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Transcriptome analysis of the *Populus trichocarpa–Rhizophagus irregularis* Mycorrhizal Symbiosis: Regulation of Plant and Fungal Transportomes under Nitrogen Starvation

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Nutrient transfer is a key feature of the arbuscular mycorrhizal (AM) symbiosis. Valuable mineral nutrients are transferred from the AM fungus to the plant, increasing its fitness and productivity, and, in exchange, the AM fungus receives carbohydrates as an energy source from the plant. Here, we analyzed the transcriptome of the *Populus trichocarpa–Rhizophagus irregularis* symbiosis using RNA-sequencing of non-mycorrhizal or mycorrhizal fine roots, with a focus on the effect of nitrogen (N) starvation. In *R. irregularis*, we identified 1,015 differentially expressed genes, whereby N starvation led to a general induction of gene expression. Genes of the functional classes of cell growth, membrane biogenesis and cell structural components were highly abundant. Interestingly, N starvation also led to a general induction of fungal transporters, indicating increased nutrient demand upon N starvation. In non-mycorrhizal *P. trichocarpa* roots, 1,341 genes were differentially expressed under N starvation. Among the 953 down-regulated genes in N starvation, most were involved in metabolic processes including amino acids, carbohydrate and inorganic ion transport, while the 342 up-regulated genes included many defense-related genes. Mycorrhization led to the up-regulation of 549 genes mainly involved in secondary metabolite biosynthesis and transport; only 24 genes were down-regulated. Mycorrhization specifically induced expression of three ammonium transporters and one phosphate transporter, independently of the N conditions, corroborating the hypothesis that these transporters are important for symbiotic nutrient exchange. In conclusion, our data establish a framework of gene expression in the two symbiotic partners under high-N and low-N conditions.

Keywords: Ammonium transporter • Arbuscular mycorrhiza • Nitrogen metabolism • *Populus trichocarpa* • *Rhizophagus irregularis* • Symbiosis • Transcriptome • Transportome.

Abbreviations: AM, arbuscular mycorrhizal; AMT, ammonium transporter; CCD, carotenoid cleavage dioxygenase; ERM, extraradical mycelium; FDR, false discovery rate; GOGAT, glutamine oxoglutarate aminotransferase; GS, glutamine synthetase; IRM, intraradical mycelium; N, nitrogen; P, phosphorus; Pi, inorganic phosphate; PR, pathogenesis-related; qPCR, quantitative reverse transcription–PCR; S, sulfur.

Introduction

Arbuscular mycorrhizal (AM) fungi belong to the order of Glomerales and form mutualistic symbiosis with about 80% of land plant species. With their complex and extensive hyphal network they are able to extract mineral nutrients and water from the soil and make them available to the plant symbiont. In exchange, the plant supplies the AM fungus with photosynthates (Smith and Read 2008). In AM symbiosis, the extracted nutrients are taken up by the extraradical mycelium (ERM), incorporated into transport molecules and transferred to the intraradical mycelium (IRM). Inside the plant cortical cells, the IRM forms tree-like structures (arbuscules) which are surrounded by the plant-derived periarbuscular membrane and are the site of nutrient exchange (Bonfante and Genre 2010). In addition to the nutritional benefit, it was reported that plants gain in fitness, and have increased growth rates and an improved disease resistance (Smith and Read 2008).

As a constituent of amino acids and nucleotides, nitrogen (N) is one of the most important elements for life. Due to the high mobility of nitrate and ammonium in soil, it was assumed for a long time that the depletion zone around the roots of plants diminished rather quickly. Consequently, AM fungi were expected to play only a minor role in N uptake for plants (Hodge and Storer 2015). However, evidence is accumulating that the mycorrhizal symbiosis is also important for the plant's N nutrition (Courty et al. 2015, Garcia et al. 2016). Depending on the plant–fungal combination and soil N sources, it was demonstrated that plants can receive up to 42% of the N from the AM symbiont (Frey and Schüepp 1993, Mäder et al. 2000, Govindarajulu et al. 2005, Walder et al. 2012).

Ammonium seems to play a main role as an N source in the AM symbiosis. In contrast to nitrate, ammonium can be

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directly assimilated by the GS/GOGAT pathway (Hodge and Storer 2015) into glutamine by glutamine synthetase (GS) and further into glutamate by glutamine oxoglutarate aminotransferase (GOGAT). In subsequent metabolic steps, N is incorporated into other amides and amino acids such as alanine, asparagine and arginine. Arginine is the most common N form transported in plants and AM fungi (Govindarajulu et al. 2005, Cruz et al. 2007). Stable isotope labeling experiments have shown that N is taken up by the ERM and incorporated into amino acids whereby arginine constitutes up to 90% of all labeled amino acids (Govindarajulu et al. 2005). In the arbuscules, arginine is metabolized in the urea cycle and the released ammonium is translocated between the fungal plasma membrane and the periarbuscular membrane, the periarbuscular space. Specialized nutrient transporters located in the fungal and plant membrane are responsible for nutrient translocation and uptake (Courty et al. 2015, Hodge and Storer 2015).

In AM symbiosis, three high-affinity ammonium transporters (AMTs) were identified in the AM fungus *Rhizophagus irregularis* ((formerly *Glomus intraradices*): GintAMT1 (López-Pedrosa et al. 2006), GintAMT2 (Pérez-Tienda et al. 2011) and GintAMT3 (Calabrese et al. 2016). GintAMT1 is expressed in the extraradical mycelium, suggesting a role in NH_4^+ acquisition from the soil, while GintAMT2 might be involved in retrieving leaked NH_4^+. Some AMTs are induced during mycorrhizal colonization in AM plants such as in *Lotus japonicus* (Guether et al. 2009), *Glycine max* (Kobae et al. 2010), *Oryza sativa* (Pérez-Tienda et al. 2014), *Medicago truncatula* (Breuillin-Sessoms et al. 2015) or *Sorghum bicolor* (Koegel et al. 2013). Some AM-inducible AMTs were detected on the branch domain of periarbuscular membranes, indicating that active NH_4^+ transfer occurs around arbuscular branches by recruiting NH_4^+ in the acidic periarbuscular space and releasing the uncharged NH_3 into the cytoplasm of the arbusculated cells (Guether et al. 2009, Kobae et al. 2010, Koegel et al. 2013).

In plants, nitrate is considered to be the main N source in fertile soils and, indeed, many plant nitrate transporters have been described (Miller et al. 2007). Some nitrate transporters are induced during mycorrhizal colonization in AM plants such as in *L. japonicus* (Guether et al. 2009) and *M. truncatula* (Hohnjec et al. 2005). In *R. irregularis*, only one nitrate transporter has been identified so far (GiNT; Tian et al. 2010) and another one has been predicted (Tisserant et al. 2012), Moreover, GiNT, expressed both in the IRM and the ERM, is only regulated by the N source in the ERM, indicating that nitrate uptake is less relevant in the symbiosis (Tian et al. 2010).

Apart from N, phosphorus (P) also is made available to plants by AM symbiosis (Karandashov and Bucher 2005). In mycorrhizal symbiosis, the amount of P transferred from the fungus to the plant can range from a small percentage up to full nutrition of the plant (Paszkowski 2006, Javot et al. 2007b). Inorganic phosphate (Pi) at the soil–hyphal interface is translocated to the fungal cytoplasm with the help of phosphate transporters and then transported into the periarbuscular space. In AM fungi, only three transporters have been described so far in the three AM fungi *Glomus versiforme*, *R. irregularis* and *Glomus mosseae* (GvPT, GintPT and GmosPt) (Harrison and van Buuren 1995, Maldonado-Mendoza et al. 2001, Benedetto et al. 2005); they all belong to the major facilitator superfamily similar to those described in plants (Javot et al. 2007b, Tatry et al. 2009). In the periarbuscular space, Pi is taken up by the plant through H^+:Pi transporters that are localized at the periarbuscular membrane and are only expressed upon mycorrhization. Specific expression and localization of these transporters has been shown in many plant species such as *M. truncatula* (MtPT4, Harrison et al. 2002), *Lycopersicum esculentum* (now *Solanum lycopersicum*), *Solanum tuberosum* (LePT4/StPT4, LePT5/StPT5, StPT3; Nagy et al. 2005) and *Populus trichocarpa* (PtPT10; Loth-Pereda et al. 2011).

