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Expression of genes involved in symbiotic carbon and nitrogen transport in *Pinus taeda* mycorrhizal roots exposed to CO_2 enrichment and nitrogen fertilization

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Abstract As atmospheric carbon dioxide (CO_2) concentrations rise, one important mechanism by which plants can gain greater access to necessary soil nutrients is through greater investment in their mycorrhizal symbionts. In this study, we tested the hypotheses that (1) plants increase C allocation to ectomycorrhizal fungi (EMF) under elevated CO_2 conditions, (2) N fertilization decreases C allocation to EMF, and (3) EMF activity at the site of symbiotic C and nutrient exchange is enhanced with CO_2 enrichment. To test these hypotheses, we examined expression levels of *Pinus taeda* genes encoding monosaccharide transport (MST) and ammonium transport (AMT) proteins thought to be involved in symbiotic C and N movement, respectively, from mycorrhizal root tips exposed to CO_2 and N fertilization. We also examined EMF ribosomal RNA expression (18S rRNA) to determine EMF activity. There was a trend toward lower relative MST expression with increased CO_2. AMT expression levels showed no significant differences between control and treatment plots. EMF 18S rRNA expression was increased in CO_2-enriched plots and there was a marginally significant positive interactive effect of CO_2 and N fertilization on expression (p=0.09 and 0.10, respectively). These results are consistent with greater C allocation to EMF and greater EMF metabolic activity under elevated CO_2 conditions, although selective allocation of C to particular EMF species and greater fungal biomass on roots are plausible alternative hypotheses.

Keywords Ectomycorrhizal fungi · Belowground carbon allocation · Nutrient transport · Monosaccharide transporter · Ammonium transporter · Symbiosis · Gene expression · Nutrient limitation

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Introduction

Increased atmospheric carbon dioxide (CO_2) concentrations can lead to short-term increases in forest productivity (Moore et al. 2006). The degree to which forests can maintain increased net primary productivity depends upon the ability of plants to acquire sufficient amounts of limiting nutrients (Finzi et al. 2007; Norby et al. 1999; Zak et al. 2003). One way in which forest tree species may increase nutrient acquisition from soil is by increasing their dependence on mycorrhizal symbionts to extract nutrients from the soil (Finzi et al. 2007). The ectomycorrhizal symbiosis is the most common type of nutritional mutualism between temperate coniferous forest tree species and fungi (Smith and Read 1997). The site of carbon and nutrient exchange between partners occurs at the interfacial apoplast, the space between opposing fungal and plant plasma membranes in the intercellular spaces of colonized fine roots. There, cell wall and membrane bound transporters shuttle sugars and nutrients between partners (Chalot et al. 2002).

Although the molecular physiology of carbon (C) and nutrient transport in the ectomycorrhizal symbiosis is not entirely understood, a great deal of progress has been made in increasing our understanding of the genes involved in the transport of nutrients, particularly C and nitrogen (N) and

of the form of nutrients transported between partners (Chalot et al. 2002). In order for fungi to take up C in the apoplast, sucrose, the primary C transport molecule in plant tissues, must first be cleaved by plant invertases into its constituent glucose and fructose monosaccharide molecules (Smith and Read 1997). Ectomycorrhizal fungi (EMF) appear to be largely incapable of acquiring sucrose directly from their plant hosts because they appear to lack invertase genes that confer sucrolytic capabilities (Deveau et al. 2008; Salzer and Hager 1991). Once plants hydrolyze sucrose into glucose and fructose, these monosaccharides can then be taken up by fungi via monosaccharide transporters (MSTs; Nehls et al. 1998). However, plants also utilize MSTs for retranslocating monosaccharides from the apoplast back into plant root cells and plant and fungal MSTs may compete for monosaccharides in the apoplastic space where these symbionts interact (Nehls et al. 2001). Work by Grunze and colleagues (2004) has shown that particular plant MSTs are upregulated in ectomycorrhizal symbiosis, but these MSTs decrease activity in symbiotic tissues when starved for nutrients, suggesting that this may be a plausible mechanism by which plants may regulate the amount of C allocated to their mycorrhizal symbionts.

Although there is no definitive evidence elucidating what the dominant molecule is for transport of N from EMF to plants, the long-standing view has been that it is some form of amino acid. However, this assumption has never been experimentally validated, and recent studies suggest that other N compounds could also be involved in symbiotic exchange (Chalot et al. 2006). The examination of N transport in the arbuscular mycorrhizal fungal symbiosis between carrot plants and *Glomus intraradices* showed that in that system N is first converted from arginine to ammonium before being transferred to the plant (Govindarajulu et al. 2005). In an ectomycorrhizal system, expression analysis of a high affinity ammonium transporter (AMT) in *Populus tremula* × *Populus tremuloides* colonized by the EMF species *Amanita muscaria* under varying N conditions was consistent with the pattern expected if ammonium is the form of symbiotically derived N taken up from the apoplast by plants (Selle et al. 2005).

As plants gain greater access to C through increasing atmospheric CO_2 concentrations, C fixation can become limited because of inadequate supply of other limiting nutrients, particularly N (Oren et al. 2001). One possible physiological response by plants to increased C supply and N demand that can be important for ectomycorrhizal fungal communities is to increase fine root production and biomass, and a number of studies have demonstrated such a response (Matamala and Schlesinger 2000; Norby et al. 2004). A second physiological strategy that plants may employ to increase nutrient acquisition when exposed to elevated CO_2 concentrations is to increase C flow to their fungal symbionts on a per root tip basis. An even more sophisticated strategy would be for plants to selectively increase C flow to those mycorrhizal roots that offer the greatest nutrient gain.

