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REGULAR ARTICLE

Transcriptomic and metabolic responses of mycorrhizal roots to nitrogen patches under field conditions

Daniel R. Ruzicka · Natasha T. Hausmann · Felipe H. Barrios-Masias · Louise E. Jackson · Daniel P. Schachtman

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Abstract
Background Arbuscular mycorrhizal (AM) fungi contribute to plant nutrient uptake in systems managed with reduced fertilizer and pesticide inputs such as organic agriculture by extending the effective size of the rhizosphere and delivering minerals to the root. Connecting the molecular study of the AM symbiosis with agriculturally- and ecologically-relevant field environments remains a challenge and is a largely unexplored research topic.
Methods This study utilized a cross-disciplinary approach to examine the transcriptional, metabolic, and physiological responses of tomato (*Solanum lycopersicum*) AM roots to a localized patch of nitrogen (N). A wild-type mycorrhizal tomato and a closely-related non-mycorrhizal mutant were grown at an organic farm in soil that contained an active AM extraradical hyphal network and soil microbe community.
Results The majority of genes regulated by upon enrichment of nitrogen were similarly expressed in mycorrhizal and non-mycorrhizal roots, suggesting that the primary response to an enriched N patch is mediated by mycorrhiza-independent root processes. However where inorganic N concentrations in the soil were low, differential regulation of key tomato N transport and assimilation genes indicate a transcriptome shift towards mycorrhiza-mediated N uptake over direct root supplied N. Furthermore, two novel mycorrhizal-specific tomato ammonium transporters were also found to be regulated under low N conditions. A conceptual model is presented integrating the transcriptome response to low N and highlighting the mycorrhizal-specific ammonium transporters.
Conclusions These results enhance our understanding of the role of the AM symbiosis in sensing and response to an enriched N patch, and demonstrate that transcriptome analyses of complex plant-microbe-soil interactions provide a global snapshot of biological processes relevant to soil processes in organic agriculture.

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Daniel R. Ruzicka and Natasha T. Hausmann contributed equally to this work.

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D. R. Ruzicka · D. P. Schachtman (✉)
Donald Danforth Plant Science Center,
975 N Warson Rd.,
St. Louis, MO 63132, USA
e-mail: dschachtman@danforthcenter.org

D. R. Ruzicka
e-mail: druzicka@danforthcenter.org

N. T. Hausmann · F. H. Barrios-Masias · L. E. Jackson
Department of Land, Air, and Water Resources,
University of California,
1 Shields Avenue,
Davis, CA 95616, USA

N. T. Hausmann
e-mail: nteutsch@gmail.com

F. H. Barrios-Masias
e-mail: fbarrios@ucdavis.edu

L. E. Jackson
e-mail: lejackson@ucdavis.edu

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Abbreviations

AM	arbuscular mycorrhiza
N	nitrogen
P	phosphate
Pi	inorganic phosphate
Zn	zinc
S	sulfur
Cu	copper
PT	phosphate transporter
NH_4^+	ammonium
NO_3^-	nitrate
AMT	ammonium transporter
NRT	nitrate transporter
PSR	phosphate starvation response

Introduction

The arbuscular mycorrhizal (AM) fungal root symbiosis occurs in the majority of land plants including most crop plants, and may play an important role in nutrient uptake in low input environments (Allen and Shachar-Hill 2009; Govindarajulu et al. 2005; Javot et al. 2007; Smith and Read 2008). The AM fungi develop a network of extra-radical mycelium that extends into the soil, expanding the effective range of the root rhizosphere (Miller et al. 1995). Inorganic phosphate (Pi) is the main soil nutrient supplied by the symbiosis and mycorrhizal-specific plant Pi transporters (PTs) are responsible for the majority of Pi transport into mycorrhizal roots (Javot et al. 2007). Much of the transcriptional response to the mycorrhizal symbiosis is likely a consequence of this additional mycorrhizal Pi uptake (Nagy et al. 2009). Mycorrhizal plants also exhibit higher levels of mineral nutrients including sulfur (S), zinc (Zn), and copper (Cu) (Cavagnaro et al. 2008; Liu et al. 2000); however the plant transporters responsible for the uptake of these mycorrhizal-supplied nutrients have not been identified beyond transcriptional studies in greenhouse-grown plants (Guether et al. 2009a).

Soil nitrogen (N) is not uniformly distributed and is often limiting in agricultural systems making it an important nutrient for mycorrhizal-mediated uptake. The varied sources of N and soil processes lead to complex dynamic N pools (Burger et al. 2005). Fates of inorganic N in the soil include direct uptake by plant roots or AM hyphae, microbial immobilization or transformation, and environmental losses to nitrate (NO_3^-) leaching below the root zone and gaseous emissions. Initial plant response to available N in the environment include the regulation of ammonium (NH_4^+) and NO_3^- transporters and root proliferation in localized areas with high N content (Granato and Raper 1989; Hodge 2004; Remans et al. 2006). While most studies have focused on a single form of nitrogen, our work has shown that roots also have the ability to respond to the increased availability of multiple forms of N during the nitrification after added NH_4^+ (Ruzicka et al. 2010).

AM fungi help plants compete for soil N that may be lost or immobilized by other soil microbes. The extraradical mycelium take up organic and inorganic N from the soil via amino acid and NH_4^+ transporters, translocate it to the intraradical mycelium as arginine, and release N to the plant in the form of NH_4^+ (Cruz et al. 2007; Govindarajulu et al. 2005; Jin et al. 2005; Leigh et al. 2009; Tanaka and Yano 2005). Recent studies have identified *Medicago truncatula* and *Lotus japonicus* mycorrhizal-induced plant NH_4^+ transporters that are likely to transport mycorrhizal-derived NH_4^+ across the periarbuscular membrane to the plant (Gomez et al. 2009; Guether et al. 2009b; Kobae et al. 2010). It has been proposed that this mycorrhizal N-uptake pathway is similar in some ways to the well characterized mycorrhizal Pi uptake pathway. The identification of significant downstream effects of the N supplied by mycorrhizal fungi on plant physiological or biochemical processes are limited (Hildebrandt et al. 2002).

Previous work in a controlled greenhouse pot study reported a strong and complex tomato root transcriptional response to an enriched N patch (Ruzicka et al. 2010). In the present study, we used the same system to explore mycorrhizal root responses to an enriched N patch. The current study was conducted in an organic farm soil in the field where plants may have been subject to greater environmental heterogeneity, where soil nutrient transformations were more complex, and where patches of applied N captured a smaller proportion of the total root system. This is the first field-based study to examine root gene expression changes in response to mycorrhizal fungi. The