Populus trichocarpa (polar) is an angiosperm tree native in temperate ecosystems with broad adaptive and genetic variability that forms symbioses with ectomycorrhizal and AM fungi. So far, not much is known about the effects of mycorrhization on N uptake by perennial plants. For *P. tremuloides*, a near relative of *P. trichocarpa*, it has been shown that it is highly capable of inorganic N uptake by high- and low-affinity uptake systems enabling the tree to grow in nitrate- and ammonium-poor or -rich soils, indicating the existence of specialized transporters for nutrient uptake and internal distribution (Min et al. 1999, Min et al. 2000). Here, we used Illumina sequencing to investigate the transcriptome of mycorrhizal and non-mycorrhizal *P. trichocarpa*, as well as the transcriptome of *R. irregularis* in poplar roots under high or low N availability. In both organisms, we analyzed the effects of N availability on gene expression in general and on the expression of N metabolism-related genes. As N and P nutrition has a major role in AM symbiosis, we focused on fungal and plant ammonium and phosphate transporters. To deepen our knowledge about nutrient transport in the AM fungus, we analyzed the transportome of *R. irregularis* in the mycorrhizal roots.

Results and Discussion

Experimental design

AM and non-mycorrhizal plants were subjected to low-N and high-N nutrition to investigate which genes, especially transporters and genes linked to N metabolism and transfer, were affected by the nutrient conditions. Root samples of three biological replicates per condition were sampled; RNA was extracted and sequenced (**Fig. 1**). Our experimental set-up allowed the comparative analysis of the *R. irregularis* transcriptome in low-N and high-N conditions of colonized plant roots. We estimated AM colonization in poplar plants (**Fig. 1**). Non-mycorrhizal poplar plants were not colonized. Root colonization in AM plants was significantly ($P < 0.004$, Student's *t*-test) higher in plants grown in high-N conditions (from 75% to 82%) than in low-N conditions (from 45% to 59%). In high-N conditions, N content in the shoots was significantly ($P < 0.005$, Student's *t*-test) higher in AM plants (from 7.6% to 8.6 mg N g^{-1} shoot DW) than in non-mycorrhizal plants (from 3.8 to 4.6 mg N g^{-1} shoot DW). In high-N conditions, the N amount in shoots was significantly ($P < 0.005$, Student's *t*-test) higher in AM plants (from 914 to 995 mg N)

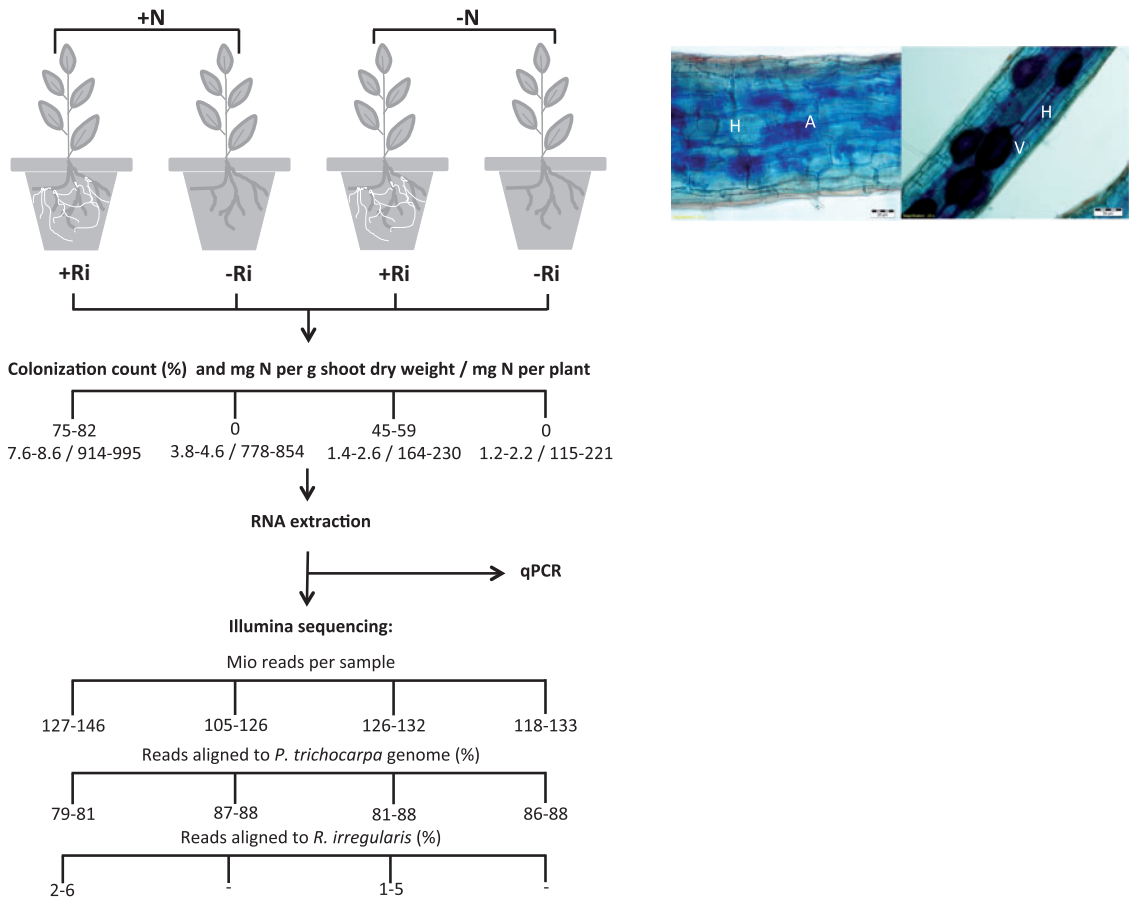

Fig. 1 Experimental set-up. *Populus trichocarpa* cuttings were inoculated with *Rhizophagus irregularis* (+Ri) or grew without the mycorrhizal fungi (−Ri). Systems were fertilized either with standard Hoagland solution (+N) or with Hoagland solution devoid of a nitrogen source (−N). Plants grew in the greenhouse for 12 weeks. At harvest time, roots were freed from substrate, one subsample was snap-frozen, and one subsample was used for colonization count (%) and one subsample was used for N measurement (mg N per g shoot DW and mg N per plant). Total RNA was extracted and mRNA sequencing was performed. Data were processed and evaluated. Key in mycorrhizal roots: A, arbuscule; H, hypha; V, vesicle.

than in non-mycorrhizal plants (from 778 to 854 mg N). In low N conditions, the quantity of N and the N amount in shoots was not significantly different in AM and non-mycorrhizal plants (**Fig. 1**). These data differ from the findings of Blanke et al. (2005), who reported in a field experiment that the colonization of *Artemisia vulgaris* was higher in N-deficient plots than in N-high plots, but under high P concentrations, and of Koegel et al. (2013), who reported in a time course experiment that the colonization of *S. bicolor* was not affected by the N nutrition using the same nutrient solution. These results highlight the fact that the influence of colonization by AM fungi on plant N is different between plant species and depends on growth conditions, confirming that nutrient availability modifies the AM colonization (Augé et al. 2014). Using Illumina technology, we sequenced about 105–133 million reads per RNA sample. About 79–88% of the reads could be aligned to the genome of *P. trichocarpa*. In AM root samples, 1–6% of the reads could be aligned to the genome of *R. irregularis*.

To validate the RNA-sequencing data, we technically confirmed the expression pattern of a subset of genes by quantitative reverse transcription–PCR (qPCR) measurements. All of the eight tested genes (genes involved in N metabolism and uptake or sugar transport) exhibited similar gene expression patterns when analyzed by qPCR and RNA-sequencing, indicating that changes of gene expression are valid and not biased by the experimental approach (Supplementary Fig. S1).