One way to test the hypotheses that C flow to EMF is greater in elevated CO_2 conditions and that the amount of C flow is positively correlated with N provided to plants is to examine the regulation of the genes responsible for transporting C and N from the apoplast to the plant cell (Wright et al. 2005). If plant uptake of glucose and fructose from the apoplast via MSTs functions as a mechanism by which plants regulate C flow to EMF, as has been suggested (Grunze et al. 2004), then we would expect plant MST expression levels to be reduced in elevated CO_2 relative to control conditions, thereby allowing greater C to pass to their EMF symbionts. Furthermore, if C allocation to EMF and N allocation to plants are positively correlated and if ammonium is one form by which N is transferred from EMF to plants, we would expect to find a negative relationship between MST and AMT expression levels in plants.

The goal of this study was to test the following hypotheses: (1) Plant MST expression levels decline in plants exposed to a CO_2-enriched atmosphere, (2) plant MST and AMT gene expression levels are negatively correlated, and (3) greater C allocation to EMF in plots subjected to elevated atmospheric CO_2 results in greater activity of EMF colonizing fine roots. To test these hypotheses, we examined the relative expression levels of several plant MST and AMT genes as a proxy for plant C allocation to EMF and fungal N transfer to plants, respectively. We also measured fungal small subunit ribosomal RNA (18S rRNA) expression levels to assess EMF activity in live, fine roots of *Pinus taeda*. Samples were collected in 2005 from the FACTS-I research site in Duke Forest, NC, USA, where three experimental plots have been exposed to a CO_2-enriched atmosphere (200 ppm above ambient) since 1996, and three reference plots have been maintained under ambient conditions. Previous nutrient addition experiments at this site has demonstrated the N-limited nature of this system (Oren et al. 2001), and trees in this experiment exposed to elevated CO_2 have increased N uptake (Finzi et al. 2007). Using a real-time polymerase chain reaction (PCR) approach, we compared the relative expression levels of plant MST and AMT genes and fungal 18S genes in ambient and elevated CO_2 plots. We also examined the effects of N fertilization, both alone and in conjunction with CO_2 enrichment, on the regulation of these genes. To our knowledge, this is the first study that has used a molecular physiological approach to

examine the effects of elevated CO_2 on C and N flow between plants and their fungal symbionts in a field setting.

Materials and methods

P. taeda EST database search and library analysis

To retrieve all putative MST and AMT genes present in the *P. taeda* genome, *P. tremula* × *P. tremuloides* MST and AMT gene sequences were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/) and queried against the DFCI *Pinus* Gene Index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=pine) and The Institute for Genomic Research (TIGR) Plant Transcript Assemblies (http://blast.jcvi.org/euk-blast/plantta_blast.cgi) using BLASTn to identify homologues. The number of expressed sequence tags (ESTs) recovered for each gene was also tallied along with plant tissue type and growth conditions from which the libraries were generated to quantify the relative abundance of MST and AMT gene transcripts in different *P. taeda* plant tissues.

P. taeda MST and AMT phylogenetic analysis

To determine the number of MST and AMT genes present in *P. taeda* and to determine their relationship to genes in these families whose functions have been characterized in other plant species, gene phylogenies were constructed for both MST and AMT gene families. Sequences from *Arabidopsis thaliana*, *Brassica nigra*, *Lotus japonicus*, *Lycopersicon esculentum*, *P. taeda*, *Picea abies*, *P. tremula* × *P. tremuloides*, *Populus trichocarpa*, and the alga *Parachlorella kessleri* were included in AMT and MST phylogenetic analyses (24 and 33 sequences, respectively). Amino acid sequences were aligned using MUSCLE (Edgar 2004) and alignments were analyzed using ProtTest version 1.3 software (Abascal et al. 2005) to determine the best-fitting amino acid model of evolution. Phylogenies and node support were assessed using maximum likelihood bootstrap (1,000 replicates) as implemented in PHYML (Guindon and Gascuel 2003), using the Whelan and Goldman amino acid model, gamma distributed substitution rates across variable sites and an estimated proportion of invariant sites.

Field site

Samples were collected from the FACTS I research site in Duke Forest, Orange County, NC, USA. These plots are located in an even-aged *P. taeda* stand that was planted in 1983. The soil at this site is an Ultic Alfisol in the Enon Series, which is somewhat acidic, N- and P-limited, and rich in clay (Schlesinger and Lichter 2001). CO_2 enrichment began in August 1996. Since that time, three circular 30-m-diameter experimental plots have been continuously fumigated to achieve CO_2 concentrations 200 ppm above ambient (i.e., 565 ppm, or 1.5× current levels). Three control plots have been maintained at ambient CO_2 concentrations. Each plot is further subdivided into four quadrats, with each quadrat further subdivided into one vegetation sector and one soil sector. Soil sectors are used for belowground sampling. Beginning in 2005, a nitrogen fertilization regime was implemented in one half (i.e., two soil sectors) of every plot. In March and April, 2005, NH_4–NO_3 pellets were broadcast by hand to achieve a total annual fertilization rate of 11.2 g N m^{-2} $year^{-1}$. To prevent migration of fertilizer into unfertilized control portions of the plots, impermeable sheets were inserted to a depth of 70 cm along the edges dividing the circular plot into four quadrats. Additional details regarding Free Air CO_2 Enrichment technology and site characteristics can be found in Hendrey et al. (1999) and Schlesinger and Lichter (2001).