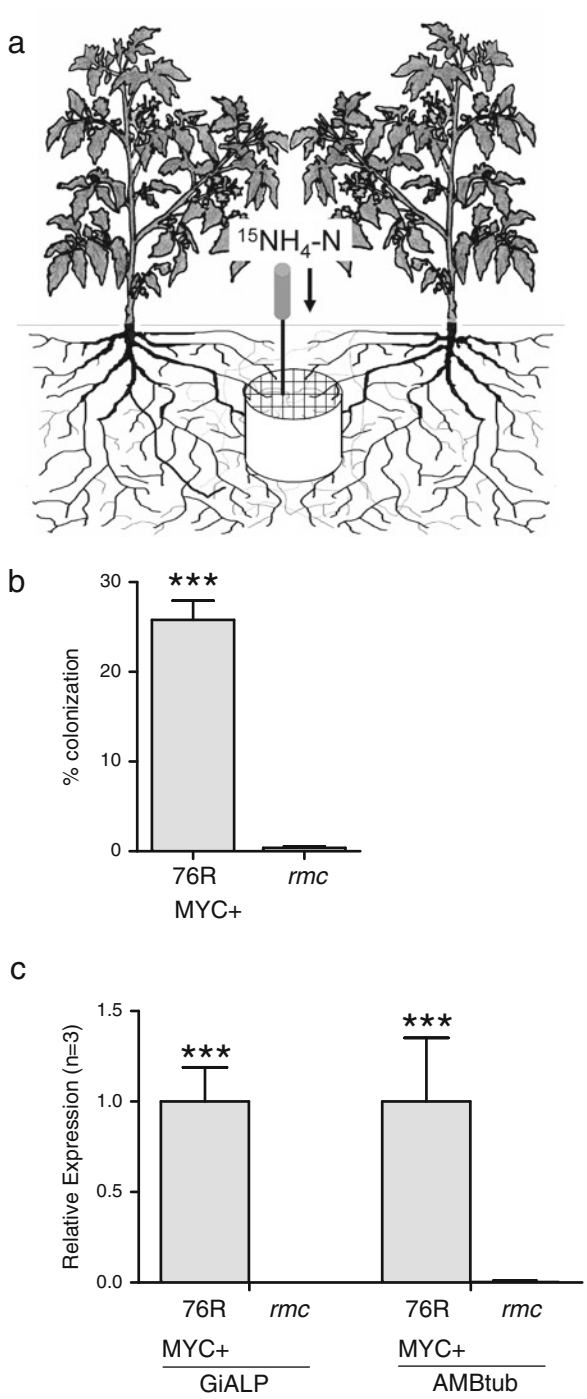


Fig. 1 Diagram of nitrogen patch experimental design and quantification of arbuscular mycorrhizal colonization. Root ingrowth rings were buried below each genotype (76R MYC+ or *rmc*) and injected with either high $^{15}NH_4^+$-N (65 μg $^{15}NH_4^+$-N g^{-1} soil), low $^{15}NH_4^+$-N (6.5 μg $^{15}NH_4^+$-N g^{-1} soil) or water to simulate a nutrient patch. Patch roots were harvested 24 h after injection. The mycorrhizal wild-type (76R MYC+) and the non-mycorrhizal mutant (*rmc*) had different colonization rates by standard line intersect methods (**b**) and by qPCR analysis of *Glomus intraradices* alkaline phosphatase (*Gi*ALP) and arbuscular mycorrhizal ß-tubulin (Amß-tubulin) fungal gene expression (**c**). Asterisks represent a significant difference between 76R MYC+ and *rmc* for each comparison (***=$P<0.001$). The light and dark grey bars represent 76R MYC+ and *rmc* respectively. The relative expression was calculated using the ΔΔCT method with LeACT as the reference control to normalize for tomato tissue input, and the 76R MYC+ group set to 1

use of a reduced mycorrhizal mutant (*rmc*) that is closely matched in growth to its mycorrhizal wild-type tomato (Cavagnaro et al. 2008) provided the opportunity to conduct this study in an agriculturally relevant field setting and avoided many of the confounding factors introduced by anti-mycorrhizal treatments. Two concentrations of $^{15}NH_4^+$ and a water control were applied to localized soil patches containing mycorrhizal wild-type or non-mycorrhizal *rmc* roots (Fig. 1a). Twenty-four hours after the NH_4^+ treatments, plants were harvested to examine the effects of the AM symbiosis on the root responses to the addition of N in the patch. We demonstrate that while the N-enriched patches had profound impacts on plant gene expression, the symbiosis resulted in additional and interactive effects. At lower concentrations of soil inorganic N, similar to those typically found in lower input organic agriculture (Mader et al. 2002; Smukler et al. 2010), AM fungi supplied additional N to the roots. In addition to changes in nitrogen responses, we found mycorrhizal-enhanced plant P, Cu, and S uptake linked to the coordinated mycorrhizal-regulation of plant Pi, Cu, and S transporters and downstream metabolism genes. We propose a conceptual model of mycorrhizal root N metabolism based on the transcription profiling data where the mycorrhizal effect on N uptake and metabolism is more pronounced and possibly promoted under lower N availability. This model highlights a transcriptional repression of direct root N uptake pathways under low N conditions where mycorrhizal-specific tomato ammonium transporters LeAMT4 and LeAMT5 are expressed.

Materials and methods

Plant material

Two different tomato genotypes (*S. lycopersicum* L.) were used: the wild-type cv RioGrande 76R (Peto

Seeds) that forms mycorrhizal associations (76R MYC+) and the *rmc* mutant (derived from 76R (Barker et al. 1998)). Plants were germinated as described in Ruzicka et al. (2010). Eight-week old seedlings were transplanted one foot apart at a tomato field on an organically managed farm, Durst Organic Growers Inc. in Esparto, Yolo County, California (field soil properties are presented in Cavagnaro et al. (2006)). In order to apply nutrients in patches and recover the roots that were directly exposed to the nutrient addition, root in-growth rings were buried at 7.5 cm below the soil surface and between the two experimental plants in each plot (see below). The rings had an internal diameter of 10 cm and were 5.2 cm tall (total volume 408 cm^3) and were filled with 457 g of field soil to a final bulk density approximately equal to that of the surrounding soil (1.2 g cm^{-3}). The broad ends of the ring were covered with a 1-mm plastic mesh to allow roots to easily grow into the ring. Plants were watered regularly using 1.9 l $hour^{-1}$ surface drip hose with emitters spaced 30 cm. Emitters were placed between plants, i.e., 15 cm on either side of every plant and directly above the buried ring. No fertilizer was applied except for the experimental N treatments.

Experimental design

Each experimental plot consisted of two experimental plants and two outer plants considered as buffers; 4 plants total per plot. There were two genotypes (76R MYC+ and *rmc*) and three nutrient treatments (water, low NH_4^+, and high NH_4^+), and six plot replicates (biological reps) for a total of 36 plots. The transplants were grown for five weeks in the field to allow sufficient root mycorrhizal colonization as well as root exploration of the buried ring. Treatments were injected at 13 evenly distributed positions in the ring in 2-mL aliquots, for a 26 mL total volume. For the low NH_4^+ treatment, 6.5 μg $^{15}NH_4^+$-N (99 atom percent) per gram of dry soil were added in the ring (2.97 mg $^{15}NH_4^+$-N per ring) which was a 10-fold increase over ambient soil levels of NH_4^+-N. For the high NH_4^+ treatment, 65 μg $^{15}NH_4$-N (99 atom percent) per gram of dry soil were added in the ring (29.7 mg $^{15}NH_4^+$-N per ring), a 100-fold increase over ambient soil levels. Water was used as a control. Additional 76R MYC+ and *rmc* plots were used to study ^{15}N uptake over time. These plots were injected with the high NH_4^+ or water control treatment. The youngest, fully emerged leaves of the two middle plants in each plot were harvested one, two, five, seven, and nine days post-treatment for ^{15}N isotope analysis.

Harvest and sample analysis

Plots were harvested 24 h after the rings were injected by cutting the two plants on either side of the ring at ground level, and later separating the shoot in leaves, fruits and stems. All aboveground biomass was dried, weighed, and ground for $\delta^{15}N$ stable isotope analysis and nutrient analysis. Immediately after severing the plants, the buried ring was carefully excavated to prevent pulling roots out of the ring, and all sides shaved with a razor blade. Two, 50–150 mg (fresh weight) subsamples of roots were promptly removed from the ring, rinsed, pat dry, and flash frozen in liquid nitrogen for RNA extraction under minimized/indirect light. A representative subsample of soil was removed from the rings, 2 *M* KCl added at a liquid: soil ratio of 2.5:1, shaken and the supernatant analyzed using a modification of Miranda et al. (2001) for NO_3^- and Foster (1995) for NH_4^+. Gravimetric soil moisture was also determined. The background ammonium and nitrate concentrations in the soil was determined by randomly collecting nine soil samples from two depths (0–15 cm and 15–45 cm) for each genotype (36 soil samples total).

From the remaining roots, a small subsample of patch roots was scored for AM colonization at 200X (McGonigle et al. 1990). All remaining roots from the ring were dried, weighed, and ground for $\delta^{15}N$ isotope analysis on a PDZ Europa ANCA-GSL elemental analyzer and a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at the UC Davis Stable Isotope Facility, USA. Leaves were analyzed for total P, S, Zn, and Cu using a nitric acid/hydrogen peroxide microwave digestion (Meyer and Keliher 1992; Sah and Miller 1992) followed by atomic absorption spectrometry and inductively coupled plasma atomic emission spectrometry at the UC Davis DANR labs. Plant hormone analysis on 4 biological replicates of each treatment/genotype group was performed on lyophilized root tissue harvested from the nutrient patch (50 mg fresh weight) by LC/MS/MS on the ABI 4000 QTRAP system at the Danforth Center Proteomics and Mass Spectrometry

Facility (See Protocol in Online Resource 1 for detailed protocol).

