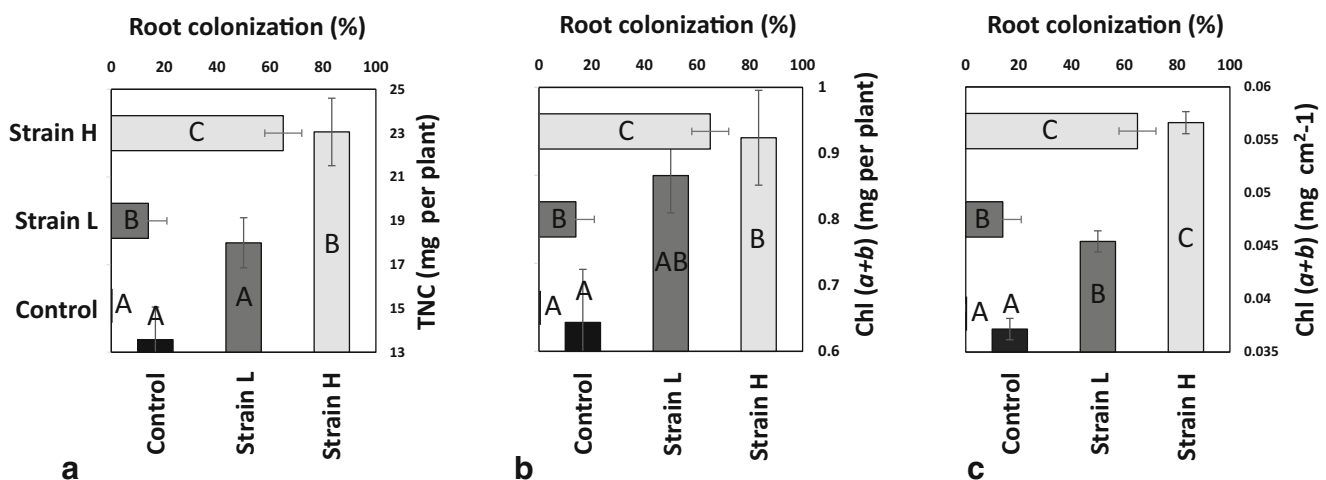


Fig. 4 Root colonization compared with the foliar biochemical traits. Foliar contents of TNC, $n = 9$ (a). Foliar contents of Chl ($a + b$), $n = 10$ (b) and Chl ($a + b$) contents per square centimeters of leaf blade area, $n = 5$ (c) (all compared with the total root colonization (%)) are shown. Mean

\pm SE values are presented; the different letters indicate significant differences between the values (one-way ANOVA, Tukey's test, $\alpha = 0.05$)

synthesis of compounds necessary for translation processes were upregulated in the leaves of plants inoculated with isolate H (Supplemental Table S2, Figs. 6 and 7). In these leaves, a large increase in translation was observed, with signals showing greater abundances of ribosome components and protein transporters (e.g., chloroplastic “presequence protease 1” up-regulation). Intense proteomic signals for increased proteasome activity were detected in plants inoculated with isolate H (Supplemental Table S2, Fig. 6). Such symptoms were generally absent in plants inoculated with isolate L (Supplemental Table S2), in which a general decrease in protein turnover was detected (Supplemental Table S2, Fig. 6), except for the up-regulation of eukaryotic translation initiation factor 5A-2, which is involved in ribosome building.

Proteins involved in photosynthesis

The affiliation of proteins in particular functional groups was highly differentiated between the treatments (Supplemental Table S2), but the most numerous proteins in both analyses were those involved in photosynthesis (sum of carbon fixation and light-dependent phase proteins; Supplemental Table S2, Fig. 7). Photosystem proteins, mainly those in photosystem II, and numerous components of the Calvin-Benson cycle were differentially abundant between the *P. involutus* isolates (Supplemental Table S2, Fig. 6). Plant leaves inoculated with isolate H exhibited strong proteomic signals for upregulated photosynthesis (Fig. 7); this finding was in contrast to the plants inoculated with isolate L, which generally had decreased abundances of photosynthetic proteins (especially those associated with carbon fixation) compared with the controls (Supplemental Table S2, Figs. 6 and 7).