Field sampling

Live fine roots were collected 16 October 2005. Three to five sampling locations were randomly selected in each soil sector, with the criterion that they had to be within 2 m of an adult *P. taeda* individual. Fine roots were manually excavated from the top 5 cm of soil, dipped in diethylpyrocarbonate (DEPC)-treated water to remove excess soil and debris, and placed in 50 ml falcon tubes filled with the RNA preservation buffer RNA*later* (Applied Biosystems, Foster City, CA, USA). Samples were then stored at 4°C for up to 3 days in RNA*later* pending processing. Roots were visually examined with a stereomicroscope and live root tips colonized with EMF were harvested to achieve 30–50 mg fresh weight of mycorrhizal material for each soil sector. Harvested root tips were transferred to microcentrifuge tubes also containing RNA storage buffer pending RNA extraction. This resulted in a total of 24 samples (2 CO_2 levels × 3 plots/level × 2 nitrogen fertilization levels per plot × 2 soil sectors per fertilization level) used in this study.

Molecular methods

RNA extraction and cDNA synthesis RNA was extracted according to the protocol of Chang et al. (1993) adjusted for use with smaller amounts of material and with two modifications: Fresh material was ground directly in 500 µl warmed extraction buffer using a micropestle and pellets

were washed in 1 ml 80% *v/v* ethanol and resuspended in 25 μl of DEPC-treated water.

RNA extracts were treated with RQ1 RNase-free DNase according to manufacturer's specifications (Promega Madison, WI, USA). Two separate reverse transcription (RT) reactions were then performed. One reaction synthesized cDNA from mRNA using a dT (14-mer) reverse primer, and the second reaction used to examine plant and fungal 18S expression generated cDNA from rRNA using the universal 18S rRNA reverse primer SR6.1 (5′-TGTTACGACTTTTASTTCCTCT-3′). cDNA synthesis used 500 and 50 ng of DNase digested RNA for oligo dT and 18S rRNA reactions, respectively, using the Omniscript RT kit according to manufacturer's protocol (Qiagen Valencia, CA, USA).

Primer design *P. taeda*-specific primers were designed using Primer 3 software (Rozen and Skaletsky 2000), with target products of 90–150 bp in length chosen to amplify regions from the 3′ end of the transcripts of interest and melting temperatures of approximately 60°C (Table 1). Primers were tested with both DNA and cDNA from *P. taeda* shoots and mycorrhizae. Amplified fragments were visualized by gel electrophoresis and sequenced using standard protocols. Sequencing results verified that all primers were shown to only amplify the genes of interest. Genes with detectable transcript levels in mycorrhizal roots were chosen for real-time PCR experiments.

Real-time PCR The expression levels for seven *P. taeda* genes (five experimental genes: MST1.1, MST3.1, MST6.2, AMT1, AMT2; two control genes: actin, 18S rRNA) and one fungal gene (18S rRNA) were examined for all 24 samples. For each of the 24 samples, three replicates of each experimental and control gene were amplified on the same plate in addition to one no-template negative control. RT-free controls were also performed using DNase digested extracts not subjected to cDNA synthesis to be certain that the samples were not contaminated with residual DNA. PCR reactions consisted of 15 μl of 2× Sybr PCR master mix (Applied Biosystems), 0.6 μl of 10 μM forward and reverse primers, and 10 μl diluted cDNA template, and the mixture was brought to a final volume of 30 μl with ddH2O. Real-time PCR reactions were carried out on an ABI 7000 thermal cycler (Applied Biosystems) according to the following conditions: an initial cycle at 95°C for 10 min, after which 45 additional cycles were performed at 95°C denaturation for 30 s, 58°C annealing for 30 s, and 72°C extension for 1 min. Dissociation curves were generated at the end of each run, which began at 60°C initial temperature and climbed to 90°C. Because the actin variant (TA1974_3352) expression levels were lower than that of most of the experimental genes, it was deemed unsuitable to use as a reference gene. Consequently, it was excluded from expression analyses and only *P. taeda* 18S rRNA was used as a control.

Table 1 Table of primers used in real-time PCR reactions for the genes analyzed in this study

Gene region	Primer name	Sequence 5′→3′	Length	T_m (°C)	Predicted product size (bp)	Primer reference
Fungal 18S rDNA	NS7.1	CCCTGCCCTTTGTACACA	18	62.2	150	
	EF3	TCCTCTAAATGACCAAGTTTG	21	58.3		Smit et al. (1999)
Pine 18S rDNA	Pine18SF	AATGATCCGGTGAAGTGTT	19	59.4	97	
	Pine18SR	TCCTTCCTCTAAATGATAAGGTTCA	25	58.1		
Pine actin	PtAct-3F	AGTTGTAGCACCCCCTGAGA	20	62.5	130	
	PtAct-3R	GTGGACAATTGAGGGACCTG	20	62.5		
Pine MST1.1	PtMST1-3F	TGGAAGAAACATTGGCTGTG	20	64	124	
	PtMST1-3R	GGGAATTATCGTAGGCTTTGG	21	63		
Pine MST3.1	PtMST3.1-3F	CTGTTTTATGCCGCCTGGA	19	66	170	
	PtMST3.1-R	GAGGATTTGACAGGCTCACC	20	64		
Pine MST6.2	PtMST6.2-3F	CGGGCTGTTTCTATTCTTCG	20	64	121	
	PtMST6.2-3R	CCAGTGTTCCTTCCACACAA	20	64		
Pine AMT1	Pine AMT1F	AGGGTATCTGGGGATGATGA	20	63	132	
	Pine AMT1R	AGAAGCCTTCAGCATGAACC	20	63		
Pine AMT2	Pine AMT2F	CAAACTTCTCCGGGTTTCTG	20	63	141	
	Pine AMT2R	TTTCGAGAGGCCTTCATCAT	20	64		