Microarray analysis

Transcriptome profiling of each 24 h-post injection RNA sample was performed using the Tomato Genome Array Chip (Affymetrix, Santa Clara, CA, USA) as described in Ruzicka et al. (2010). The data were filtered to remove probesets called absent by the MAS5 summarization in all 30 arrays. For each probeset, which represents the combined expression data from all relevant probe pairs on the chip, the generalized linear model $Y_{ijk}=\mu+\alpha_i+\beta_j+(\alpha\beta)_{ij}+\varepsilon_{ijk}$ was fit. In each ANOVA, Y_{ij} is the log2 normalized transcript level for the i^{th} treatment, the j^{th} genotype, and the k^{th} replicate, μ is the overall mean expression for the probeset, α represents the i^{th} treatment (water, low nutrient, and high nutrient), β represents the j^{th} genotype (76R MYC+ and *rmc*), and $(\alpha\beta)$ the interaction of the i^{th} treatment and j^{th} genotype. F-tests were used to test the null hypothesis for each main effect and the interaction effect. We examined the model for conformation to the assumption of normality of the residuals testing the null hypothesis that the residuals for each gene were normally distributed using the Shapiro-Wilkes Test. All analyses were performed in JMP Genomics 4.0 (SAS Institute, Cary NC). An FDR level of 5% was used for declaring main effect or pairwise findings significant (Verhoeven et al. 2005). The list of statistically significant transcripts was annotated as described in Ruzicka et al. (2010) and functionally classified according to Bevan et al. (1998). Microarray data was plotted in tables as a fold-change, indicating the difference in average signal intensity between groups, or plotted graphically as relative signal intensity with the 76R MYC+ control treatment (water) group set to 1. This array data (Online Resource 2) has been made available on the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/projects/geo/) accession GSE30270 .

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was performed including primer testing as described in Ruzicka et al. (2010). Online Resource 3 contains sequences of gene-specific primer pairs first reported here. qRT-PCR primers were designed at www.idtdna.com, and all amplicon sizes were between 80 and 200 nucleotides. The tomato actin gene (BT013707, LeACT) was used as the reference control gene as it did not exhibit differential expression among N treatments or between wild-type and *rmc*. Relative expression was analyzed according to the $\Delta\Delta$CT method, and standard error was computed from the average of the ΔCT values for each biological sample.

Calculations and statistics

The atom percentage of ^{15}N tracer in each plant part (leaves, stems, fruits and ring roots) was measured by first calculating the amount of excess ^{15}N above background. Background ^{15}N was calculated as the average atom% ^{15}N in the water samples (mean atom percent ^{15}N±SE in leaves: 0.369%±0.0006, stems: 0.369%±0.0005, fruits: 0.3688%±0.0005, ring roots: 0.381%±0.005, no significant differences between the two genotypes). This background ^{15}N was then subtracted from total atom% ^{15}N.

All soil and ^{15}N data was analyzed with a three-way, fixed factor analyses of variance with genotype, nutrient addition, and block as the main effects or a two-way fixed factor analysis of variance without the nutrient main effect if the treatment had not yet been applied. All assumptions of ANOVA were checked and data transformed as needed. Tukey-Kramer HSD test was used to compare means ($P<0.05$). All data were analyzed using R.

Results

76R MYC+ plants exhibited AM colonization and active symbiosis

Colonization rates of wild-type roots (76R MYC+) were 23%, while the reduced mycorrhizal mutant (*rmc)* roots were largely uncolonized (<1%) (Fig. 1b) as determined by trypan blue staining and microscopy. To estimate AM abundance or activity (Aono et al. 2004; Lammers et al. 2001) using molecular markers, *Glomus intraradices* alkaline phosphatase (ALP) and AM fungal ß-tubulin gene expression were assayed in the root samples using quantitative RT-PCR (qPCR). Both fungal genes were highly expressed in all 76R MYC+ root samples but were not detected in *rmc* root samples (Fig. 1c).

The transcriptome of patch roots was assayed using Affymetrix tomato GeneChips. Across all samples in the main experiment, 9022 of the 10038 probesets on the microarray were detected, indicating the expression of the corresponding genes. The array analysis identified 174 genes that were differentially regulated between mycorrhizal wild-type and non-mycorrhizal *rmc* roots irrespective of the N treatment (>1.5 fold-change, FDR corrected *P*-value<0.05) (Fig. 2a and Online Resource 4). To test whether these expression differences were due to the AM symbiosis or genetic differences between the 76R wild-type and *rmc* genotypes, we performed microarray analysis on wild-type and *rmc* root RNA samples from plants grown in soil lacking AM spores. None of the 174 genes reported here were differentially expressed when wild-type and *rmc* transcript profiles were compared in a pot study with sterile soils in the absence of AM (data not shown). This confirmed that the 174 regulated genes were not due to the difference in genotype.

There were 361 genes differentially regulated between the high NH_4^+ and water control treatment groups, and 98 genes were differentially regulated between the high NH_4^+ and low NH_4^+ treatment groups. Surprisingly, the arrays did not identify any genes differentially regulated between the low NH_4^+ and water control treatments. The differentially regulated genes were classified into diverse functional categories based on Bevan et al. (1998) (Fig. 2b and c, and Online Resource 5). The effect of a localized N patch on tomato root transcription was previously reported (Ruzicka et al. 2010), and 61 genes (13%) were similarly regulated by the N treatments in the previous greenhouse pot experiment and this field experiment in both wild-type and *rmc* roots (Online Resource 6).

The effect of AM symbiosis on P and other nutrients/micronutrients

Mycorrhizal plants contained significantly higher total P concentrations in leaves (Fig. 3a) and therefore may have been less phosphate limited than the *rmc* plants. The microarray results indicate that *rmc* roots were likely to be more phosphate limited as indicated by the induction of a strong multigene phosphate starvation response (PSR) (Table 1). PSR genes such as SYG1/Pho81 domain containing proteins (BI205943, AJ249999, and AJ715789), acid phosphatase TPSl1 (BI922966), ribonuclease RNAlx (X793338), and three phosphoric monoester hydrolases (AF305968.1, AJ459817.1, AJ459816.1) were all more highly expressed in *rmc* compared to mycorrhizal roots. Since the characterized phosphate transporter genes were not all present on the tomato microarray, quantitative PCR (qPCR) was used to test whether the mycorrhizal symbiosis also resulted in the dynamic regulation of the phosphate transporter (PT1, 2, 3, 4, and 5) genes (Fig. 3b). PT1 was not differentially expressed between

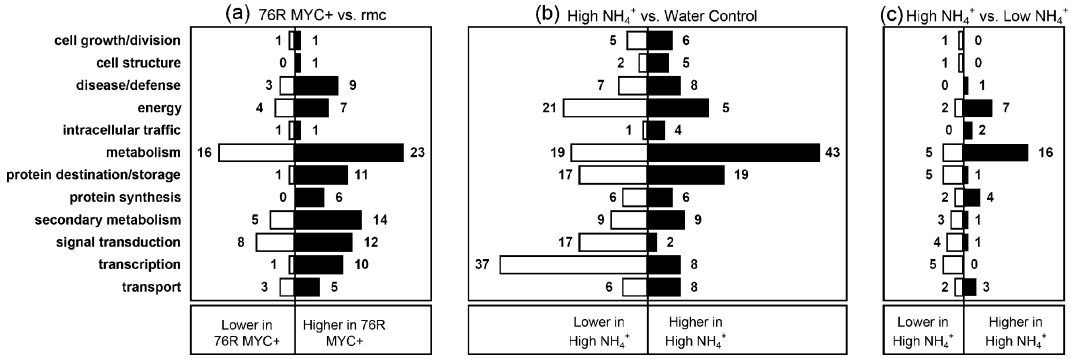


Fig. 2 Functional classification of tomato genes regulated by the mycorrhizal symbiosis and nitrogen treatments. Affymetrix probeset sequences were matched to publicly available Genbank accession identifications and categorized according to tomato or Arabidopsis orthologue gene annotations. 174 genes were differentially regulated by the AM symbiosis (**a**). 361 genes were differentially regulated between the high NH_4^+ and water control treatments (**b**). 98 genes were differentially regulated between the high NH_4^+ and low NH_4^+ treatments (**c**). Black bars represent genes more highly expressed in 76R MYC+ (**a**), or high NH_4^+ (**b** and **c**) roots. White bars represent genes more highly expressed in *rmc* (**a**), water control (**b**), or low NH_4^+ (**c**) roots. No differentially expressed genes were identified in the comparison between the low NH_4^+ and water control treatments. Unclassified genes or with unknown function are not represented on the figure