One exception was the most frequently detected protein - RuBisCo activase (Rca). Thirteen different identified spots (Supplemental Table S2, Figs. 5 and 6) showed alterations in “carbon fixation in photosynthetic organisms” pathway in leaves of all inoculated poplars, according to DAVID analysis (Fig. 7a and b). Rca was one of the most abundant proteins in poplar leaf cells (Fig. 5). Two Rca isoforms were detected in the 2DE profiles, and both were represented by numerous spots characterized by slightly different pI values (Fig. 5). For all inoculated plants, Rca spots were either less or more abundant compared with the controls (Supplemental Table S2, Fig. 5), but all identified spots with a theoretical mass near that of Rca had a decreased abundance (Supplemental Table S2, Fig. 7). By contrast, the spots characterized by a mass higher or lower than the theoretical MW of Rca (except spot no. 518) were more abundant in plants inoculated with either *P. involutus* isolate (Supplemental Table S2, Fig. 5). Among all 13 spots, Rca was the only identified protein (multiple identifications were not observed; Online Resource 2).

Proteins involved in carbohydrate metabolism

The abundances of proteins involved in carbohydrate metabolism (excluding elements of the Calvin-Benson cycle) were higher than in controls only for plants inoculated with isolate H (Supplemental Table S2 and Fig. 6). This phenomenon was reflected in alterations of numerous cellular processes associated with carbohydrate metabolism in leaves of plants inoculated with isolate H (Fig. 7). Among the more abundant proteins, phosphoglucomutase was detected, an enzyme that is crucial for carbohydrate synthesis in plants, catalyzing the reversible isomerization of glucose-1-phosphate to glucose-6-phosphate. Increased levels of this protein typically result in increased starch production (Uematsu et al. 2012; Fig. 7), a known end-product of the triose-P synthesized during the Calvin-Benson cycle (Bahaji et al. 2014). Next, glucose-1-phosphate adenylyltransferase, which is directly associated with starch synthesis, similar to alanine aminotransferase, was upregulated in the leaves of plants inoculated with isolate H; UDP-glucose-1-phosphate uridylyltransferase, an enzyme involved in UDP-glucose synthesis, was also upregulated (Kleczkowski et al. 2011).

Proteins involved in the stress response

In the leaf proteomes of the inoculated plants, numerous differentially abundant proteins related to the stress response were detected, such as several HSPs. These proteins were more abundant in plants inoculated with isolate H. In addition, redox-regulated proteins, superoxide dismutase, and 2-Cys peroxiredoxin were upregulated in plants inoculated with isolate H and downregulated in plants inoculated with isolate L, all compared with the noninoculated controls (Supplemental Table S2 and Fig. 6).

Other proteins

The nonclassified proteins (group “Other”) were identified only in plants inoculated with isolate H (Supplemental Table S2), reflected in the larger number of functional categories determined by DAVID analysis (Fig. 7).

In the leaves of plants inoculated with isolate H, the enzyme glutamine synthetase and S-adenosylmethionine synthase were more abundant (Supplemental Table S2, Fig. 6). Several proteins with increased abundances in the leaves of plants inoculated with isolate H remain “unknown” (Supplemental Table S2, Fig. 6).

Discussion

One of the best-known consequences of fungal symbiosis is nutrient-carbon exchange between the fungus and its plant