Gene expression analysis of *Rhizophagus irregularis* in mycorrhizal roots

Effect of N deficiency on R. irregularis gene expression. From the 30,282 predicted *R. irregularis* gene models, 27,030 were considered as expressed based on the presence of reads. A total of 1,295 genes were differentially expressed in low-N compared with high-N conditions.Of these differentially expressed genes, 107 (about 0.4% of total expressed genes) exhibited expression changes of >5-fold (Supplementary Table S1). Twenty-five of these highly differentially expressed genes could be identified and assigned to specific functional groups. Interestingly, virtually all differentially expressed genes were up-regulated under N deficiency, indicating that N starvation led to a general induction of gene expression within the AM fungus. It has already been observed in other organisms that N starvation

induced transcriptional activity (Scheible et al. 2004, Bi et al. 2007, Voigt et al. 2007, Krapp et al. 2011).

Most of the genes with highly induced expression in the low-N condition encoded proteins involved in cellular processes and signaling (i.e. signal transduction, post-translational modification and intracellular trafficking) as well as in metabolic processes (i.e. lipid transport and inorganic ion transport metabolism). We also observed a high representation of genes belonging to the functional classes of cell growth, membrane biogenesis and cell structural components. These changes may indicate that N starvation activated stress-induced signaling cascades, leading to up-regulation of nutrient sensing, uptake and transport systems as well as induction of defense mechanisms (Supplementary Table S1).

Expression changes in N metabolism-related genes in R. irregularis. The expression of most genes related to fungal N metabolism was not affected under low-N conditions (83 out of 90; Supplementary Table S2). The low-N conditions induced expression of only seven N metabolism-related genes, among them a nitrate transporter and AMTs, to which we refer later. High expression of genes coding for enzymes involvd in N metabolism (i.e. GS and GOGAT) suggested that there was a high metabolic rate of N in the IRM, as proposed by Gomez et al. (2009) and Guether et al. (2009).

High expression of a gene encoding an arginase is another indicator for a rapid turnover of N in the fungal IRM. Arginine is one of the main N carriers from the ERM to the IRM (Govindarajulu et al. 2005, Cruz et al. 2007). Interestingly, apart from transcripts coding for the GS/GOGAT pathway, we detected similar transcript levels of nitrilases in low- and high-N conditions. Nitrilases hydrolyze nitrile compounds into carboxylic acids and ammonia, but their biological role is largely unknown. However, there is evidence that they play a role in the microbial colonization process of plants (Howden and Preston 2009). In bacteria, it was also shown that nitrilases are involved in the conversion of indole-3-acetonitrile to IAA, which is involved in many physiological processes such as cell elongation, cell division, lateral root formation and tissue differentiation, all processes that might facilitate colonization of plant tissue by pathogens or mycorrhizal fungi (Pace and Brenner 2001, Spaepen et al. 2007, Kumari et al. 2015, Shao et al. 2015). Furthermore, it has been shown that treatment of plant roots with synthetic auxin stimulates mycorrhizal root colonization, in particular the formation of arbuscules (Etemadi et al. 2014). In several ectomycorrhizal fungi, an IAA pathway was identified and the fungus-derived IAA increased hyphal growth and mycorrhizal root colonization (Ek et al. 1983, Krause et al. 2015). Taken together, the constitutive expression of most N metabolism-related genes indicated that adaptation of the fungal metabolism to N deprivation did not involve the transcript level. Instead, it may rather have involved changes in protein turnover or enzyme activity, and metabolite interconversion and reallocation.

Differentially expressed nutrient transporters of R. irregularis. Apart from ammonium and phosphate transporters, which we discuss below, we identified 18 additional differentially expressed transporters, all induced under low-N conditions (**Table 1**). Amongst them are nitrate and amino acid transporters and transporters of the ABC superfamily. Our data suggest that under N-limiting conditions the AM fungus offers more N to the host plant. However, a direct link of transporter transcript abundances and the amount of actually transferred nutrients could not be proven as yet (Walder et al. 2015). It is assumed that N is transferred from the AM fungus to the host plant in the form of ammonium. However, increased expression of fungal amino acid transporters may indicate an export of amino acids serving as an N and sulfur (S) source for the plant.

Furthermore, genes encoding transporters actively involved in intracellular trafficking and carbohydrate transport, an urea transporter and a zinc transporter were up-regulated in low-N conditions.

Since colonization by AM fungi is known to increase plant P uptake (Smith et al. 2003), we also investigated the expression pattern of fungal phosphate transporters. We measured the transcript levels of the first characterized phosphate transporter gene in *R. irregularis*, *GiPT1* (here named *RiPT1*; Maldonado-Mendoza et al. 2001) and of the six additional recently identified phosphate transporter geness (from *RiPT2* to *RiPT7*) (Walder et Al. 2016). Here, the seven phosphate transporters were expressed; *RiPT4* was only marginally expressed compared with the six other transporters (**Table 2**). The significant induction of *RiPT5* and *RiPT7* in the low-N condition suggested that they could be involved in the transfer of P from the fungus to the plant and that the AM fungus could deliver more P. Alternatively, N starvation may signal general nutrient deficiency, resulting in the increased expression of nutrient transporters. By alleviating nutrient shortage for the plant, the AM fungus may ensure efficient return of essential carbohydrates from the plant (Olsson et al. 2002, Kiers et al. 2011 Fellbaum et al. 2014).

Gene expression analysis in *Populus trichocarpa*

Out of the 41,335 *P. trichocarpa* genes, 1,914 genes (approximately 4.6% of all expressed genes) were differentially expressed in poplar roots in the low-N condition or upon mycorrhization, or both (**Fig. 2**). Of interest, only a few genes were significantly regulated by both AM symbiosis and N conditions (e.g. Vapyrin) (**Fig. 3**; Supplementary Table S3).

Effect of mycorrhization on genes of the AM symbiotic pathway and on genes involved in arbuscule development. In AM symbiosis, many genes essential for the establishment of symbiosis (e.g. Vapyrin and the GRAS-type transcription factors RAM1 and RAM2), for arbuscule development (e.g. ABC transporters STR and STR2) and for the functioning of AM symbiosis (e.g. the phosphate transporter homologous to PT4 from *M. truncatula*) were induced in AM fungal symbiosis only, as already shown previously (Delaux et al. 2013), except for Vapyrin, also regulated by the N status. Of interest, these genes were not regulated in the ectomycorrhizal symbiosis *Laccaria bicolor–P. trichocarpa* (**Fig. 3**; Plett et al. 2015) whereas other genes such as the carotenoid cleavage dioxygenases CCD7

Table 1 Differentially expressed transporter genes of *R. irregularis*

Transcript ID	Eukaryotic orthologous groups				Mean RPKM	
	Description	Class	Group	Log2 ratio	−N	+N
147773	Protein transporter of the TRAM[a] superfamily	Intracellular trafficking, secretion and vesicular transport	Cellular processes and signaling	0.7	55	35
349661	Protein transporter of the TRAM[a] superfamily			1.1	73	34
339691	Vesicular amine transporter			1.2	110	49
34969	Amino acid transporters	Amino acid transport and metabolism	Metabolism	1.0	25	12
40376	Amino acid transporters			1.6	94	30
94248	Urea transporter			1.8	85	24
67708	GDP-fucose transporter	Carbohydrate transport and metabolism		1.0	28	14
289764	GDP-fucose transporter			1.2	158	70
218287	Ammonia permease (AMT)	Inorganic ion transport and metabolism		1.7	87	26
67530	Na^+/dicarboxylate, Na^+/tricarboxylate and phosphate transporters			1.8	128	36
30566	Nitrate transporter (MFS[b])			2.5	40	7
29953	Predicted nitrate transporter (MFS[b])			4.7	44	2
334075	Predicted divalent cation transporter			0.9	62	34
291068	Predicted divalent cation transporter			1.2	153	69
67368	Putative Zn^{2+} transporter MSC2 (cation diffusion facilitator superfamily)			0.9	36	20
286345	Zn^{2+} transporter			1.2	73	31
344948	Nucleoside transporter	Nucleotide transport and metabolism		0.8	35	19
21303	Transporter, ABC superfamily (Breast cancer resistance protein)	Secondary metabolites biosynthesis, transport and catabolism		2.5	20	4
341277	Long-chain acyl-CoA transporter, ABC superfamily (involved in peroxisome organization and biogenesis)	General function prediction only	Poorly characterized	0.8	77	44
9468	Predicted transporter ADD1 (MFS[b])			1.9	18	5

Gene expression in the low-N condition was tested against that in the high-N condition.
[a] TRAM translocating chain-associating membrane.
[b] MFS, major facilitator superfamily.

or CCD8 were only regulated in the early stage of the ectomycorrhizal symbiosis (4 weeks). This highlights clearly that the establishment and the functioning of the ectomycorrhizal and AM symbiosis are different.