T_m melting temperature of primer in degrees Celsius, *Length* nucleotide length of primer sequence

Real-time PCR data analysis

Cycle threshold (Ct) values were transformed to relativized concentrations using the following equation:

$$\text{Relative expression} = 10^{-(\Delta Ct/3.9383)} \times \text{ng ribosomal gene cDNA/ng experimental gene cDNA}$$

where ΔCt = experimental gene Ct−mean *P. taeda* 18S ribosomal Ct. The value 3.9383 was experimentally determined from a standard curve of Ct versus log concentration of *P. taeda* 18S ribosomal real-time PCR dilution series, where −3.9383 is the slope of the line. Replicates for each sample were then averaged, excluding any extreme outliers, considered to be replicates that deviated more than 1.5 Ct from the other two replicates during the log-linear phase of PCR. Therefore, the expression levels for each sample are expressed as the mean concentration of real-time PCR product per molecule of pine ribosomal RNA. To determine effects of elevated CO_2 and N fertilization on MST and AMT gene expression levels, analysis of variance was performed for each gene separately. Data were log-transformed prior to analysis when necessary to achieve normality.

Results

MST and AMT gene abundance in *P. taeda*

Results from the phylogenetic analyses of all available clade I MST (Lalonde et al. 2004), or AMT expressed sequence tag assemblies (TAs), estimate that the *P. taeda* genome contains 11–14 sugar transport proteins in this subfamily (Fig. 1) and five to six AMT proteins (phylogeny not shown). We provide ranges rather than an exact number because the TAs vary in length and they do not always overlap in sequence to allow homology assessment. Three TAs were removed from the MST alignment and one from the AMT alignment prior to analysis due to a lack of overlap. MST 3.1 is the most closely related of the *P. taeda* MST genes to *P. tremula* × *P. tremuloides* MST3.1 (Grunze et al. 2004). Two *P. taeda* TAs (Pt MST1.1–2) appear to be orthologs of *Arabidopsis* STP13, and four (Pt MST4.1–4) are orthologs of *Arabidopsis* STP7. The phylogeny of AMT gene sequences is consistent with a previously published AMT phylogeny (Selle et al. 2005).

Tissue and treatment-specific expression of MST and AMT genes in *P. taeda* EST libraries

Public databases currently hold 92 EST libraries comprised of 327,484 EST sequences for *Pinus*, primarily from *P. taeda* (DFCI *Pinus* gene index). Forty-five percent of ESTs were generated from root tissue, 12% are derived from needles or a combination of needle and stem material, 22% from xylem, 20% from embryos, and the remainder from other tissue types. Some of the libraries represent plants grown in control conditions, but many were generated from plants exposed to a variety of environmental stresses. Among all sequences, 70 MST and 24 AMT transcripts were detected from *Pinus* EST libraries, representing less than 1% of total ESTs.

All but one of the *P. taeda* MST sequences were represented by ESTs obtained from root tissue, although the relative abundances of the different genes in root versus other tissues varied (Table 2). MST1.1 was most prevalent overall in the EST libraries (Table 2). MST5 was not found in the root libraries but was most abundant in embryonic tissue. AMT1 and AMT5 were the most abundant of all AMT genes in the EST libraries, with nine and seven ESTs recovered, respectively, all from root libraries.

Gene expression of *P. taeda* fine roots exposed to elevated CO_2 and N fertilization

Expression levels of Pt MST4.4 and Pt MST5 were too low to detect in roots and those genes were therefore omitted from the expression profile analyses (data not shown). Expression levels from the field samples mirrored results from the EST library analysis, with MST 1 and AMT1 showing the highest expression levels of the transcripts investigated in the field collected roots tips as well as greater frequencies in the EST libraries derived from roots than the other MST and AMT genes (Fig. 2). All three MST genes examined showed lower relative expression levels in elevated CO_2 plots than in the control plots (Fig. 2). Furthermore, both ambient and elevated CO_2 half plots subjected to fertilization had lower MST relative expression levels than their unfertilized counterparts, but these differences are not statistically significantly different. AMT relative expression levels were also lower in elevated CO_2 plots and the fertilized control plots had lower levels than unfertilized plots. However, in the elevated CO_2 plots, the fertilized halves possessed fine roots with AMT expression levels that were marginally higher than the unfertilized halves (Fig. 2), but none of these trends were statistically supported.