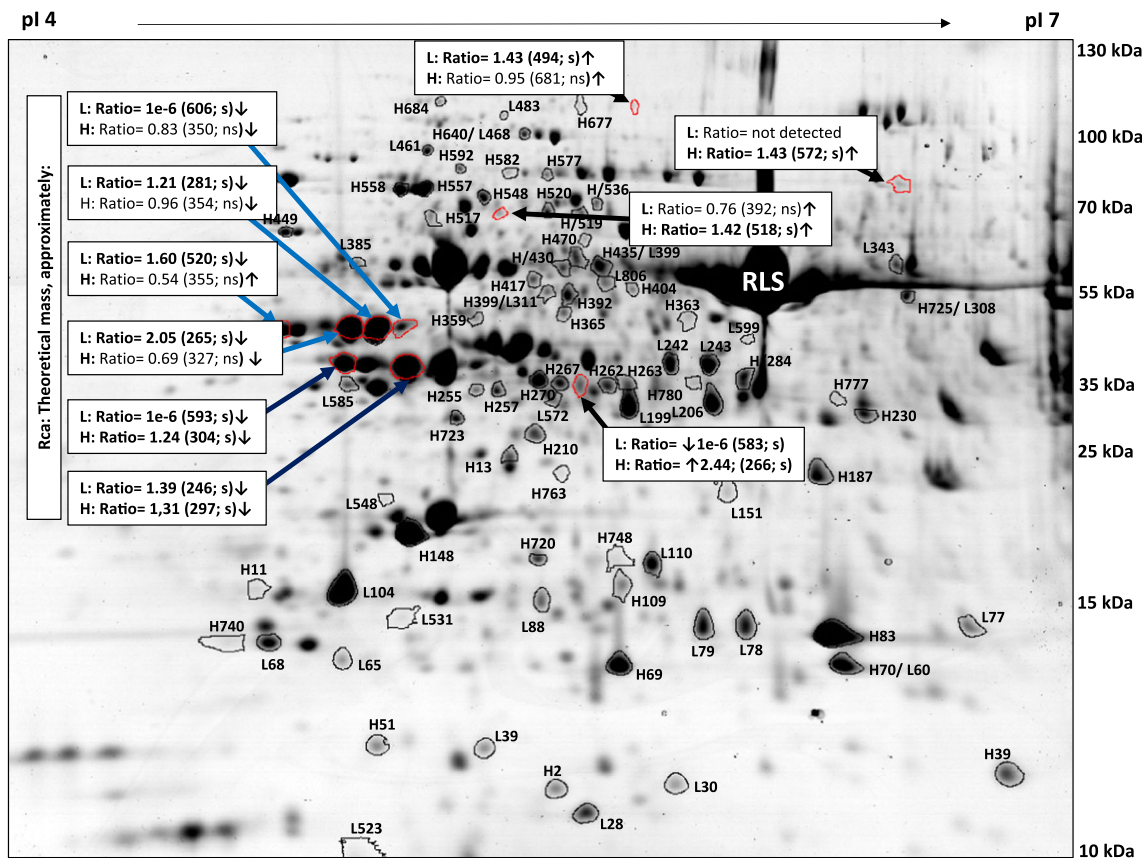


Fig. 5 Representative image of a 2DE protein profile for isolates H and L with the spots selected for the marked identification. The spot numbers are defined in Fig. 6 and Supplemental Table S2 (Online Resource 2). Spots identified as RuBisCo activase (Rca) are highlighted in red. Ratio and ANOVA values are provided for Rca identified spots and their corresponding spots. s significant difference in spot abundance between

control and inoculated treatments ($\alpha = 0.05$, ns no significant difference). Blue arrows: Rca spots characterized with the experimental MW similar to the theoretical MW of Rca (light blue arrows: bigger Rca isoform, dark blue arrows: smaller Rca isoform); black arrows: Rca spots characterized with experimental MW different than the theoretical Rca MW. RLS: RuBisCo large subunit

partner (Nehls et al. 2007; Willmann et al. 2014). Increased plant nutrition, especially in terms of the most “limiting” nutrient, N, has usually been considered a key benefit of the ECM symbiosis, with positive effects on plant physiology (Reid et al. 1983; Hobbie and Colpaert 2003; Nehls et al. 2007). The increased abundance of glutamine synthetase detected in the leaves of plants colonized with isolate H may be related to the insignificantly increased foliar (and significantly increased in stems and roots) contents of N found in those plants (Fig. 3; see also Online Resource 2). Glutamine synthetase is a ubiquitous enzyme in plants involved in ammonium assimilation (Castro-Rodríguez et al. 2011), and its upregulation has been reported previously in response to AM colonization (Smith et al. 1985). In contrast, in the leaves of plants inoculated with isolate L, there were no increases in glutamine synthetase abundance nor trends towards an increase in foliar N content. An unchanged or even decreased foliar N status under ECM colonization has been reported surprisingly frequently, even in plants characterized by increased growth (Hobbie and Colpaert 2003; Koele et al. 2012; Näsholm et al. 2013; Szuba et al. 2017). In all inoculated plants,