Effect of N availability on gene expression in non-mycorrhizal poplar. In the absence of a symbiotic interaction partner, 1,295 genes were differentially regulated by N availability in poplar roots (**Fig. 2**). Among those, N deficiency induced expression of 342 genes (Supplementary Table S4). A total of 106 of the 342 genes were assigned to functional classes of the eukaryotic orthologous groups. Most of the genes overexpressed in the low-N condition were involved in metabolic processes (i.e. secondary metabolite synthesis, transport and catabolism, transport of inorganic ions and transport and metabolism of carbohydrates). Within the group of secondary metabolite synthesis, we observed that transcript abundances of many members of the Cyt P450 and 2-oxoglutarate- and Fe (II)-dependent oxygenase superfamily members were affected by low-N conditions (Supplementary Table S4). Both gene families encode proteins involved in reduction or incorporation of oxygen (www.uniprot.org; Bolwell et al. 1994). CCD1, CCD7 and CCD8 involved in apocarotenoid biogenesis and in strigolactone biosynthesis (Auldridge et al. 2016), respectively, are up-regulated in low-N conditions (**Fig. 3**). Consistent with their role in activating AM fungal symbiosis, biosynthesis of trigolactones is up-regulated by nutrient deficiency (Jamil et al. 2011). We could suggest an increase in the number and outgrowth of

Table 2 List of ammonium, nitrate and phosphate transporters in *R. irregularis*

Name	Transcript ID	Eukaryotic orthologous groups				Mean RPKM	
		Description	Class	Group	Log2 ratio	−N	+N
GintAMT1	337137	Ammonia permease (AMT)	Inorganic ion transport and metabolism	Metabolism	1.4	67	26
GintAMT2	314321				0.9	74	40
GintAMT3	218287				**1.7**	87	26
GintNT1	30566	Nitrate transporter (MFS[a])			**2.5**	40	7
GintNT2	29953	Predicted nitrate transporter (MFS[a])			**4.7**	44	2
RiPT1	345640	Inorganic phosphate transporter			−1.3	90	223
RiPT2	22848	Inorganic phosphate transporter			−0.4	18	25
RiPT3	7378	Inorganic phosphate transporter			0.4	7	6
RiPT4	13201	Inorganic phosphate transporter			−3.0	0	1
RiPT5	346370	Na^+/Pi symporter			2.7	84	13
RiPT6	49664	Na^+/Pi symporter			−2.1	2	8
RiPT7	67530	Na^+/tricarboxylate and phosphate transporters			**1.8**	128	36

Gene expression in the low-N condition was tested against that in the high-N condition.
Significant values are highlighted in bold (FDR corrected P-value < 0.05).
[a] MFS, major facilitator superfamily.

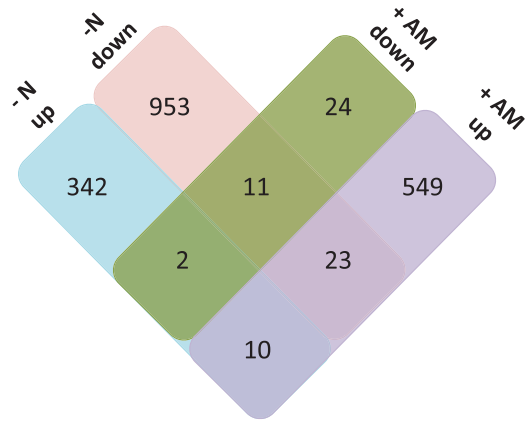

Fig. 2 Venn diagramm of nitrogen- and *R. irregularis*-regulated transcripts of *Populus trichocarpa* (>5 fold, FDR P-value < 0.05). Abbreviations: −N down: down-regulated in low-N conditions; −N up: up-regulated in low-N conditions; +AM down: down-regulated in AM symbiosis; +AM up: up-regulated in AM symbiosis.

lateral roots as shown under low phosphate (Ruyter-Spira et al. 2011). Further, several genes encoding UDP-glycosyltransferases were up-regulated in low-N conditions; they are known to be fundamental for the biosynthesis of natural compounds (Supplementary Table S4). By transferring activated sugar moieties to their acceptor molecule they are affecting the bioactivity of secondary metabolites, amino acids, phytohormones, etc., functional groups which we found over-represented when comparing low-N and high-N conditions using MAPMAN annotations (**Fig. 4**). Consistent with a role for glycosyltransferases in many fundamental processes, they are also presumed to be involved in stress responses (reviewed in Ross et al. 2001, Lim and Bowles 2004, Wang 2009). Low-N conditions further induced the expression of members of the major facilitator superfamily and ammonium and nitrate transporters to which we refer further below.

N deficiency significantly reduced expression of 953 genes, as defense-related genes (i.e. ethylene-forming enzymes and carboxylesterase, Supplementary Table S5) were down-regulated in low-N conditions. Consistent with previous observations (Liu et al. 2003, Güimil et al. 2005), the lowered defense may contribute to maintain and stimulate symbiotic interaction to improve its nutrient supply. Further, we observed a down-regulation of genes involved in S metabolism and of sulfate transporters in low-N conditions (**Fig. 4**). A co-regulation of S metabolism and N limitation has already been observed previously: low-N conditions reduced expression of genes encoding ATP sulfurylases and APS reductases, both normally induced upon sulfur starvation (Leustek et al. 2000, Nocito et al. 2007).

Effect of mycorrhization on gene expression in poplar roots upon N deficiency. Comparison of transcript abundance in mycorrhizal vs. non-mycorrhizal roots in low-N conditions yielded 320 differentially expressed genes (Supplementary Table S6). Many of these genes encoded transcription factors involved in control of secondary metabolism and cell morphogenesis (Dubos et al. 2010). A total of 271 genes were overexpressed upon mycorrhization, e.g. Vapyrin, a gene essential for intracellular progression of arbuscular mycorrhozal symbiosis (Pumplin et al. 2010); six genes encoded the orthologs of the Arabidopsis gibberellic acid signaling-related DELLA transcription factors RGA-like 1. Interestingly, upon mycorrhization, RGA-like 1 was nearly 700-fold overexpressed. Further, transcription factors, including one gibberellic acid signaling-related GRAS transcription factor, were down-regulated. Gibberellic acid is a key player in the regulation of mycorrhizal symbiosis as down-regulation of DELLA at the protein level resulted in decreased mycorrhization (Floss et al. 2013, Gutjahr 2014, Gobbato 2015, Heck et al. 2016). Taken together, induced expression of DELLA protein