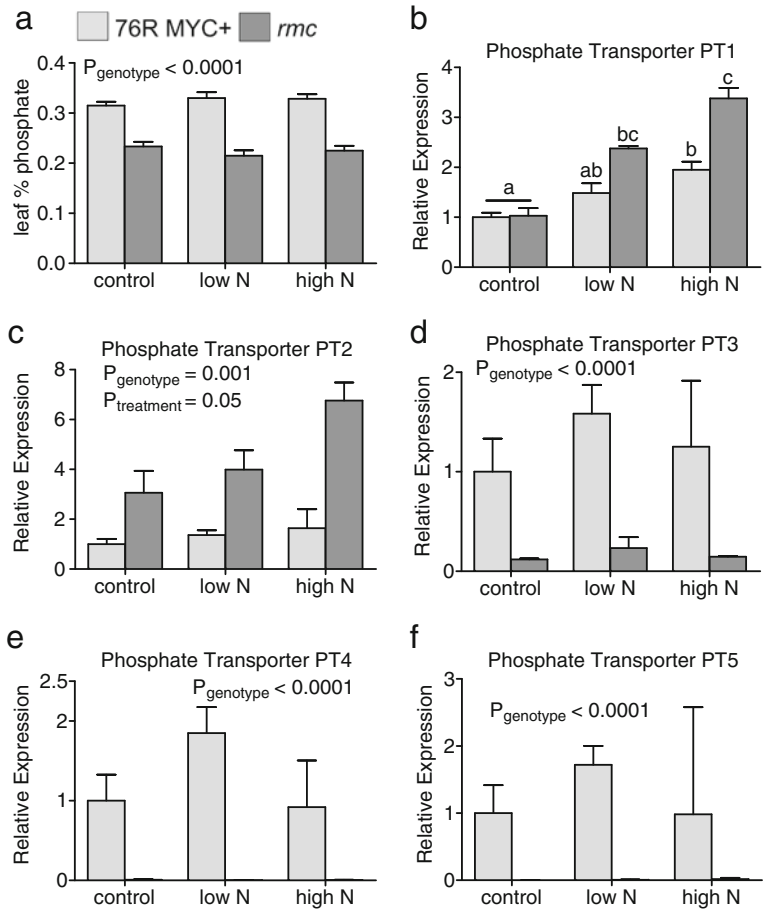


Fig. 3 Leaf phosphate levels and root expression of phosphate transporter genes in response to the nitrogen treatments and mycorrhizal symbiosis. Leaf phosphate concentrations were measured in 76R MYC+ and *rmc* plants (**a**). Expression levels of phosphate transporters PT1 (**b**), PT2 (**c**), PT3 (**d**), PT4 (**e**), and PT5 (**f**) were assayed using qRT-PCR. Relative expression was calculated using the ΔΔCT method with actin (LeACT) as the reference control, and the 76R MYC+ water control group normalized to 1. For PT1 (**b**), different letters indicate significant differences among groups (2-way ANOVA, Tukey HSD). The P values for significant main effects in PT2, 3, 4, and 5 (**c**–**f**) are noted (2-way ANOVA). The light and dark grey bars represent 76R MYC+ and *rmc* respectively

Table 1 Tomato phosphate metabolism and transporter genes regulated by the AM symbiosis between all 76R MYC+ and *rmc* samples

Phosphate transport and metabolism genes		mycorrhizal vs. *rmc*	
Affy Probe Set ID[a]	Putative Annotation[b]	Fold Change[c]	*P*-value[d]
Les.3799.1.S1_at	SPX domain-containing protein	−1.77	0.000
Les.5914.1.A1_at	SPX domain-containing protein	−2.33	0.003
Les.102.1.S1_at	inorganic phosphate transporter	−2.40	0.000
Les.2012.1.S1_at	myo-inositol-1-phosphate synthase	−2.50	0.003
LesAffx.18686.1.S1_at	phosphatase	−2.95	0.04
Les.50.1.S1_at	ribonuclease (rnalx)	−2.98	0.001
LesAffx.59441.1.S1_at	SPX domain-containing protein	−5.93	0.000
LesAffx.8748.1.A1_at	acid phosphatase TPSI1	−6.66	0.000
Les.4024.1.S1_at	phosphoric monester hydrolase	−9.42	0.000
Les.2672.2.S1_at	phosphoric monester hydrolase	−21.91	0.000

[a] Probe Set ID; Affymetrix identifier for each microarray probeset. [b] Putative Annotation; functional annotation based on tomato protein function or function of Arabidopsis orthologues identified with BLAST searches. [c] Fold Change; linear fold change (threshold >1.5 fold different) in mycorrhizal roots compared to *rmc* roots. [d] *P*-value; FDR adjusted *P* value threshold <0.05

mycorrhizal and *rmc* roots in the water control treatments, but was induced in *rmc* and not mycorrhizal roots in the low and high N patches. PT2 expression levels were at least three-fold higher in *rmc* compared to mycorrhizal roots under all N conditions. Conversely, mycorrhizal-specific PT 3, 4, and 5 were more highly expressed in mycorrhizal roots compared to *rmc* and did not exhibit any changes due to N treatment.

In addition to total P, the concentrations of Cu and S were significantly higher in mycorrhizal wild-type leaf tissue ($P<0.001$ and $P=0.007$) (Fig. 4a and b). Microarray analysis detected higher expression levels of at least one Cu transporter COPT2 (LesAffx.37692.1.S1_at) and a Cu chaperone protein (LesAffx.68294.1.S1_at) as well as S transporters ST1 (Les.3480.1.S1_at) and ST2 (Les.3479.1.S1_at) in mycorrhizal roots (Fig. 4c–f). Leaves of the mycorrhizal wild-type plants also exhibited higher concentrations of Zn and lower concentrations of manganese (Mn) (data not shown). Transcriptome analysis did not identify any differentially expressed Zn or Mn transporters. Additional physiological analysis of the field grown plants including biomass, photosynthesis, and water relations are presented in Online Resource 7. 76R MYC+ plants were slightly larger than *rmc* plants (mean ± SE: 63.5±2.4 g and 53.8±2.7 g respectively, $P=0.01$). Nutrient analysis was also performed on 76R MYC+ and *rmc* tomato fruit from a parallel plot (see Online Resource 8 for methods and results) from which roots were not harvested. Tomato fruit from 76R mycorrhizal plants had significantly higher concentrations of total P, iron (Fe), and Cu. Fruit from non-mycorrhizal *rmc* plants had higher concentrations of Mn.

AM symbiosis and hormone metabolism and signaling

In addition to the effects on nutrient metabolism and uptake, the mycorrhizal symbiosis resulted in the differential expression of hormone signaling and biosynthesis genes. The methyl salicylate esterase Les.5920.1.S1_at (61%ID Arabidopsis MES1 At2g23620) was more highly expressed in mycorrhizal roots (Table 2). Additional orthologous methyltransferases including LesAffx.69609.1.S1_at and Les.2504.1.A1_at were also

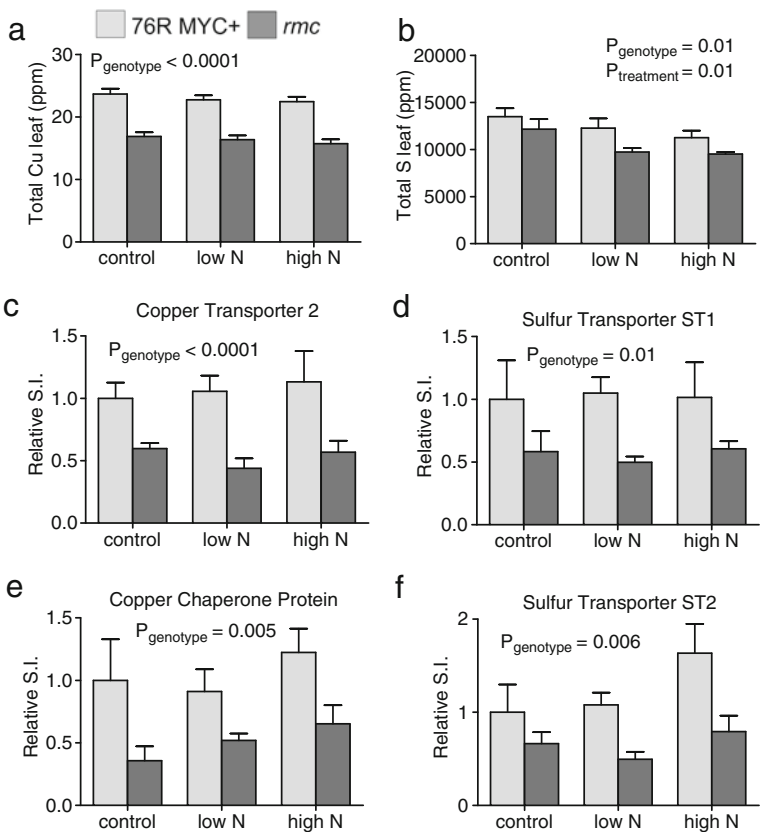


Fig. 4 Shoot copper and sulfur levels and root expression of copper and sulfur transporter genes in response to the nitrogen treatments and mycorrhizal symbiosis. Leaf copper (**a**) and sulfur (**b**) concentrations were measured in 76R MYC+ and *rmc* plants. Expression levels of copper transporter COPT2 (**c**) and copper chaperone (**e**), and sulfur transporters ST1 (**d**) and ST2 (**f**) were analyzed by Affymetrix microarray. Microarray relative signal intensities were calculated in reference to the 76R MYC+ water treatment group and plotted as an average of 5 replicates. The P values for significant main effects are noted (2-way ANOVA). The light and dark grey bars represent 76R MYC+ and *rmc*, respectively