especially in the leaves of poplars inoculated with isolate H, higher C/N ratios were observed compared with the controls. In fact, the reciprocal C and N exchange between the host and fungi (Kytöviita 2005; Kiers et al. 2011; Garcia et al. 2016) may be disrupted during the initial stages of ectomycorrhization (Corrêa et al. 2011). *Populus* × *canescens* inoculated in vitro with *P. involutus* has a greater investment in C acquisition relative to its gain in the form of increased N uptake to leaves (and other nutrients; Szuba et al. 2017). This “additional C” may be transferred to the fungus, but it is also clearly visible when foliar C and N contents (g per whole plant) are compared (Fig. 3c). Moreover, the foliar contents and concentrations (see also Online Resource 1) of carbon, but not of nitrogen, were significantly positively related to the root colonization ratio, and only plants inoculated with isolate H revealed massively increased stem contents of both C and N, which may suggest increased resource exchange. This finding was strongly reflected in the leaf proteome response.

The most important differences in the leaf proteomes of ECM-inoculated poplars indicate alterations in photosynthesis, resulting in different carbon fixation levels. In our

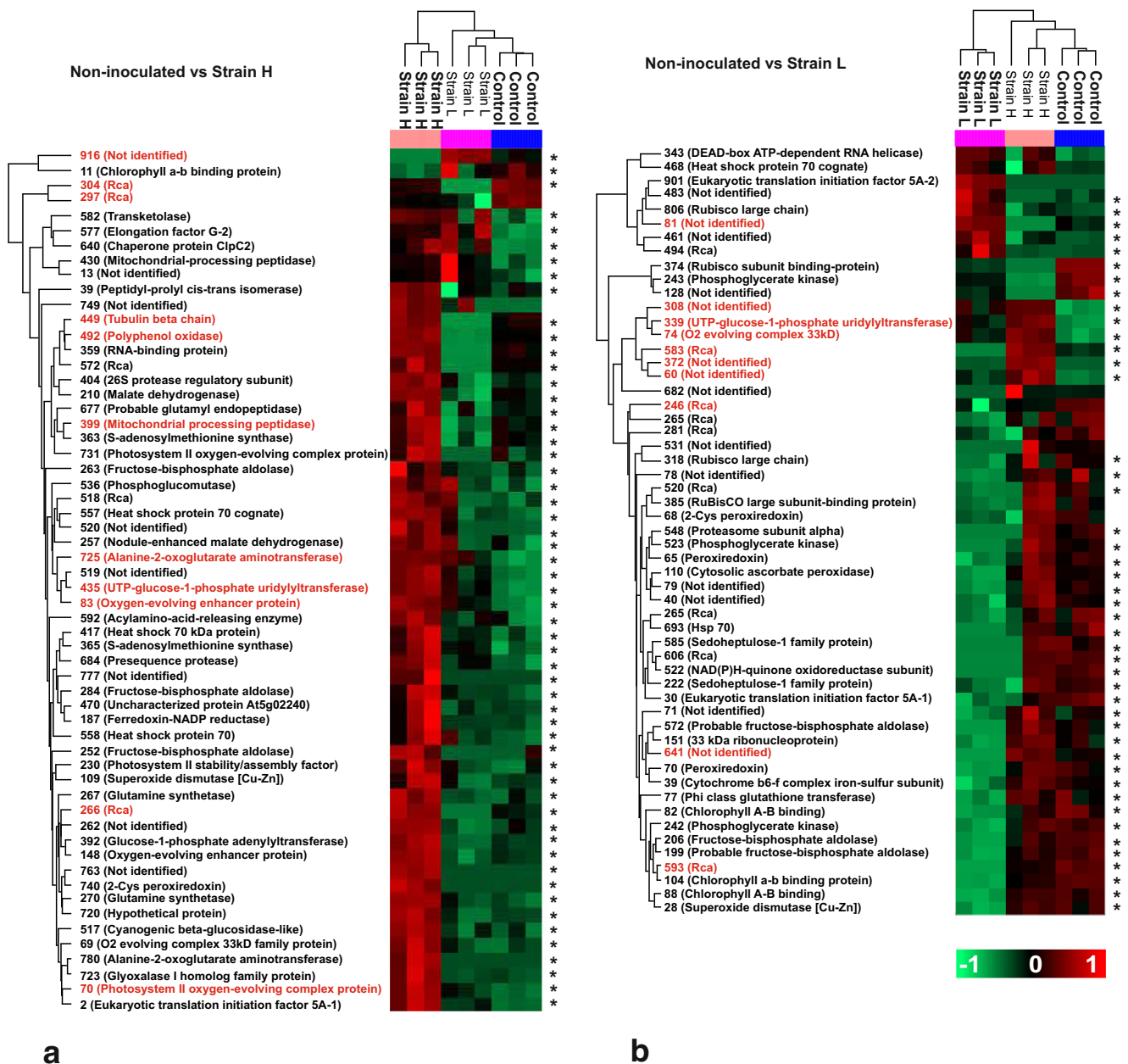


Fig. 6 Protein abundance. Heat map analysis combined with the hierarchical cluster analysis showing proteins identified in IMP 7.0 software as differentially abundant between control vs isolate H (**a**) and control vs isolate L (**b**). Spot normalized volumes (%Vol) of spots were transferred into PERSEUS software. Additionally, the %Vol values of the corresponding spots (%Vol of isolate L for (a) and %Vol of isolate H for (b)) were used for the hierarchical cluster analysis. Proteins identified as