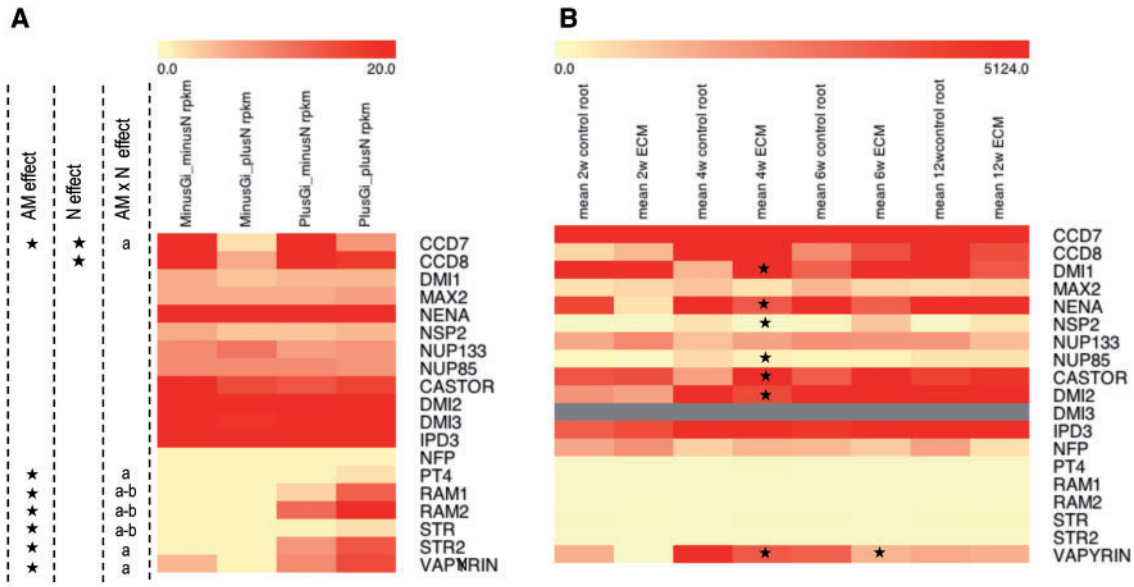

Fig. 3 Expression of selected AM marker genes. (A) Expression in *R. irregularis*-infected poplar roots with high or low N compared with non-inoculated control roots. (B) Expression in poplar roots inoculated with the ectomycorrhizal fungus *Laccaria bicolor* for 2, 4, 6 and 12 weeks (according to Plett et al. 2015). Abbreviations: ★, positive AM or N effect (FDR *P*-value < 0.05) (A) or significantly regulated (FDR *P*-value < 0.05) with *L.bicolor* compared with control roots (B) **a**, indicates a significant AM effect in +N samples; **b**, indicates a significant AM effect in −N samples.

expression and Vapyrin suggests that N deficiency leads to induction and maintenance of symbiosis. Similarly, germin-like proteins, involved in mycorrhization and root nodule formation (Doll et al. 2003, Güimil et al. 2005, Puppo et al. 2013), were also overexpressed in AM symbiosis under low-N conditions in our study.

Interestingly, we also found the gene expression of four putative chitinase to be highly induced upon mycorrhization. Specific expression of chitinase was reported in arbusculated cells (Bonanomi et al. 2001, Hogekamp et al. 2011), where it is thought to contribute to the initiation of plant immunity (Tromas et al. 2012). Chitinase was also up-regulated in a recent RNA-sequencing analysis on AM roots of *L. japonicus* (Handa et al. 2015).

Effect of mycorrhization on gene expression in poplar roots under high-N conditions. Under high-N conditions, a total of 316 genes were differentially regulated upon mycorrhization (Supplementary Table S7). A total of 271 genes exhibited induced expression upon mycorrhization. Among them were genes encoding transcription factors, serine carboxypeptidases and, interestingly, also yellow stripe like (YSL) proteins. The latter are involved in developmental processes and biotic stress responses (Lou and Baldwin 2006, Ham et al. 2012, Hofstetter et al. 2013). *Pseudomonas syringae*, for instance, has been shown to secrete virulence factors into the host cell using YSL metal ion transporters (Conte and Walker 2012, Hofstetter et al. 2013). Therefore, it may be possible that YSL transporters also play a role in AM colonization processes.

Among the 45 down-regulated genes upon mycorrhization were genes encoding proteins with functions in carbohydrate transport and metabolism, as well as in post-translational modification, protein turnover and as chaperones (Supplementary Table S7). Consistently, MAPMAN classification-based over-representation analysis found transcripts with functions in carbohydrate metabolism, glycolysis, and tricarboxylic acid (TCA) cycle/organic transformation over-represented upon mycorrhization under full nutrient conditions (**Fig. 4**). Down-regulation of these genes might indicate that under full nutrient conditions the plant reduces transfer of the valuable carbon as it has access to all essential nutrients by itself. It was already shown that carbon supply of the host plant triggers N transfer in mycorrhizal symbiosis and it was also shown that the fungus rewards the plant with the better carbon source. However, in both cases, it has been assumed that the fungus is the driving factor of the symbiotic nutrient exchange (Fellbaum et al. 2012, Fellbaum et al. 2014).

Two phosphate starvation-induced genes were also up-regulated. Under phosphate starvation, it was shown that the plant triggered expression of the microRNA species miR399 in the shoots (Bari et al. 2006, Chiou et al. 2006) which is then transported to the roots targeting PHO2 for degradation (Chiou and Lin 2011). PHO2 encodes an E2 ubiquitin-conjugating enzyme located in the endomembrane system targeting Pht1 members (phosphate–H^+ symporter) for degradation. (Bari et al. 2006, Lin et al. 2008). It was also shown that with these regulatory steps a class of non-coding RNA was induced, AT4, and induced phosphate starvation (Aung et al. 2006, Bari et al. 2006). Both mimic the target of miR399, scavenge it and lessen the phosphate starvation response (Franco-Zorrilla et al. 2007). Here, we observed that high-N conditions led to increased expression of two phosphate starvation-induced genes, suggesting that the N status of the plant determines the P demand. The fact that mycorrhization reduced expression