Fungal 18S relative expression was enhanced in the elevated CO_2 plots, and this difference had marginal statistical support (p=0.09; Fig. 3). Similarly, there was marginal statistical support for a CO_2 × fertilization interactive effect on fungal 18S expression (p=0.10), but no effect of N fertilization alone (p=0.8). In order to compare the relationship between fungal 18S and *P. taeda* 18S expression levels, samples were normalized to

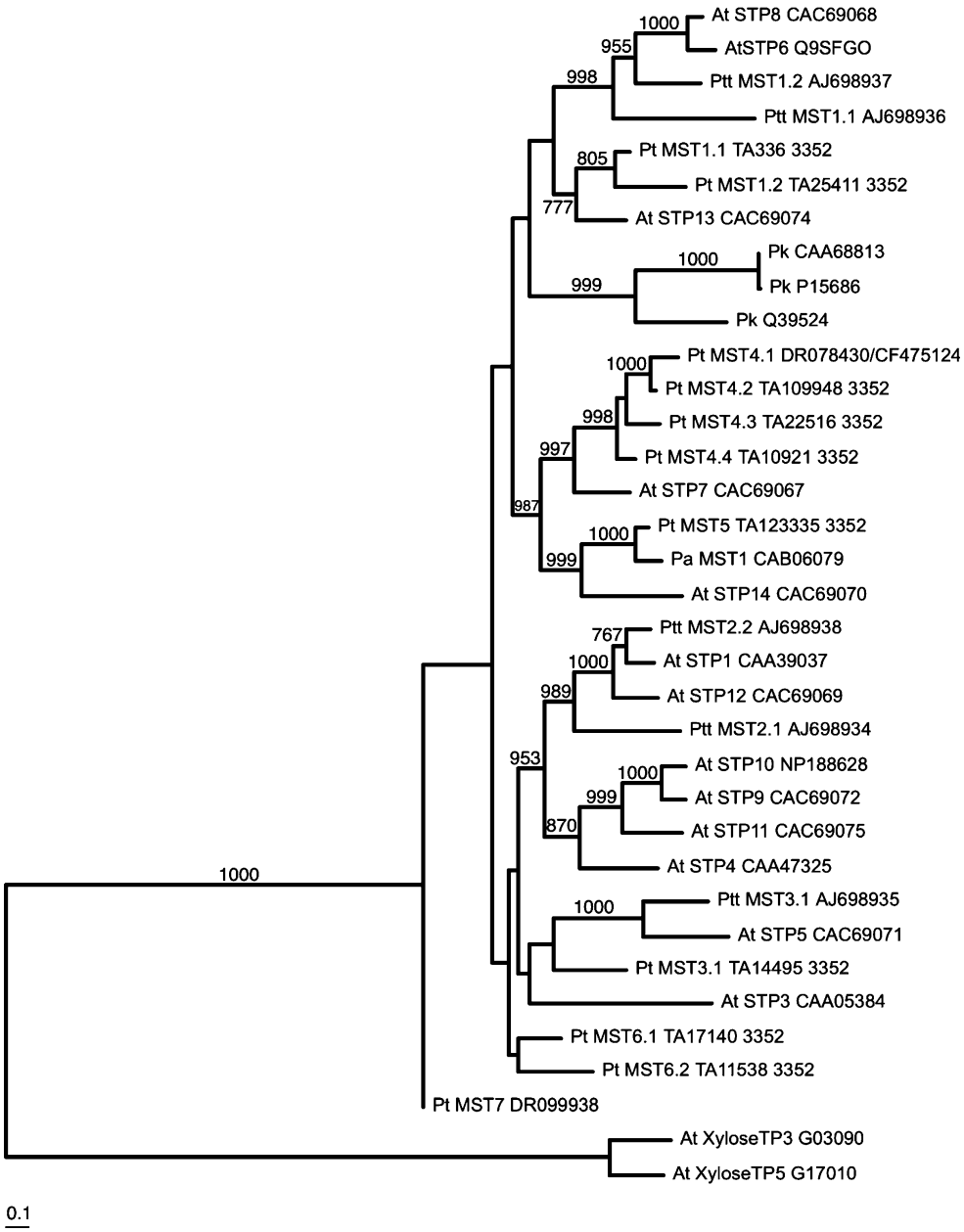

Fig. 1 Maximum likelihood phylogeny for *P. taeda* MST genes derived from EST libraries. The phylogeny is outgroup rooted with *A. thaliana* xylose transporter genes (Johnson et al. 2006). Bootstrap values from 1,000 bootstrap replicates are shown. *At A. thaliana*, *Ck Chlorella kessleri*, *Pk Parachlorella kessleri*, *Pa Picea abies*, *Pt Pinus taeda*, *Ptt Populus tremula* × *Populus tremuloides*. EMBL accession numbers are listed for all species, except for *P. taeda*, for which EST accession numbers from the TIGR plant transcript assembly are given

one sample with intermediate expression levels for both fungal and pine 18S expression. Note that in this case, the fungal 18S data are not being relativized to the pine 18S data, but rather to the fungal 18S Ct value of this intermediate sample, and the same is the case for the plant 18S data. The resulting normalized values were then plotted against each other. If pine and fungal 18S expression were perfectly congruent in every sample, all points would fall along a 1:1 line, and deviation from this ratio suggests greater expression of fungal 18S than pine 18S, or vice versa. This was not observed, but instead, a large variation in the ratio of fungal/plant 18S transcript abundance was observed (Fig. 4). Notably, the six samples with the greatest fungal 18S expression levels all came from elevated CO_2 plots (Fig. 4).