differently abundant in both analyses (a and b; “common spots”) are indicated in red font. Intensity values were log₂-transformed, batch-corrected and Z-scored row wise. Green, minimal abundance; red, maximal abundance. Asterisk indicates significant differences between H and L treatment (Student’s *t* test, $\alpha = 0.05$). The spot numbers are defined in Fig. 5 and Supplemental Table S2 (Online Resource 2)

analyses, inoculation with isolate H (but not isolate L) positively affected numerous light-dependent phase proteins as well as the chlorophyll content. This phenomenon also had a strong effect on proteins in the Calvin-Benson cycle (Supplemental Table S2, Fig. 6), which is consistent with reports showing that symbiotic fungal inoculation causes an increase in abundance, rate of regeneration, and turnover of RuBisCo (Choi et al. 2005; Rozpądek et al. 2015). The protein most frequently detected

during our study was RuBisCo activase (Rca). Rca is a crucial enzyme for the maintenance of carbon fixation, and it coordinates and regulates photosynthesis, particularly dark-light carbon transitions (Portis et al. 2008). Changes in Rca abundances were similar for both *P. involutus* isolates, which is surprising considering the contrasting protein abundance trends. In its oligomeric form, Rca is active not only as a hexamer (Hazra et al. 2015) but also as an alternative form of 2–4 subunits, which were possibly

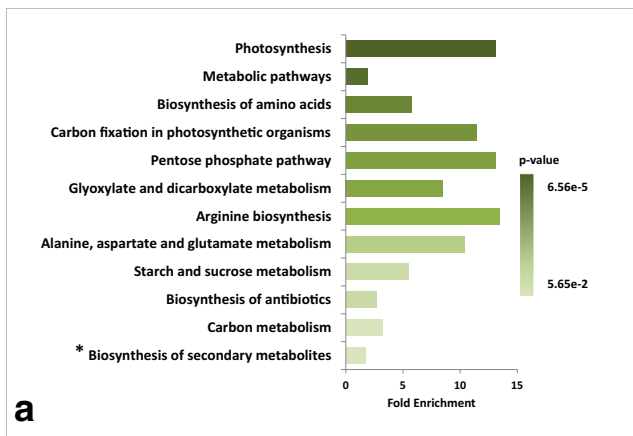
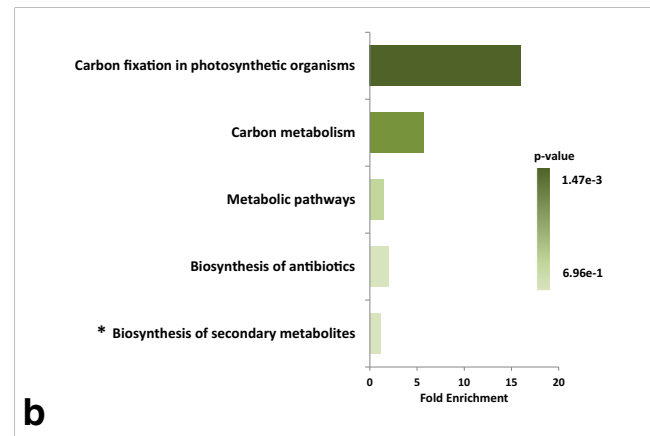