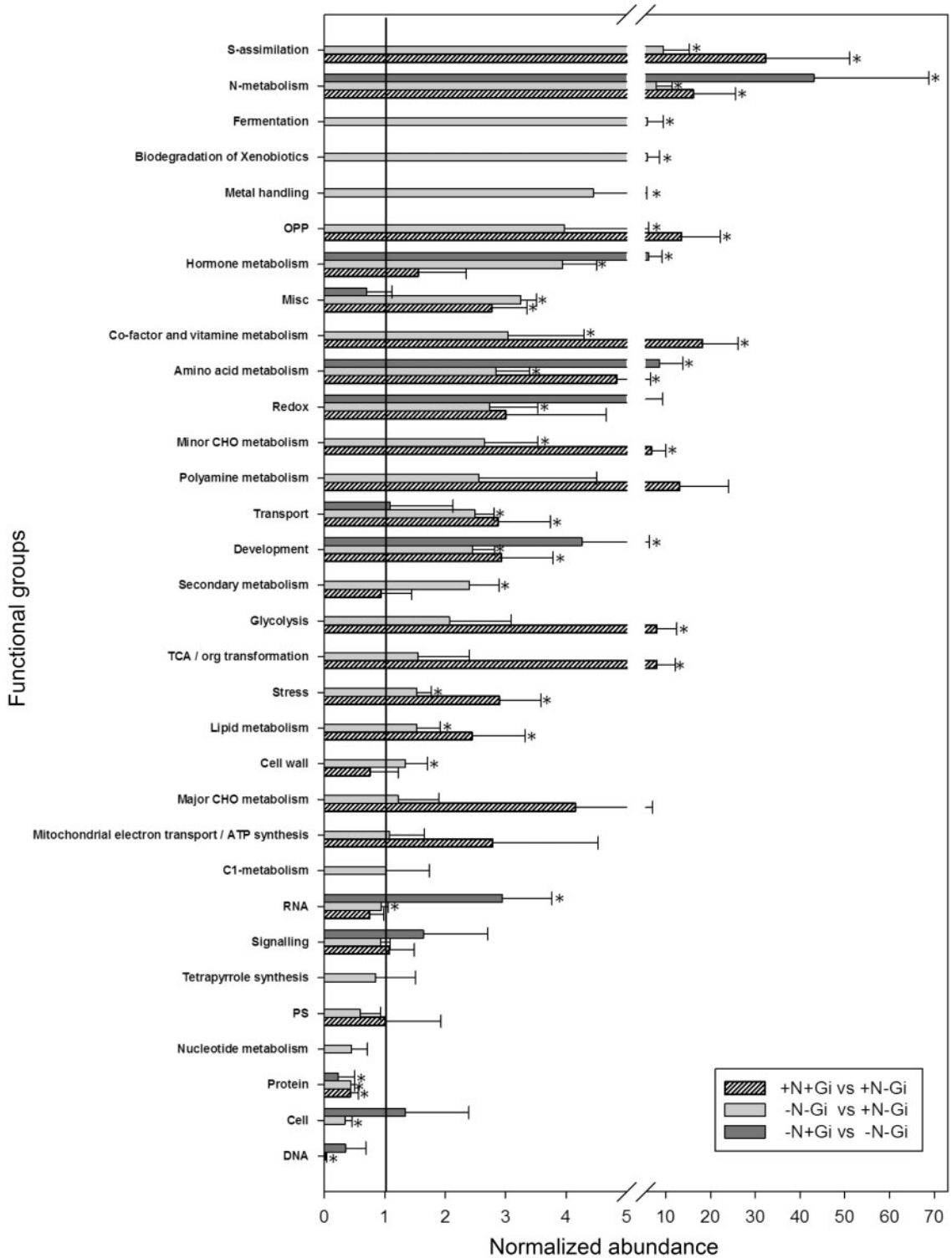

Fig. 4 MAPMAN-based functional classification of the poplar genes shown in Supplementary Tables S3, S5 and S6. Poplar genes were classified into their biological processes according to their Arabidopsis homolog using the Classification SuperViewer tool (Provart and Zhu 2003). Classification was based on MAPMAN classes (Ath_AGI_LOCUS_TAIR10_Aug2012). The relative abundance of genes was normalized to the frequency of class in the Arabidopsis reference data set. Mean and SD were calculated for 100 bootstrap repetitions. Asterisks indicate significantly under- or over-represented sequences ($P < 0.05$).

of the P starvation-induced genes suggested further that the fungus alleviates phosphate stress of the plant. Specific up-regulation of mycorrhiza-inducible phosphate transporters (see section below) corroborates our hypothesis.

Consistent with the induction of S assimilation-related genes (**Fig. 4**), we found genes involved in S metabolism down-regulated in AM plants (Supplementary Table S7). With respect to S metabolism, recent studies have shown

that AM fungi increased S supply and content of the AM plants (Casieri et al. 2012, Sieh et al. 2013, Giovannetti et al. 2014, Gerlach et al. 2015). It is thought that amino acids are transported from the AM fungus to the plant, and that methionine and cysteine may cover, in this way, the plant's demand for S (Whiteside et al. 2012).

The role of the regulation of defense genes in AM symbiosis still needs to be investigated. Comparative transcriptome analysis showed that AM fungi as well as pathogenic fungi induced expression of a common set of genes at early stages of interaction, showing that there are similarities in symbiotic and pathogenic infection pathways (Güimil et al. 2005). It has been shown that the mycorrhization level correlates with the expression of defense-related genes. Upon mycorrhization, defense-related genes were shown either to be suppressed or to become induced in early stages of AM colonization (Liu et al. 2003). High amounts of jasmonic acid as well as up-regulation of the pathogenesis-related (PR) family and other defense-related genes led to decreased mycorrhization rates (Ruiz-Lozano et al. 1999, Jung et al. 2012, Gutjahr et al. 2015). On the other hand, it was postulated that the AM fungus is able to bypass the immune response of the plant by suppressing or counteracting actively the immune response of the plant (Bennett et al. 2009, Campos-Soriano et al. 2010, Kloppholz et al. 2011). In agreement with Güimil et al. (2005), we observed a down-regulation of the PR gene expression which might underpin the hypothesis that PR genes are directly involved in regulation of the mycorrhization rate.

Effect of mycorrhization and N availability on poplar N metabolism. As we were interested in N metabolism, we screened for differentially expressed genes in mycorrhizal and non-mycorrhizal conditions. We found differentially expressed genes in all three comparisons (i.e. −N +Gi vs. −N −Gi, −N −Gi vs. +N −Gi and +N +Gi vs. +N −Gi; **Fig. 4**).

In non-mycorrhizal conditions, 24 genes were differentially expressed upon N deprivation (Supplementary Table S8). Of those, 14 genes exhibited reduced expression. Most of these genes encoded GSs, GOGATs and asparagine synthetases, as well as one predicted AMT. On the other hand, the genes encoding four AMTs and two possible urease accessory proteins, which are necessary for activation of ureases (Witte et al. 2005), were also induced.

The comparison between mycorrhizal and non-mycorrhizal plants in low-N conditions revealed expression changes in only four genes (Supplementary Table S8), indicating that mycorrhization plays no role in the regulation of the expression of N metabolism-related genes.

Effect of mycorrhization and N availability on poplar ammonium and phosphate transporters. In addition to the 14 described AMTs in *P. trichocarpa* (Couturier et al. (2007), we identified six more AMTs. By performing phylogenetic analysis, we could assign one gene to the AMT1 transporter family, three to the AMT3 family and two more genes clustered to the AMT4 transporters (Supplementary Fig. S2). Transcripts of PtrAMT1-2, PtrAMT1-3 and PtrAMT1-4 were induced upon mycorrhization by the ectomycorrhizal fungi *Paxillus involutus* and *Amanita muscaria* (Selle et al. 2005, Couturier et al. 2007). However, here, PtrAMT1-2 was one of the most expressed AMTs under all experimental conditions, but not significantly regulated in mycorrhizal conditions (**Table 3**). The other two transporters were constitutively, although barely, expressed. Interestingly, the five members of the AMT4 family (PtrAMT4-1 to PtrAMT4-5) were specifically induced upon mycorrhization, independently of the N supply. Consistent with our observation, an induction of AMT4 members has been observed in several plant species (Guether et al. 2009, Kobae et al. 2010, Ruzicka et al. 2012, Koegel et al. 2013), making these genes good general markers for mycorrhiza, whatever the plant species. Specific induction of AMT4 transporters suggested that they may be specifically located at the plant–fungal interface, i.e. the periarbuscular membrane, and are probably important for a functional symbiosis. Consistent with our hypothesis, it has been shown that an *M. truncatula* phosphate transporter (MtPT4) and an AMT (MtAMT2;3) were specifically localized at the periarbusuclar membrane. Silencing of MtPT4 led to premature arbuscule degeneration and therefore to an insufficient symbiosis (Javot et al. 2007a). Moreover, Breuillin-Sessoms et al. (2015) have shown that MtPT4 and MtAMT2;3 influence arbuscule life span and that their relative importance depends on the N status. However, to confirm the specific localization of the populus AMT4 transporters at the periarbuscular membrane, further expreriments need to be conducted.

Further, we found five more AMTs differentially expressed (**Table 3**). AMT1-5 and AMT3-1 were significantly induced in low-N conditions. Moreover, the newly identified Potri.013G049600.1 was induced in the non-mycorrhizal high-N condition and Potri.013G040400.1 showed a similar expression pattern to AMT4, members with induced expression in the non-mycorrhizal low-N condition.

As AM fungi are known to be important for N and P transfer to the host plant, we also investigated the expression levels of plant phosphate transporters upon N starvation and mycorrhization. A comprehensive study by Loth-Pereda et al. (2011) already investigated the expression of 12 Pht1 phosphate transporters in poplar species. Three Pht1 transporter genes (*PtrPht1-1*, *PtrPht1-6* and *PtrPht1-11*) were down-regulated during AM symbiosis. However, *PtrPht1-10* was specifically induced in AM roots, while *PtrPht1-8*, a close homolog of *PtrPht1-10*, was not expressed at all. The authors proposed that phosphate transporters of poplar have distinct roles in the acquisition and translocation of their substrate. Specific induction of transporters, especially induction of mycorrhiza-specific transporters, enables them to extract P from deprived soils. In our experimental conditions, all transporters were expressed. Specific induction of *Pht1-8* is in line with the hypothesis of Loth-Pereda et al. (2011) that phosphate transporters of clade I are mycorrhiza inducible (**Table 4**). Specific induction of mycorrhiza-inducible phosphate transporters has also been demonstrated in other plant species (Rausch and Bucher 2002, Glassop et al. 2005, Nagy et al. 2005, Loth-Pereda et al.