Table 2 Differential regulation of tomato hormone metabolism genes in root tissue by the AM symbiosis

Hormone metabolism genes		mycorrhizal vs. *rmc*	
Affy Probe Set ID[a]	Putative Annotation[b]	Fold Change[c]	*P*-value[d]
Les.4335.2.S1_at	gibberellin 2-oxidase	9.82	0.000
Les.10.1.S1_at	gibberellin 3b-hydroxylase	5.70	0.000
Les.64.1.S1_at	gibberellin 20-oxidase-1	4.94	0.000
LesAffx.70667.1.A1_at	gibberellin requiring 3	2.90	0.003
Les.27.1.S1_at	copalyl diphosphate synthase	2.79	0.021
Les.5920.1.S1_at	methylesterase	2.75	0.000
LesAffx.69609.1.S1_at	S-adenosyl-L-methionine:carboxyl methyltransferase	2.50	0.047
Les.2504.1.A1_at	S-adenosyl-L-methionine:carboxyl methyltransferase	2.27	0.005
LesAffx.52427.1.S1_at	GDSL lipase 1	−1.60	0.016
Les.3225.2.S1_at	1-aminocyclopropane-1-carboxylate oxidase	−2.40	0.012

[a] Probe Set ID; Affymetrix identifier for each microarray probeset. [b] Putative Annotation; functional annotation based on tomato protein function or function of Arabidopsis orthologues identified with BLAST searches. [c] Fold Change; linear fold change (threshold >1.5 fold different) in mycorrhizal roots compared to *rmc* roots. [d] *P*-value; FDR adjusted *P* value threshold <0.05

more highly expressed in mycorrhizal roots. A salicylic acid-repressed GDSL-lipase (LesAffx.52427.1.S1_at) was more highly expressed in *rmc* roots. Genes involved in gibberellin (GA) synthesis, signaling, and degradation were induced in 76R MYC+ roots (Table 2). These included GA 2-β dioxygenase, GA 20-oxidase-1, GA 3-β hydroxylase, GA-requiring 3, and GA-requiring 1 copalyl diphosphate synthase (Les.4335.2.S1_at, Les.64.1.S1_at, Les.10.1.S1_at, LesAffx.70667.1.A1_at, and Les.27.1.S1_at respectively). Patch root samples were analyzed for changes in hormone concentrations with liquid chromatography- tandem mass spectrometry (LC/MS/MS) to test whether transcriptional changes in hormone biosynthesis and secondary metabolism genes correlated with phytohormone concentrations. With these methods we were able to analyze indole acetic acid (IAA), abscisic acid (ABA), jasmonic acid (JA), jasmonic acid—isoleucine (JA-Ile), and salicylic acid (SA) (Fig. 5). IAA and ABA concentrations were not significantly different among N treatment and mycorrhizal colonization status (Fig. 5a and b). SA concentrations were ~1.5-fold higher in mycorrhizal roots compared to *rmc* ($P<0.001$, Fig. 5e). The N treatments also affected root hormone levels including higher levels of JA and JA-Ile in the low N treatment (Fig. 5c and d) which was independent of the colonization status of the roots. Mycorrhizal roots exhibited slightly higher levels of JA-Ile compared to *rmc* roots specifically in the low N treatment ($P=0.07$, Fig. 5d).

Soil N transformations and root uptake

Twenty-four hours after $^{15}NH_4^+$-N injection into the rings, soil NH_4^+ concentrations were nearly 60- and 3.35-fold higher in the high NH_4^+ and low NH_4^+ treatment patches compared to the water control, respectively (treatment $P<0.001$, Fig. 6a). However, soil NH_4^+ concentrations were approximately 30% and 60% of the calculated applied amounts of the high NH_4^+ and the low NH_4^+ treatments, respectively. This indicates that rapid changes in plant-microbe-soil NH_4^+ transformations had occurred during this time period. Soil NO_3^- levels were 5-fold and 2.75-fold higher in the high NH_4^+ and low NH_4^+ treatment patches compared to the water control indicating substantial nitrification in 24 h (Fig. 6b).

Tomato roots took up significant amounts of labeled ^{15}N from the soil patches and translocated this N to shoot tissue over time. Patch roots from the low and high NH_4^+ treatment groups contained higher atom% ^{15}N compared to the water controls at the 24-hr harvest (Fig. 6c). The rapid increase in root N uptake corresponded with the large effect on root gene expression (Fig. 2b and c). Less than 7% of the added ^{15}N was recovered in the shoots of either genotype after 24 h (data not shown). Interestingly, *rmc* showed a trend towards higher atom% ^{15}N in the ring roots compared to 76R MYC+ ($P=0.08$) in both the low and high N treatments (Fig. 6c). When uptake of ^{15}N was followed over the course of a week in a parallel