Fig. 7 Enrichment of cellular processes. Prediction of the affected processes (KEGG_PATHWAYS; both up- and downregulated) in response to mycorrhiza using the DAVID tool. Prediction based on the list of identified proteins according to a comparison of proteins isolated from

found in our study (Fig. 5; Keown et al. 2013). Rca oligomerization (and thus activity) is dependent on posttranscriptional modifications such as dithiol/disulfide exchange (Portis et al. 2008) or tyrosine phosphorylation (Boex-Fontvieille et al. 2014). The shifts in pIs of Rca monomers of both known poplar Rca isoforms found in our 2DE gels (Hozain et al. 2010; Rablroud and Lelong 2011) may indicate that *P. involutus* colonization affected the aforementioned posttranscriptional modifications. Rca monomer abundances were significantly lower in all inoculated *Populus × canescens*. Decreased (Ghabooli et al. 2013) or unchanged (Martins et al. 1997) Rca monomer levels, despite increased photosynthesis rates, have been found previously in response to mycorrhiza. This phenomenon could explain the Rca shifts observed in the inoculated poplars in the face of the numerous proteomic indications of increased photosynthesis in the ECM association with isolate H. Concomitantly, the abundances of spots with MWs higher than those of Rca monomers increased under ECM inoculation in our experiment, especially in plants inoculated with isolate H. Such shifts in the MWs of Rca proteins suggest polymerization or, more likely (due to the protein denaturation step during the 2DE experiment), aggregation of Rca monomers. Indeed, similar shifts in Rca abundances have been previously observed in *Pisum sativum* under heat stress, and the authors related this phenomenon to the extreme susceptibility of Rca to aggregate (Salvucci et al. 2001). Rca aggregation under ECM inoculation could be a consequence of the stress response (see below), as well as increased Rca turnover (also manifested by higher Rca degradation, especially in isolate H treatment, where higher protein turnover was detected). In contrast, polymerization of Rca is strongly positively dependent on the protein concentration (Keown et al. 2013), which is known to increase in response to mycorrhizal colonization (Martins et al. 1997; Latif 2013) and which clearly increased in the leaves of the colonized poplars. The modulation of Rca under ECM symbiosis together with the important role



control leaves and the leaves of plants inoculated with isolates H (a) and L (b). * KEGG_PATHWAYS selected during DAVID analysis and characterized by $p > 0.05$

of Rca in plant functioning (Portis et al. 2008) make Rca an interesting subject for further analyses of ECM plants.

The main output of photosynthesis is the delivery of substrates for subsequent carbohydrate biosynthesis. The leaves of plants inoculated with isolate H responded to root colonization by up-regulating many enzymes involved in sugar synthesis (as has been reported previously for AM plants; Lingua et al. 2012), including an increase in malate dehydrogenase abundance. Previous studies have detected proteomic signals relating this role of malate under various types of endophytic fungus symbiosis (e.g., increased levels of the aforementioned malate dehydrogenase; Lingua et al. 2012; Rozpadek et al. 2015); malate may be responsible for increased fixed carbon export to the cytosol and its subsequent use (Gietl 1992).