Discussion

In this study, we tested the hypotheses that CO_2-enrichment leads to greater C allocation to EMF and that C allocation is positively correlated with N transfer to plants by EMF, using MST and AMT expression levels as a proxy for C and N allocation, respectively. The study was conducted in the field to recapitulate the naturally high levels of EMF diversity of these terrestrial forests by

Table 2 Number, tissue source, and treatments of MST and AMT sequences recovered from publicly available *P. taeda* EST libraries

Gene	Tissue			
	Root	Stem/shoot	Embryo	Xylem
MST1.1	10	5	0	2
MST1.2	0	0	2	0
MST3.1	2	0	0	0
MST4.1	2	0	0	0
MST4.2	3	1	3	0
MST4.3	2	0	0	0
MST4.4	3	0	9	0
MST5	0	0	7	2
MST6.1	2	0	2	0
MST6.2	8	0	5	0
MST7	0	1	0	0
AMT1	9	0	0	0
AMT2	0	2	0	1
AMT3	1	0	0	1
AMT5[a]	7	0	0	0
AMT6	0	2	0	1

[a] AMT1 and AMT5 EST sequences differ by only 3/165 aligned amino acids and may represent allelic variants

taking advantage of an ongoing experimental plot of *P. taeda* subjected to CO_2 and N addition. We also tested whether fungal activity measured as fungal ribosomal RNA expression level was greater under elevated CO_2 conditions. We determined that MST expression levels in elevated CO_2 plots are lower relative to control plots, and expression was lower still in plots that also received N fertilization. Although these differences were not statistically significant, the pattern was consistent with what is expected if C allocation to EMF is increased under elevated CO_2. Fungal 18S expression levels were also increased with increased CO_2 conditions, supporting the hypothesis that CO_2 enrichment leads to greater EMF activity. In contrast, AMT expression levels were not consistent with the prediction that increased CO_2 would lead to increased N transport activity and instead showed a positive correlation with MST transcript levels, except in elevated CO_2 half-plots that also received fertilization, where AMT levels were marginally greater than their unfertilized companion plots.

In the following paragraphs, we will discuss the implications of greater fungal 18S expression levels for mycorrhizal metabolism and biomass under CO_2 enrichment. We will also discuss MST and AMT expression results from this study in the context of current knowledge regarding symbiotic C and N transport between plants and fungi and alternative hypotheses to explain the observed patterns in the data.

Increased EMF expression in elevated CO_2 and N fertilization plots

Fungal 18S rRNA expression levels increased in plots exposed to greater CO_2 and N fertilization levels. This increase in 18S expression could be interpreted as either greater metabolic activity or fungal mantle biomass. Measurements of soil heterotrophic respiration in these

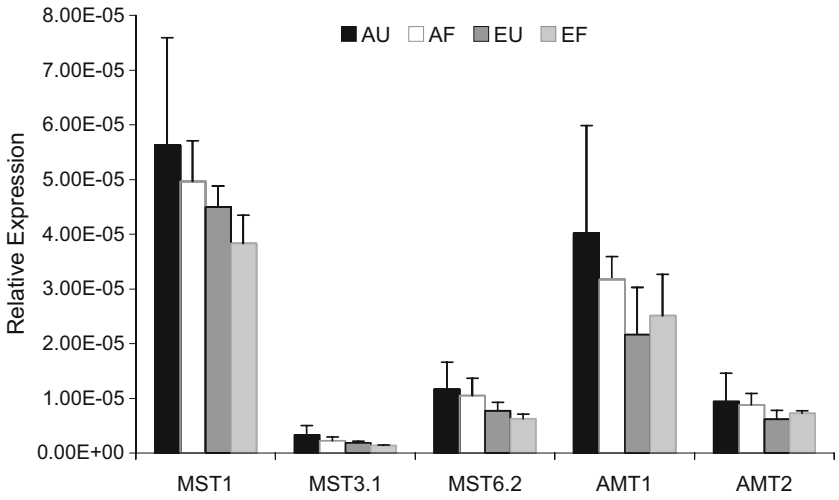

Fig. 2 Mean per sector relative expression levels of *Pinus taeda* monosaccharide transporter (*MST*) and ammonium transporter (*AMT*) genes in EMF colonized fine roots exposed to elevated CO_2 and N fertilization treatments. Gene expression levels for each gene were relativized to *P. taeda* 18S ribosomal RNA expression. *Black bars* are ambient CO_2 unfertilized sectors (*AU*), *white bars* represent ambient CO_2 fertilized sectors (*AF*); *dark gray bars* show elevated CO_2 unfertilized sectors (*EU*), *light gray bars* represent elevated CO_2 fertilized sectors (*EF*). Fertilized sectors received two applications of NH_4–NO_3 in spring 2005 prior to sampling in October 2005. Error bars represent 1 standard error above the mean

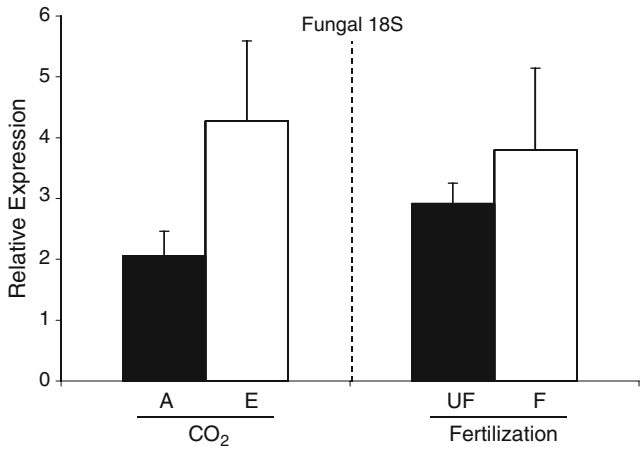

Fig. 3 Mean fungal 18S ribosomal gene expression per sector from mycorrhizae exposed to elevated CO_2 concentrations and N fertilization. Values were relativized to *P. taeda* 18S control gene expression. *A* ambient CO_2, *E* elevated CO_2, *UF* unfertilized sectors, *F* fertilized sectors. Error bars represent 1 standard error above the mean