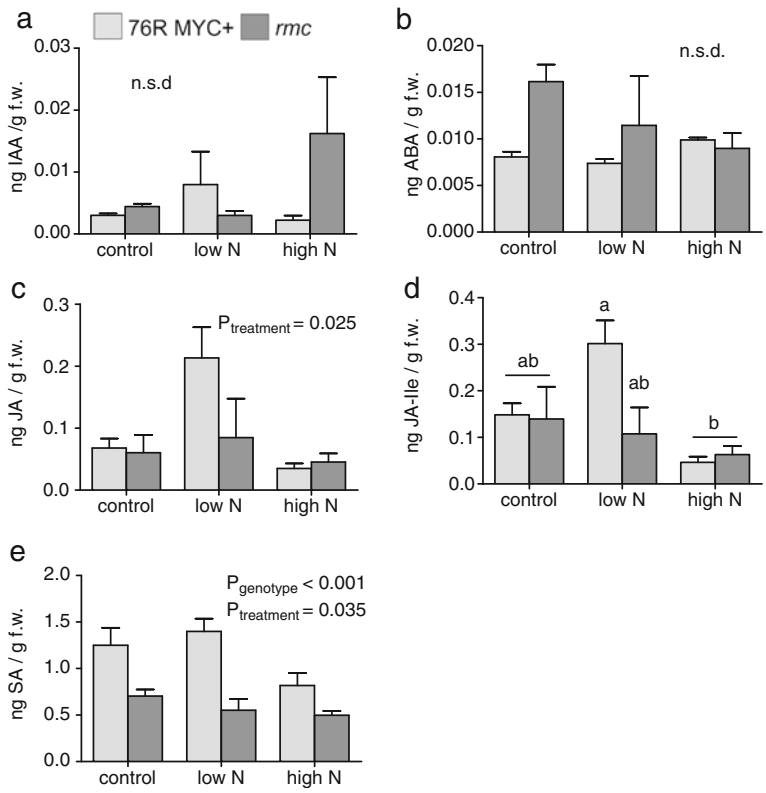


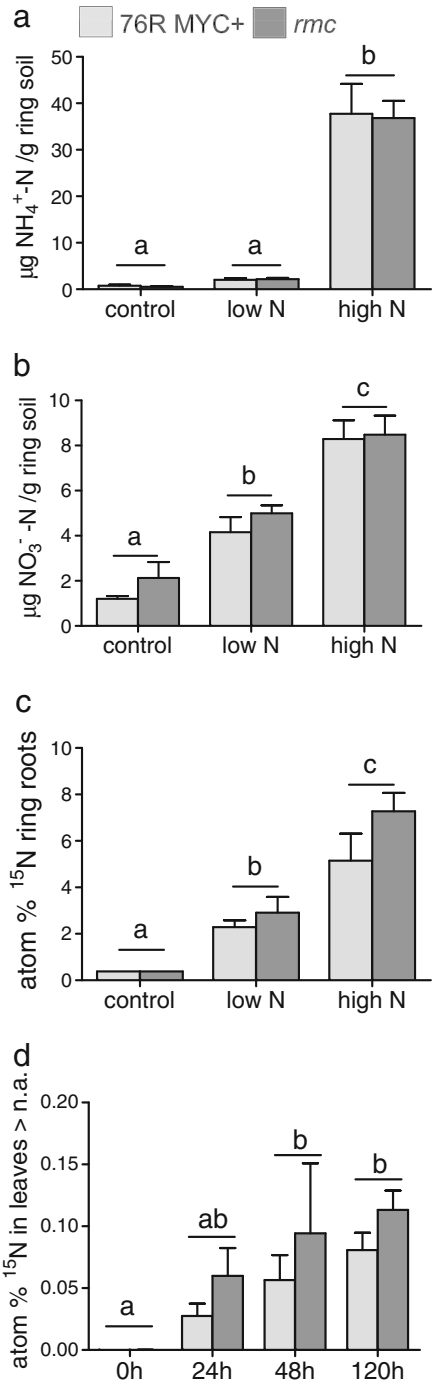
Fig. 5 Root plant hormone concentrations in response to the nitrogen patch and mycorrhizal symbiosis. Concentrations of plant hormones indole acetic acid (IAA) (**a**), abscisic acid (ABA) (**b**), jasmonic acid (JA) (**c**), jasmonic acid—isoleucine (JA-Ile) (**d**), and salicylic acid (SA) (**e**) were measured by LC/MS/MS in 76R MYC+ and *rmc* roots from the various N treatment plots, and calculated by dividing by the fresh weight of the root sample. The P values for significant main effects in JA (**c**) and SA (**e**) are noted (2-way ANOVA). For JA-Ile (**d**), different letters indicate significant differences among groups (2-way ANOVA, Tukey HSD). The light and dark grey bars represent 76R MYC+ and *rmc*, respectively. n.s.d; no significant difference. g f.w; grams fresh weight

set of high NH_4^+ treatment and water control plants, the atom% ^{15}N in the leaves increased significantly over time in the high NH_4^+ treatment plants, but no differences were found between mycorrhizal and *rmc* leaf tissue (time P=0.006, Fig. 6d).

General transcriptional responses to the N patch

Despite the trend of higher short-term ^{15}N uptake from the nitrogen patch by *rmc* roots, mycorrhizal plants had higher total N concentrations in shoot tissue (Fig. 7a). A search of the tomato genome sequence (http://solgenomics.net/about/tomato_project/) identified a novel tomato ammonium transporter gene (AC225328_5.1.1, LeAMT4) on chromosome 9 that was 68% identical to mycorrhizal root specific *L. japonicus* LjAMT2.2 (ABO83202). Expression analysis with qPCR showed that LeAMT4 was exclusively expressed in mycorrhizal roots, although was not significantly regulated by the NH_4^+ treatments (Fig. 7b). A second tomato ammonium transporter (AC214210_4.1, LeAMT5) that shares 64.4% amino acid identity with LeAMT4 was also exclusively expressed in mycorrhizal roots and not significantly regulated by the NH_4^+ treatments (Fig. 7c). Phylogenetic analysis of the tomato AMT gene family confirmed that LeAMT4 and LeAMT5 both fit into an arbuscular mycorrhizal-specific clade that includes mycorrhizal root specific AMTs *L. japonicus* LjAMT2.2 (ABO83202) and *M. truncatula* MtAMT (ABO83202) (Online Resource 9).

We searched for patterns in the microarray and qRT-PCR data that might indicate a differential response to the NH_4^+ treatments depending on the mycorrhizal colonization of the roots (2-way ANOVA, see materials and methods). The majority of genes regulated by the NH_4^+ treatments were not affected by the symbiosis (Online Resource 5). However, a number of N metabolism genes were found to be differentially regulated between mycorrhizal and *rmc* roots specifically under the most limiting N conditions (water control treatment) (Fig. 7d–k), and these expression differences between mycorrhizal and *rmc* roots were less pronounced or absent when N (6.5 or 65 μg NH_4^+-N g^{-1} soil) was added to the patch in the NH_4^+ treatment groups. Glutamine dehydrogenase GDH (Les.2649.1.S1_at) (Fig. 7d), cytosolic glutamine synthetase GS1 (Les.224.1.A1_at) (Fig. 7e) and asparagine synthetase AS



◀ **Fig. 6** Soil and root N dynamics and uptake in response to the nitrogen treatments and mycorrhizal symbiosis. Ring soil was analyzed for NH_4^+ (**a**) and NO_3^- (**b**) concentrations. Plants were analyzed for percent recovery of ^{15}N in the roots (**c**). Atom% recovery ^{15}N differed in roots by treatment ($P<0.001$) and showed a trend toward higher atom% ^{15}N in *rmc* compared to 76R MYC+ ($P=0.08$). ^{15}N uptake was also measured in individual leaflets over time on a parallel set of plants and displayed as the atom% excess of ^{15}N in the treatment group above the water control natural abundance (n.a.) ^{15}N level (**d**). Letters indicate significant differences among treatments ($P<0.05$). Light and dark grey bars are for 76R MYC+ and *rmc*, respectively

(Les.4317.1.S1_at) (Fig. 7f) were induced in both 76R MYC+ and *rmc* roots by the N treatments, but under the water control conditions, all three were more highly expressed in 76R MYC+ roots compared to *rmc* roots. Nitrate reductase NR (Fig. 7g) and nitrite reductase Nii2 (LesAffx.45315.4.S1_at) (Fig. 7h) were also induced by the N treatments, but were specifically more highly expressed in *rmc* roots compared to 76R MYC+ roots in the water control treatment. Furthermore, while the expression levels of nitrate transporters NRT1.1-like (TC207669) (Fig. 7i) and NRT3.2 (LesAffx.69264.1.S1_at) (Fig. 7j) did not respond to the N treatments, both genes were expressed more highly in *rmc* roots compared to 76R MYC+ specifically in the water control treatment. The NH_4^+ transporter AMT2 (Les.3640.1.S1_at) (Fig. 7k) was induced by the N treatments in mycorrhizal roots, but not in *rmc* roots. Under the lower N conditions of the water control AMT2 was more highly expressed in *rmc* roots as compared to the wild-type roots. These expression differences between 76R MYC+ and *rmc* roots under the water control conditions are summarized and put into the context of N uptake, assimilation and metabolism in Fig. 8.

Discussion

Transcriptome and metabolome changes in response to the AM symbiosis

The complex relationship between plant roots and mycorrhizal fungi necessitates the continuous exchange of signals and the regulation of plant genes to control downstream changes in plant development and physiology. The higher concentration of P in mycorrhizal plants observed in this study is consistent with previous field observations (Cavagnaro et al. 2008). In this study the non-mycorrhizal mutant (*rmc*) exhibited a more pronounced phosphate starvation response which included the increased expression of root P transporters PT1 and PT2. These findings are in agreement with the well accepted model of the suppression of the general phosphate starvation