Interestingly, symbiosis with isolate H also induces increased starch biosynthesis. A previous study found that AM plants have increased starch synthesis together with an increase in soluble sugar concentrations (Krishna et al. 1981), which the authors explained to be a result of increased metabolic activity under mycorrhizal inoculation. The accumulation of polysaccharides such as starch or cellulose is driven by UDP-glucose (which is synthesized by UDP-glucose-1-phosphate uridylyltransferase, found to be more abundant in plants inoculated with isolate H) and is regarded as a major factor driving increases in biomass (Kleczkowski et al. 2011). The increased carbohydrate contents in *Populus × canescens* leaves (especially the increase in soluble sugars, which are transferred to the fungal partner) were positively related to root colonization and may confirm our thesis that the fungal-triggered C sink was the major effector of the observed differences between the analyzed treatments, including the ECM-induced biomass growth in isolate H, which was probably partly caused by nonstructural carbohydrate accumulation. Interestingly, all inoculated poplars were taller, but only the DW of plants inoculated with isolate H was increased compared with the controls. This result may also be explained by the thicker

cell walls detected during SEM analysis in the leaves of plants inoculated with isolate H. Because *in vitro* leaves often have highly reduced levels of extracellular waxes (Hazarika et al. 2006), it can be speculated that the thicker cell walls were the result of increased amounts of cell wall (CW) carbohydrates. Increased carbon sequestration and biosynthesis of larger amounts of carbohydrates, both nonstructural and cell wall polysaccharides, require increased leaf transpiration (Jarvis and Davies 1998). Such processes would also result in increases in the dry weight percentage and plant biomass, exactly as observed in plants inoculated with isolate H compared with noninoculated plants.

Despite the possibility that both the fungus and roots assimilated the monosaccharides present in the growth medium, which could have potentially affected the leaf photosynthesis rates, our results suggest that the photosynthetic protein abundances were also related to the root colonization ratio (a fungal-triggered carbon sink; Dosskey et al. 1990; Nehls et al. 2007). Increased levels of photosynthesis (Choi et al. 2005; Luo et al. 2011; Ghabooli et al. 2013) or abundances of photosynthetic proteins (until now only reported for AM and fungal endophytes; Lingua et al. 2012; Rozpądek et al. 2015) have been commonly observed during fungal symbioses and usually result in improved plant growth. However, various ECM fungi differentially impact the photosynthetic rates of plants (Dosskey et al. 1990), as shown by isolates L and H with different effects on photosynthetic proteins. Most photosynthetic proteins were less abundant, and there were no increases in abundance of leaf enzymes involved in carbohydrate biosynthesis in these plants, in contrast to the leaves of plants inoculated with isolate H.

The unchanged plant biomass and foliar carbohydrate contents (no C cost compared with the controls) found in plants inoculated with isolate L may have been partly due to the residual saprophytic properties of ECM fungi, which can use heterotrophic C sources from the growth media (Doré et al. 2015; Heinonsalo et al. 2015). They could also have been partly due to the preferential assimilation by *P. involutus* of glucose present in the MMN medium (Nehls et al. 2007, 2010). The combination of these factors could have resulted in mycelium self-sufficiency. This thesis may also be supported by the increased C contents found in plant roots inoculated with this L isolate, which was possibly caused by the presence of the fungal hyphae and/or by increased C allocation to the roots by the plant to meet the fungal C demands.

In the leaves of plants inoculated with isolate H, an increased stress response and plant defense signals were observed, revealing costs associated with the establishment of the ECM symbiosis (Corrêa et al. 2006). These symptoms were not found in the leaves of plants inoculated with isolate L. The HSPs detected for isolate H have been shown to be induced under various stress conditions (Wang et al. 2004), confirming the occurrence of stress conditions during the mycorrhization process (Szuba et al. 2017). However, because of the crucial role of these