plots show that respiration rates do increase with CO_2 enrichment (A.C. Oishi and J.S. Pippen, personal communication), which could result from either greater fungal activity or biomass. Fungal exposure to high concentrations of inorganic N can also lead to increased production of C skeletons in EMF cells in order to incorporate ammonium into amino acids and reduce the concentration of ammonium ions in the surrounding soil, which can have negative effects on fungal cells (Wallenda and Kottke 1998). Previous research at this site found no statistically significant increase in EMF hyphal biomass in soil from elevated CO_2 plots (Parrent and Vilgalys 2007). However, it is possible that EMF hyphal biomass in the soil could remain unchanged, while biomass forming the mantle ensheathing fine roots is increased with CO_2 enrichment. This could result from either greater C investment by all EMF species at the site of C uptake (the root tip), or could be a result of observed shifts in EMF community composition (Parrent et al. 2006), such that a higher proportion of roots are now colonized by EMF species that form thicker mantles.

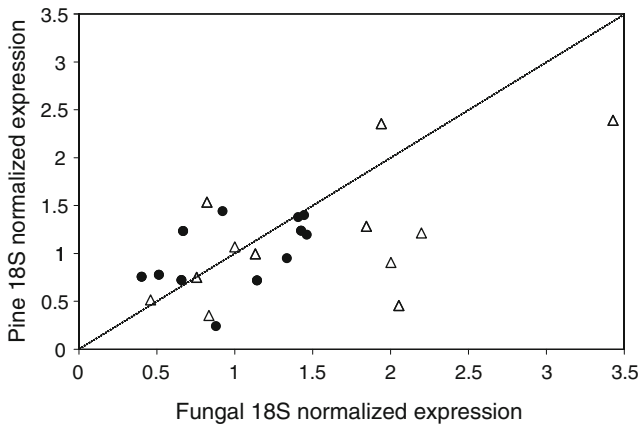

Fig. 4 Fungal 18S gene expression vs. *P. taeda* 18S gene expression levels. Expression levels for fungal and pine 18S values were normalized to the fungal and pine 18S expression levels, respectively, of the sample point 3s4, which exhibited intermediate expression levels for both pine and fungal 18S. *Dashed line* represents the 1:1 line. *Filled circles* ambient CO_2 sectors; *open triangles* elevated CO_2 sectors

Carbon transport and MSTs

Carbon movement in plants not only is a complex and dynamic process accomplished in part by passive movement of solutes along concentration gradients but also requires the active transfer of molecules into and out of phloem and plant cells by transport proteins (Kuhn et al. 1999). Plants possess a large number of sugar transporters, *Arabidopsis* and *Oryza*, for which there are completed genomes, possess 53 and 22 monosaccharide transporters, respectively, that cluster into seven subfamilies (Johnson et al. 2006; Lalonde et al. 2004). Among the *Arabidopsis* MST genes, 14 belong to the STP subfamily of plasma membrane H^+ symporters, energy-dependent proteins that cotransport H^+ and hexoses and that are responsible for monosaccharide uptake from sink tissues (Lalonde et al. 2004; Toyofuku et al. 2000). These genes are of great interest in mycorrhizal research because their expression levels have been shown to respond to plant–fungal interactions in a number of plant species, including *A. thaliana* interactions with plant pathogens (Truernit et al. 1996), *Medicago truncatula* interactions with arbuscular mycorrhizal fungi (Harrison 1996), and *P. tremula* × *P. tremuloides* interactions with EMF (Grunze et al. 2004; Harrison 1996).

Searching the *Pinus* gene index EST libraries, we detected 11–14 putative STP subfamily genes in *P. taeda*. This estimate is lower than values reported for *P. taeda* by other investigators (21 unique expressed loci; Johnson et al. 2006). However, our analysis of their sequence data revealed seven of their EST loci that are of fungal origin (CF386531, CF477109, CF471613, DR181890, CF393850, CF386615, DR112350), which we removed from our dataset prior to analysis. The fungal contaminant sequences were all from EST libraries constructed from root tissue, demonstrating the need for caution when working with sequence data derived from plant materials that often harbor symbiotic fungi. Estimates of gene number derived from EST libraries are valuable when complete genome sequences are unavailable for the organism of interest as is the case with *P. taeda*. However, EST library analysis does not allow for precise enumeration of gene number and thus, the estimate of 14 STP genes in *P. taeda* should be considered a conservative approximation.