Table 3 Ammonium transporters (AMT) of *P. trichocarpa*

Name	Transcript ID	Log2 ratio					Mean RPKM			
		−N +Ri vs. +N +Ri	−N +Ri vs. −N −Ri	+N +Ri vs. +N −Ri	−N −Ri vs. +N −Ri	Myc vs. NM	−N +Ri	+N +Ri	−N −Ri	+N −Ri
PtrAMT1-1	Potri.010G063500.1	0.2	−0.4	0.3	0.9	−0.2	26.72	23.67	36.36	19.25
PtrAMT1-2	Potri.019G023600.1	0.2	−0.8	−1.6	−0.6	−1.5	110.36	93.25	189.95	289.46
PtrAMT1-3	Potri.008G173800.1	1.0	0.5	0.4	0.9	0.3	0.23	0.11	0.16	0.09
PtrAMT1-4	Potri.002G255100.1	0.3	2.8	0.7	−1.9	1.2	0.03	0.02	0.00	0.01
PtrAMT1-5	Potri.002G255000.1	−0.4	−0.6	**3.4**	**3.6**	0.2	0.13	0.17	0.20	0.02
PtrAMT1-6	Potri.009G045200.1	0.1	0.0	−1.8	−1.7	−1.2	1.20	1.14	1.21	3.99
PtrAMT2-1	Potri.006G102800.1	−0.4	0.4	0.8	0.1	0.9	26.48	34.51	19.84	19.15
PtrAMT2-2	Potri.016G121400.1	1.2	−0.5	0.1	1.8	−0.4	10.81	4.82	15.36	4.39
PtrAMT3-1	Potri.001G305400.1	2.1	−0.3	**2.6**	**5.0**	−0.1	76.61	18.14	93.03	2.98
PtrAMT4-1	Potri.002G047000.1	−0.4	**4.1**	**4.5**	0.1	**4.1**	16.91	21.77	0.98	0.93
PtrAMT4-2	Potri.018G033500.1	−0.4	**10.2**	**9.9**	−0.6	**9.8**	34.26	45.97	0.03	0.05
PtrAMT4-3	Potri.005G216000.1	−0.6	**7.5**	**6.8**	−1.2	**6.8**	51.57	76.98	0.29	0.67
PtrAMT4-4	Potri.T103600.1	−0.8	**5.2**	**7.1**	1.1	**5.8**	1.11	1.91	0.03	0.01
PtrAMT4-5	Potri.005G106000.1	0.6	**6.8**	**8.2**	2.0	**7.0**	9.46	6.37	0.08	0.02
	Potri.013G049600.1	−2.7	−1.0	−1.6	**−3.3**	−2.0	0.76	4.97	1.55	15.06
	Potri.019G000800.1	−1.6	−0.3	2.2	0.9	1.1	1.73	5.14	2.16	1.13
	Potri.T000600.1	−2.6	−0.5	2.2	0.1	1.5	3.03	17.91	4.36	3.94
	Potri.T000200.1	1.7	−0.6	1.7	**4.0**	−0.4	6.73	2.11	10.04	0.65
	Potri.013G040400.1	−0.4	**3.9**	**7.5**	3.1	**4.7**	0.94	1.27	0.06	0.01
	Potri.006G247800.1	−0.5	0.4	2.9	1.9	1.1	0.05	0.07	0.04	0.01

Significant values are highlighted in bold (FDR corrected *P*-value <0.05).

Table 4 Phosphate transporters of the Pht1 family of *P. trichocarpa*

Name	Transcript ID	Log2 ratios					Mean RPKM			
		−N +Ri vs. +N +Ri	−N +Ri vs. −N −Ri	+N +RI vs. +N −Ri	−N −Ri vs. +N −Ri	Myc vs. NM	−N +Ri	+N +Ri	−N −Ri	+N −Ri
PtrPht1.1 c	Potri.010G072000.1	−0.6	0.2	−1.5	−2.3	−1.3	2.06	3.19	1.77	8.79
PtrPht1.2 c	Potri.010G071700.1	−1.2	−0.1	−0.3	−1.5	−0.4	19.40	46.92	20.59	56.69
PtrPht1.3 c	Potri.010G071500.1	0.2	−0.3	1.8	2.3	0.5	1.58	1.32	1.88	0.38
PtrPht1.4 c	Potri.005G223500.1	0.9	−0.1	−0.7	0.3	−0.5	67.50	36.89	73.30	61.34
PtrPht1.5 c	Potri.002G038900.1	−1.8	−0.3	−2.9	**−4.5**	−3.1	0.14	0.49	0.18	3.93
PtrPht1.6 c	Potri.005G175500.1	1.3	0.6	1.4	2.0	0.7	0.03	0.01	0.02	0.00
PtrPht1.7 c	Potri.005G223600.1	−0.1	−0.2	−1.7	−1.7	−1.2	2.51	2.77	2.79	9.18
PtrPht1.8 a	Potri.019G061900.1	**−2.9**	**7.4**	**10.8**	0.5	**9.3**	15.56	121.06	0.09	0.06
PtrPht1.9 c	Potri.002G005500.1	0.5	−0.1	−0.3	0.3	−0.3	3.11	2.18	3.28	2.74
PtrPht1.10 a	Potri.015G022800.1	**−5.2**	2.8	**9.9**	1.9	**7.1**	0.05	1.81	0.01	0.00
PtrPht1.11 b	Potri.005G256100.1	2.5	0.4	−1.3	0.8	−0.1	21.60	3.84	16.91	9.43
PtrPht1.12 c	Potri.001G318500.1	−2.2	−3.4	1.1	2.3	−1.3	0.00	0.01	0.02	0.00

Significant values are highlighted in bold (FDR corrected *P*-value < 0.05).
Pht transporters belongs to different subfamilies of H^+–Pi symporters: (a) cluster of the AM-inducible Pi; (b) some proteins from both monocots and dicots fall into a highly divergent subfamily; (c) cluster of dicots Pi, according to Walder et al. (2015).

2011, Walder et al. 2015). As shown by Harrison et al. (2002), the mycorrhiza-inducible MtPT4 is located at the periarbuscular membrane and is essential for the establishment of a functional symbiosis. We hypothesize that this might also be true for Pht1-8.

Comparing high- and low-N conditions, we found the transporters Pht1-1, Pht1-2 and Pht1-1 induced in the high-N conditions. In contrast, expression of Pht1-11 was increased in the low-N condition in mycorrhizal poplar roots, whereas *PtrPht1-8* and *PtrPht1-10* were down-regulated. Taken together, these

data indicate that the expression of phosphate transporters depends on the N status of the plant. High-N conditions may allow stronger expression of phosphate transporters as high-N conditions may signal a good nutritional status of the plant. Induction of the expression of phosphate transporters in low-N conditions may be explained when we assume that these transporters are involved in intercellular phosphate transport as nutrient deficiency generally leads to a shift in metabolic processes (Voigt et al. 2007, Krapp et al. 2011, Garapati et al. 2015, Jost et al. 2015).

Conclusion

Here, we demonstrate that N availability has significant effects on plant and mycorrhizal gene expression. In mycorrhizal roots, N starvation caused major changes in the expression of AM fungal genes belonging to the functional categories of cell growth, membrane biogenesis and cell structural components. Moreover, the newly characterized mycorrhizal AMT and one of the newly identified mycorrhizal phosphate transporters were significantly induced upon N limitation. We hypothesize that these two fungal transporters are key features of AM nutrient transfer and that in the low-N condition, more N but also more phosphate is transferred to the plant symbiont.