chaperonins during protein folding (Wang et al. 2004), the high HSP abundances may also be a consequence of the significantly increased protein turnover in ECM plants (as in AM plants: Aloui et al. 2011; Lingua et al. 2012). Similarly, redox-regulated proteins were detected in these plants, such as superoxide dismutase and 2-Cys peroxiredoxins, which may be involved in the stress response as ROS scavengers. Some groups of peroxiredoxins are involved in anti-pathogen defense (Dietz et al. 2006), but it is more likely that the upregulation of these enzymes is connected to the increased photosystem activity (Ghabooli et al. 2013) in response to mycorrhizal inoculation and is possibly connected with the detoxification of photochemically produced radicals (Ghabooli et al. 2013). The observation that plants inoculated with isolate L had decreased abundances of 2-Cys peroxiredoxins together with reduced amounts of photosynthetic proteins may provide further evidence for this thesis. An anti-oxidative role may also be played by S-adenosyl-L-methionine (SAM) synthase, which catalyzes the formation of SAM from L-methionine and adenosine triphosphate. SAM synthase has been found to play a role in the alleviation of oxidative stress in AM mycorrhizal *Medicago truncatula* plants (Aloui et al. 2009), and in our study, it was most abundant in the leaves of plants inoculated with isolate H.

Finally, our research has confirmed that the ECM symbiosis affects the leaf proteome and increases protein turnover. Some of the signals involved in increased ribosome activity supported that the effects of isolate L on the leaf proteome may even have occurred in plants characterized by a low root colonization level, but strong signals of increased protein turnover were only detected in plants inoculated with isolate H. Therefore, leaf proteome changes during ECM symbioses are probably dependent on the progress of root colonization, as has been previously reported for AM (Lingua et al. 2012).

Conclusions

The many similarities between the proteome alterations in plants colonized with isolate H in our study and the available proteomic data on the leaves of AM plants (Aloui et al. 2011; Lingua et al. 2012) indicate that the increased protein turnover; stress response; and, most importantly, photosynthesis enhancement and carbohydrate biosynthesis are a universal leaf response to mycorrhizal colonization. Even the general decrease in the abundances of these proteins in response to inoculation with isolate L does not contradict this general conclusion. The observed dissimilarities in the leaf proteomes may have resulted from various foliar C/N statuses (which could have contributed to the observed differences in photosynthetic protein abundances), delayed mycorrhization processes (Timonen and Sen 1998; Lingua et al. 2012), and/or different carbon sinks (Dosskey et al. 1990; Nehls et al. 2007; Heinonsalo et al. 2015). Similarly, Lingua et al. (2012), who studied leaf proteomes at various stages of the AM

colonization process, observed that most spots were downregulated (~68%) during early stages of the interaction: Among others, they observed the downregulation of RuBisCo subunits, ATP synthase and HSPs, which is similar to the observations of the present study. Thus, such a downregulation of proteins may be a common result of the early phase of mycorrhization. The promotion of host growth prior to the establishment of massive functional mycorrhizae, which we observed for isolate L (increased plant height and root biometrics; data not shown), has also been reported in axenic studies (Felten et al. 2009; Sarjala et al. 2010) and may be related to the activity of polyamines, auxins, or cytokines that are known to be released into the medium at high levels by *P. involutus* mycelia (Rudawska and Kieliszewska-Rokicka 1997; Sarjala et al. 2010). These factors may also be partly responsible for the relatively small differences in the detected proteome alteration levels (9.13% vs 6.83%) compared with the greater differences in root colonization ratios ($65 \pm 7\%$ vs $14 \pm 7\%$) observed for isolate H and L, respectively. Isolate characteristics such as differences in compatibility with juvenile plants or with poplars generally, the root colonization progress, and the release of secondary metabolites by fungal mycelia are other factors that influence plant biochemistry (Timonen and Sen 1998; Gafur et al. 2004). Given that the observed changes in biochemistry and plant morphology are the result of changes in the leaf proteome, extensive proteome analyses of a variety of ECM systems that link proteins with other plant traits are required to fully understand the complex effects triggered by ECM colonization.

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Author contribution statement AS designed and conducted the experiments, analyzed the data, and wrote the manuscript. LM conducted the MS/MS experiments, analyzed the data, and edited the manuscript. LK conducted the root colonization analysis and edited the manuscript. JM and DT conducted the microscopy analysis and edited the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interests.

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