Comparison of the STP homologs from *P. taeda* to *Arabidopsis* and *Populus* proteins using phylogenetic analyses provides a measure of relatedness among genes and may provide greater insight into their molecular function (Fig. 1). Gene expression profiles of *A. thaliana* and *P. tremula* × *P. tremuloides* paralogs in the STP subfamily vary in their degree of tissue specificity and the kinds of external stimuli to which they respond (Büttner 2007; Büttner and Sauer 2000; Grunze et al. 2004). Of the three *P. taeda* STP genes examined in the present study, Pt MST3.1 was most closely related to Ptt MST3.1, which showed a 12-fold increase in expression levels in *P. tremula* × *P. tremuloides* when colonized by the EMF species *A. muscaria* (Grunze et al. 2004). Interestingly, At STP3, which is also closely related to these two genes, has also been reported to have low affinity for glucose and to respond to attack by pathogens and to wounding (Büttner 2007; Büttner et al. 2000). The response of genes within the MST3.1 clade to mutualistic and pathogenic interactions, the low affinity for glucose and their expression in roots of *P. tremula* × *P. tremuloides* and *P. taeda*, together suggests the potential for Pt MST3.1 to play a role in selectively allocating C to EMF in *P. taeda*. However, unlike PttMST3.1, Pt MST3.1 did not show the greatest relative expression of the three MST genes examined here, nor did Pt MST1 demonstrate a pattern of expression consistent with that of its homolog in *P. tremula* × *P. tremuloides*. While there are a number of explanations for discrepancies in gene regulation among studies, it is also the case that homology does not necessitate functional equivalence, and this must be considered when using such an approach to infer gene function, particularly when comparing evolutionarily divergent taxa as we are here between the conifer *P. taeda* and the angiosperm *P. tremula* × *P. tremuloides*.

Plant N uptake from EMF and the role of ammonium and AMTs

Nitrogen transport is also a complex process in plants and is perhaps more complicated than C transport due to the fact that N compounds are translocated in both phloem and xylem channels and to the heterogeneity of N containing compounds (e.g., NH_4, amino acids, peptides) in plants and soil. Until recently, it has been thought that N is transported from mycorrhizae to plants in the form of an amino acid (Smith and Read 1997). Due to the structure of organic N compounds such as amino acids, organic N transport from mycorrhizae to plant would result in C loss for the fungus. Recent research has shown that N transported as arginine in the mycelium of *G. intraradices* to the interfacial apoplast is taken up by plant roots as ammonium (Govindarajulu et al. 2005). Consistent with these results, other research on this arbuscular mycorrhizal fungal system has found enzymatic activities and intermediate breakdown products in the apoplast that would result from arginine conversion to inorganic N prior to plant uptake (Bago et al. 2001; Johansen et al. 1996). Though these data provide compelling evidence for ammonium uptake by plants in the AMF symbiosis, similar data have not been generated in ectomycorrhizal systems. However, work by Selle et al. (2005) with *Populus* roots demonstrated that several high affinity ammonium transporters were strongly upregulated in ectomycorrhizal symbiosis. These results in conjunction with additional research using dually labeled N sources (Hodge et al. 2000) point to the possibility that ammonium may be directly transferred to plants from ectomycorrhizal symbionts.

Variation in MST and AMT expression in response to CO_2 enrichment

Although there were consistent trends among the expression levels of MST and AMT genes exposed to CO_2 and N fertilization, there was a large degree of among plot variability in transcript profiles of these genes. While some of this variation may be due to differences among plots in background soil nutrient characteristics (Finzi et al. 2002), data from this site show that increased CO_2 concentrations have led to significant increases in plant productivity, greater plant N demand, and greater N uptake from the soil in all elevated CO_2 plots relative to ambient plots (Finzi et al. 2007; Norby et al. 2005), and analyses of N content of *P. taeda* needles after 1 year of fertilization already showed statistically significantly greater needle N content in trees from fertilized plots than unfertilized plots (Maier et al. 2008). We therefore expected experimental conditions to be sufficiently strong to detect differences due to treatment effects. We conducted our experiments in this field setting to mimic a realistic environment where hosts interact with numerous symbionts, but difference in fungal species composition in these plots may also have increased the variability in MST and AMT expression levels. Fine root samples were not sorted with respect to particular fungal species or mantle morphology, and each sector sample was comprised of several dozen colonized fine roots, representing a heterogeneous mix of EMF taxa in each sample that reflected the distribution of EMF in those sectors, which previous work (Parrent et al. 2006; Parrent and Vilgalys 2007) has shown to be affected by both CO_2 and background nutrient concentrations. Therefore, if plants differentially allocate C to EMF taxa, sample variance in MST expression may be enhanced due to variation among samples in EMF composition, making it more difficult to isolate the CO_2-dependent effect on MST expression. Future studies, either in controlled greenhouse experiments with plants singly inoculated with a range of EMF species

or in field experiments where roots are sorted by fungal species prior to gene expression analysis are necessary to determine whether or not plant MST expression levels vary with respect to the EMF species with which they are associated and whether CO_2 concentration modulates the magnitude of such variation.

With respect to AMT expression levels, there are a number of possible reasons why the data do not match the prediction that AMT expression would be negatively correlated with MST gene expression. First, there are a number of mechanisms by which N in the form of ammonium may be taken up by plants root cells other than uptake by group I AMTs, the family of transporters examined in this study. Plants also possess a second group of high affinity AMTs, low affinity AMT proteins, and several other nonspecific import pathways, such as aquaporins and voltage-dependent cation systems, through which ammonium may also pass into plant root cells (Chalot et al. 2006). Therefore, it is possible that plants do import greater amounts of ammonium from EMF in elevated CO_2 plots, but that they are using one or all of these other mechanisms to do so. Second, just as EMF taxa vary in their capacity to take up different N containing compounds from soil, it stands to reason that they may similarly vary in the form of N they transfer to plants, with some providing N as amino acids and others transferring N as ammonium. Because these samples are aggregates of multiple EMF taxa, variation in AMT expression may also result from differences among samples in the proportion of EMF species that provide N to *P. taeda* as ammonium.

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