As regards the plant, we found that N deficiency had significant effects on metabolic processes. Gene expression of key enzymes of the GS/GOGAT pathway were down-regulated upon N limitation as well as genes involved in N translocation and transport such as AMTs, urease and arginase, suggesting that N metabolism is tightly regulated. We identified six new *Populus* AMTs. Among the ammonium and phosphate transporters, we identified three AMTs and one phosphate transporter, the genes of which were mycorrhiza inducible and could therefore be used as molecular markers for functional mycorrhiza. It is tempting to speculate that these mycorrhiza-inducible transporters are localized at the periarbuscular membrane as demonstrated for MtPT4 (Harrison et al. 2002), which would make them key elements of symbiotic nutrient exchange and essential components of a functional symbiosis. Specific induction of plant ammonium and phosphate transporters has already been shown in previous studies (Harrison et al. 2002, Paszkowski et al. 2002, Nagy et al. 2005, Javot et al. 2007a, Koegel et al. 2013). In the future, it will be interesting to elucidate the contribution of the transporters to nutrient transfer and nutritional status of the plant and especially if the expression of phosphate transporters depends on the N status of the plant.

Materials and Methods

Growth conditions

Populus trichocarpa (derived from cuttings, clone 10174, Orléans, France) grew in an autoclaved (120°C, 20 min) quartz sand (Alsace, Kaltenhouse, Trafor AG) : zeolithe (Symbion) substrate (1 : 1, w/w). Plants grown under high-N conditions were fertilized once a week with 10 ml of Hoagland standard solution, modified after Gamborg and Wetter (1975). Plants grown under low-N conditions received a solution in which the $Ca(NO_3)_2 \cdot 4H_2O$, KNO_3, $NH_4H_2PO_4$ and $(NH_4)_2MoO_4$ from the original solution were replaced by $CaCl_2 \cdot 2H_2O$, KCl, KH_2PO_4 and Na_2MoO_4 (Supplementary Table S9). To analyze the fungal effect on plant nutrition under N stress, plants grown simultaneously in the same conditions were inoculated with 1 ml of liquid inocula of *R. irregularis* (formerly *Glomus intraradices*, abbreviated as *Gi*), strain BEG75 (Inoculum Plus), dissolved in 0.01 M citrate buffer (pH 6), containing approximately 110 spores (+N +Gi, −N +Gi). Plants grew for 12 weeks in a greenhouse at about 28/15°C day/night temperature.

Harvest and colonization measurements

Root systems were freed from substrate and washed thoroughly under the tap. Primary roots and thick roots were removed. Subsamples of the fine roots were randomly taken for colonization measurements. For RNA extraction, subsamples were snap-frozen in liquid nitrogen and stored at −80°C.

For colonization measurements, fresh root samples were immersed in 10% KOH and stored at 4°C for 18 h. Roots were rinsed and kept for 1 h at room temperature in 2% HCl. After cleaning the root with tap water, they were stained in 0.005% trypan blue (w/v in lactic acid : glycerol : water, 1 : 1 : 1, by vol.) at 4°C overnight. The next day the roots were rinsed with water and destained in lactic acid : glycerol : water (1 : 1 : 1, by vol.) for several days. Colonization was estimated by the grid line intersection method (Brundrett et al. 1984). Statistics were done using a one-way analysis of variance (ANOVA) in the program SPSS Statistics, version 22 (IBM).

RNA isolation

RNA was extracted from three biological replicates per condition. Lyophilized samples were processed using the Qiagen RNeasy kit according to the manufacturer's protocol. DNA was removed using the DNA-free™ Kit, and DNase Treatment and Removal Reagents (AMBION® by Life Technologies). The quantity and quality of the extracted RNAs were verified using a bioanalyzer with RNA picochips (Agilent).

Data analysis and bioinformatics

Preparation of 12 libraries and 2 × 100 bp Illumina HiSeq mRNA sequencing (RNA-Seq) was performed by Beckman Coulter Genomics. Raw reads were trimmed for quality and aligned either to the *R. irregularis* reference transcripts available at the JGI database (http://genome.jgi-psf.org/Gloin1/Gloin1.home.html) or to the *P. trichocarpa* v3 reference transcripts (primary transcripts) from Phytozome 10.3 (http://phytozome.jgi.doe.gov/pz/portal.html) using CLC Genomics Workbench v7 (Supplementary Table S10). For mapping, the minimum length fraction was 0.9, the minimum similarity fraction 0.8 and the maximum number of hits for a read was set to 10. The unique and total mapped reads number for each transcript was determined, and then normalized to RPKM (reads per kilobase of exon model per million mapped reads). Intact pairs were counted as two; broken pairs as one. To identify differentially regulated *Rhizophagus* transcripts, a pairwise

t-test comparison, the Baggerley test (Baggerly et al. 2003), implemented in CLC Genomic workbench was applied to the data. The Baggerley test compares the proportions of counts in a group of samples against those of another group of samples. Samples are given different weights depending on the total amount of counts in each sample. The weights are obtained by assuming a Beta distribution on the proportions in a group, and estimating these, along with the proportion of a binomial distribution, by the method of moments. The result is a weighted *t*-type test statistic. In addition, Benjamini and Hochberg multiple-hypothesis testing corrections with false discovery rate (FDR) were used. To identify differentially regulated *Populus* transcripts, the LIMMA package (Smyth 2005) in R was used as described by Wilkins et al. (2009). Nitrogen effect and the effect of *Rhizophagus* inoculation were analyzed. One sample (AMCT1 meaning +N +Gi) was not used in the current analysis since it was very different from the two other biological replicates and therefore was considered as an outlier (Supplementary Fig. S3). For our analysis, transcripts with an FDR-corrected *P*-value < 0.05 and a >5-fold change were considered as differently regulated. KOG groups and classes were used for functional classification in combination with a Fisher test (in R) for enrichment. A Venn diagram was generated using http://bioinformatics.psb.ugent.be/webtools/Venn/.

Functional classification of differentially expressed genes in poplar was performed in the Classification SuperViewer tool (Provart and Zhu 2003) (http://bar.utoronto.ca/) using the best corresponding Arabidopsis TAIR10 hit name.

cDNA synthesis and qPCR

cDNA synthesis was performed on RNA extracts from the same three biological replicates per condition as used for HiSeq analysis using the iScript™ cDNA SynthesisKit (Bio-Rad). Gene-specific primers (Supplementary Table S11) for qPCR were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and tested with amplify 3.1 (http://engels.genetics.wisc.edu/amplify). For normalization, *P. trichocarpa* ubiquitin was used as a reference gene. Three biological and two technical replicates were analyzed for each gene. PCR was conducted with the following settings: initial denaturation at 95°C for 3 min, and 45 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 30 s. No DNA contamination was detected: we carried out either a PCR using an RNA sample as template or a no reverse transcription control reaction (in which no reverse transcriptase was added). The gene that we controlled was the AM-inducible Pht1 transporter (PtPht11: Potri.015G022800).

Phylogenetic analysis

The Neighbor–Joining tree was made using amino acid sequences of AMTs of *P. trichocarpa* of >400 amino acids in length. Sequences were aligned using ClustalW of the MEGA6.06 package (Tamura et al. 2013) with the following multiple alignment parameters: gap opening penalty 15, gap extension penalty 0.3, Gonnet protein weight matrix and a delay divergent cut-off value of 30%. The phylogenetic tree was computed using the Neighbor–Joining method, using the Poisson correction model with pairwise deletion option. Bootstrapping was performed with 1,000 replicates.

N analysis

The remainder of the shoot material was dried at 80°C for 72 h and weighed. These samples were ground in 1.5 ml Eppendorf tubes using 1.1 mm diameter tungsten carbide balls (Biospec Products, Inc.) in a Retch MM301 vortexer (Retch Gmbh and Co.). Total N was measured using an online continuous flow CN analyzer coupled with an isotope ratio mass spectrometer (ANCA-SL MS 20-20 system, Sercon Ltd.).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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