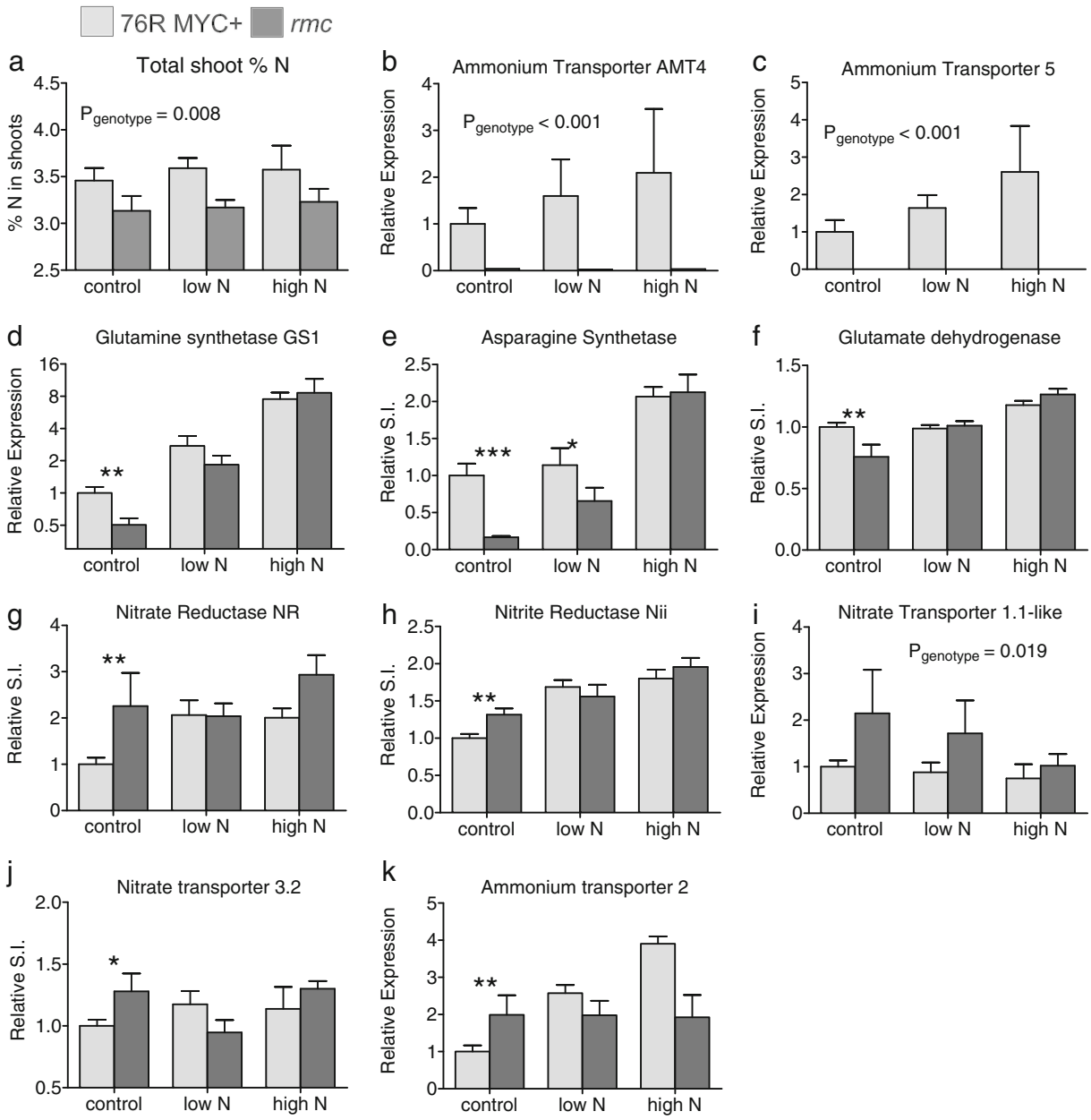


Fig. 7 Shoot N levels and root expression of N transporter, assimilation and metabolism genes in response to the nitrogen treatments and mycorrhizal symbiosis. Leaf N concentration was measured in 76R MYC+ and *rmc* plants (**a**). qPCR and/or microarrays were used to assay the expression level of NH_4^+ transporter LeAMT4 (**b**), NH_4^+ transporter LeAMT5 (**c**), glutamine synthetase (GS) (**d**), asparagine synthetase (ASN1) (**e**), glutamate dehydrogenase (GDH) (**f**), nitrate transporter NRT1.1-like (**g**), nitrate transporter 3.2 (**h**), nitrate reductase (**i**), nitrite reductase (**j**), and ammonium transporter AMT2 (**k**). qPCR relative expression was calculated using the ΔΔCT method with actin (LeACT) as the reference control, and the 76R MYC+ water treatment group normalized to 1. Microarray relative signal intensities were calculated in reference to the 76R MYC+ water treatment group and plotted as an average of 5 replicates. The P values for significant main effects are noted and asterisks represent a significant difference between 76R MYC+ and *rmc* at the given pairwise comparison (***<0.001, **<0.01, *<0.05) (2-way ANOVA, tukey HSD). Light and dark grey bars are for 76R MYC+ and *rmc*, respectively

response and certain root Pi transporters mycorrhizal roots (Bucher 2007).

Previous studies have also shown that the mycorrhizal symbiosis increases both plant macro- and

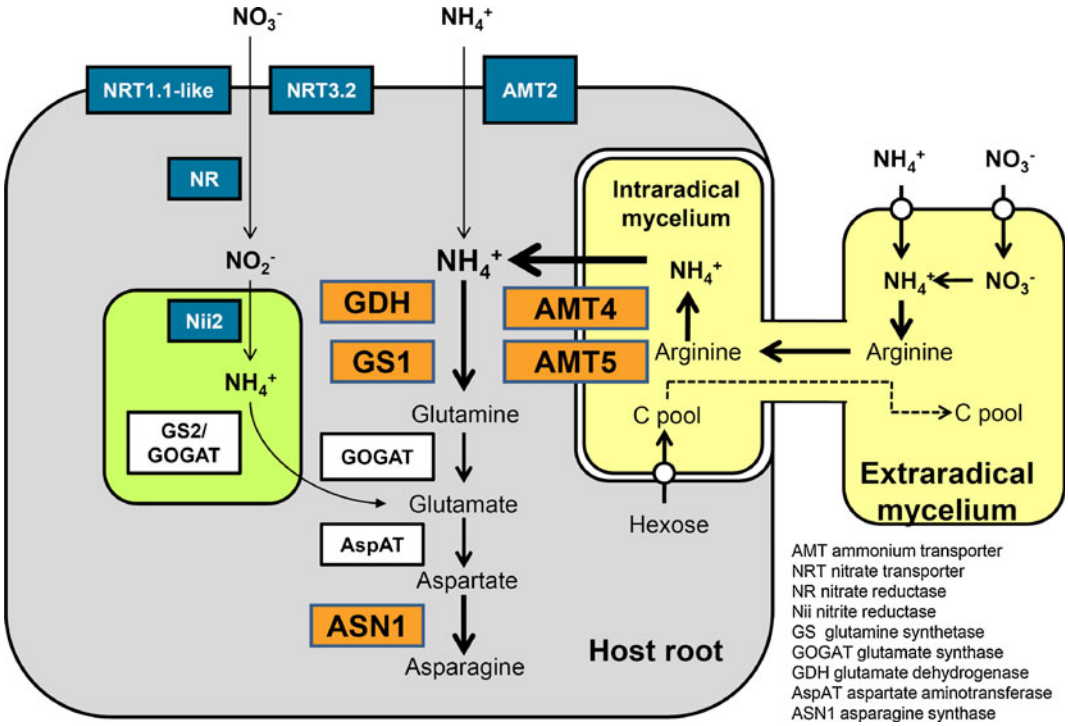


Fig. 8 Model of nitrogen uptake and assimilation gene regulation in response to the mycorrhizal symbiosis under lower soil N conditions. Mycorrhizal roots exhibit a dramatically altered N uptake, assimilation and amino acid metabolism transcriptional profile compared to non-mycorrhizal roots in the control treatment, suggesting increased mycorrhizal-mediated N uptake and assimilation and decreased direct root-mediated N uptake and assimilation. *Yellow*: arbuscular mycorrhizal fungal extraradical mycelium and intraradical mycelium compartments. Grey: Plant root cell. *Green*: plastid. Gene colors indicate expression level differences between 76R MYC+ and *rmc* samples in the water control treatment conditions (*blue* with *white* text = higher in *rmc*, orange with *black* text = higher in 76R MYC+). Thicker arrows suggest a shift toward specified metabolic pathway based on the transcriptional data. Abbreviations: *GS* glutamine synthetase, *GOGAT* glutamate synthase, *ASN* asparagine synthetase, *AMT* ammonium transporter, *NRT* nitrate transporter, *NR* nitrate reductase, *NRT* nitrite reductase, *GDH* glutamate dehydrogenase, *AspAT* aspartate aminotransferase

micronutrient concentrations (Cavagnaro et al. 2008; Liu et al. 2000). When soil S availability is low, the AM symbiosis can increase S levels in plant tissues (Banerjee et al. 2003; Guo et al. 2006). Recent work demonstrated the role of AM in increasing root S content through the transcriptional regulation of a putative fungal sulfate permease (Allen and Shachar-Hill 2009). Studies on mycorrhizal regulation of copper and ammonium transporters in *Medicago truncatula,* and NH_4^+, S, and K transporters in *Lotus japonicus* identified putative transporters but did not assay corresponding plant tissue nutrient levels (Gomez et al. 2009; Guether et al. 2009b). This study and previous work with these tomato genotypes grown on the same organic farm soil showed higher concentrations of Cu and S in shoot tissue (Cavagnaro et al. 2008) of the mycorrhizal roots compared to non-mycorrhizal mutant *rmc*. This work provides a link between the mycorrhizal-induced plant S and Cu transporters and the increased S and Cu concentrations in leaf tissue of the mycorrhizal wild-type plants. Further work will be necessary to establish a causal relationship between these candidate mycorrhizal-induced transporters and increased shoot nutrient levels.

Plant hormones and the AM symbiosis

Hormones regulate many plant responses and are thus strong candidates for regulation of physiological processes during colonization. AM fungal colonization has been shown to induce numerous hormone biosynthesis and signaling genes and alter hormone levels (García Garrido et al. 2010; Hause et al. 2007; López-Ráez et al. 2010; Shaul-Keinan et al. 2002). In this study multiple genes were induced in mycorrhizal

roots involved in GA metabolism (gibberellin 20-oxidase, gibberellin 2-oxidase, gibberellin 3-oxidase, GA-requiring 3 cytochrome P450 ent-kaurene oxidase, and GA-requiring 1 copalyl P synthase). Studies in *M. truncatula* and tobacco have also shown that the GA biosynthesis pathway is induced by AM symbiosis (Hohnjec et al. 2005; Shaul-Keinan et al. 2002), suggesting a conserved role for GA in the AM symbiosis across plant species and ecological settings. GA may play a role in repressing P starvation response (Jiang et al. 2007), which is also repressed in the wild-type mycorrhizal tomato roots (Table 1). Higher concentrations of GA in mycorrhizal roots may increase the strength of the carbohydrate sink that is created and utilized by the fungus (Blee and Anderson 1998). The simultaneous up-regulation of both GA biosynthesis and degradation enzymes in the present study also suggests a dynamic role for this plant hormone in the AM symbiosis that may vary by root cell type or throughout the course of arbuscule development.

Mycorrhizal colonization also impacts plant hormones involved in defense and stress responses such as SA (Gutjahr et al. 2008; Hause et al. 2007). Mycorrhizal plants had significantly higher concentrations of SA in their roots and significantly higher expression of multiple methyltransferases that may function in SA metabolism (Vlot et al. 2009); however genes in the SA biosynthesis pathway were not differentially expressed. Previous studies also support the idea that SA levels increase in roots at early stages of AM interaction when AM may trigger a defense response in the root (Blilou et al. 1999; 2000). Considering that 12 of the 174 genes differentially regulated between mycorrhizal and *rmc* roots function in disease/defense response (Fig. 2a), it is possible that the AM symbiosis induced an SA-mediated defense response in mycorrhizal roots (Liu et al. 2003).

Nitrogen transformations and metabolism

Fertilizer application on conventional and organic farms often occurs in pulses and patches similar to the nutrient additions applied in this experiment. Rapid N uptake occurs with a backdrop of highly complex soil processes; the roots must rapidly sense N and compete successfully with soil microbial and passive soil processes such as soil microbial N assimilation and nitrification, leaching and denitrification (Burger and Jackson 2005; Raun and Johnson 1999). N transformation in the soil was rapid with much of the added NH_4^+ being transformed to NO_3^- within 24 h. Because soil moisture was < 50% water filled pore space, the loss of NO_3^- via denitrification in this experiment was assumed to be negligible (Linn and Doran 1984). Over 24 h, plants were able to rapidly capitalize on the nutrient patch by taking up approximately seven percent of the applied ^{15}N. The high competition between roots and soil microbes, the relatively low root length densities in the rings, and the short 24 h treatment time may all contribute to the lower recovery rates of ^{15}N compared to other studies (Burger and Jackson 2005).

In an earlier greenhouse study (Ruzicka et al. 2010), we reported on the transcriptional response to an enriched N patch after 53 h of mycorrhizal roots of tomato plants grown in pots under greenhouse conditions. That response included more than 550 genes, and suggested an active and sustained response to both NH_4^+ and NO_3^- in the patch after 53 h. In this present study, we identified sixty one genes as being similarly regulated between the current field study and the earlier greenhouse pot study (Online Resource 6), highlighting the value of field experiments to validate transcriptional responses measured under controlled environment conditions. Many of the overlapping genes encode proteins that function in nitrogen metabolism, protein synthesis and turnover, and transcriptional regulation. These genes may represent a core transcriptional response to N uptake and patch utilization across variable environmental or ecological conditions. Future work could utilize these genes as markers to identify roots that are responding to soil N availability, even when soil microbial processes of nitrification and immobilization are rapidly changing.

AM-mediated N uptake and metabolism

Putative ammonium transporters LeAMT4 and LeAMT5 were expressed exclusively in wild-type mycorrhizal roots and may be the key plant mycorrhizal NH_4^+ transporters in tomato. Orthologous *Lotus* and *Medicago* AMT genes are also induced by the symbiosis, and LjAMT2.2 was shown to function as *bona fide* ammonium transporter (Gomez et al. 2009; Guether et al. 2009b). In contrast to other non-

symbiosis specific tomato ammonium transporters that are regulated by the presence or absence of NH_4^+ and NO_3^- (Ruzicka et al. 2010; Von Wirén et al. 2000), mycorrhizal-specific LeAMT4 and LeAMT5 were similarly expressed under all N treatment conditions. This suggests that the mycorrhizal symbiosis may supply roots with additional N in a constitutive manner regardless of soil N status in contrast to the mycorrhizal Pi uptake mechanism that is repressed by plant and soil Pi (Nagy et al. 2009). It is also a possibility that these mycorrhizal-specific N transporters could be regulated by plant N status, which was similar in all of the treatments in this study presumably due to the short 24 h exposure to the ammonium added to the soil patch.

The transcriptional regulation of plant N uptake, assimilation, and amino acid metabolism genes in this study contribute new information to the plant response to AM symbiosis N uptake (Fig. 8). Previous models of the AM symbiosis N uptake mechanism have detailed the role of the arbuscular mycorrhizal fungus partner (Govindarajulu et al. 2005; Jin et al. 2005; Tian et al. 2010). The higher total N concentration of mycorrhizal wild-type shoot tissue (Fig. 7a) suggested that mycorrhizal plants were taking up and assimilating more N than the non-mycorrhizal plants throughout the growing season. Based on tomato root transcriptome analysis, this increase appears to be facilitated through the mycorrhizal-specific AMT4 and AMT5 ammonium transporters, although due to the partial genome coverage of the tomato GeneChip it is possible that other transporters may play a role. Consequently, cytosolic NH_4^+ supplied via these mycorrhizal-specific transporters could act as a signaling molecule in the root cell under relatively low external N conditions, triggering the induction of N assimilation and amino acid metabolism enzymes glutamate dehydrogenase, cytosolic glutamine synthetase, and asparagine synthetase (Fig. 8, orange boxes). This agrees with the previous observation of increased glutamine synthetase expression in carrot upon treatment of fungal extraradical mycelium with KNO_3 (Tian et al. 2010). Perhaps more intriguing are the effects of the mycorrhizal symbiosis on non-symbiosis specific NO_3^- and NH_4^+ transporters NRT3.2, NRT1.1-like, and AMT2, as well as NO_3^- reductase and NO_2^- reductase (Fig. 8, blue boxes). These genes were all repressed in mycorrhizal roots in the water control, suggesting the down-regulation of the direct root NO_3^- and NH_4^+ uptake pathways in the presence of an active supply of NH_4^+ derived from mycorrhizal fungi. Cumulatively, these expression patterns fit into a model where additional NH_4^+ supplied via the mycorrhizal-mediated uptake pathway (i.e. LeAMT4 and LeAMT5) induces N assimilation gene expression to utilize the additional cytosolic NH_4^+ and reduces the need for other direct root-mediated inorganic N uptake pathways. The apparent shift away from direct root-mediated NO_3^- uptake to mycorrhizal-mediated NH_4^+ uptake may represent an energetically advantageous avenue for N uptake (Bloom et al. 2002). Alternatively, it may balance the different uptake pathways and increase the NH_4^+:NO_3^- ratio, which may result in an enhanced growth response (Lopes and Araus 2008). Given the more mobile nature of NO_3^-, it is interesting to speculate that non-mycorrhizal roots depend more on encounters with mobile NO_3^- while mycorrhizal roots utilize fungal N uptake from organic N, NH_4^+ or NO_3^- (Hodge and Fitter 2010; Tian et al. 2010).

This study quantified changes in roots after a short pulse of nitrogen. However, it would be interesting to test how mycorrhizal N uptake pathways and mycorrhizal colonization are affected when plants are grown for long periods under high N. Previous studies have demonstrated that sufficient Pi reduces both AM colonization and the positive effects of the symbiosis on plant growth (Bruce et al. 1994; Mendoza and Pagani 1997), but studies under high or sufficient N availability report both positive and negative effects on AM (Jackson et al. 2002; Olsson et al. 2005; Takacs et al. 2007).

Implications for sustainable agriculture

Mycorrhizae clearly play a key role in plant nutrition, particularly N and Pi, and yet they are largely absent from conventional farms because of the use of fertilizers and pesticides. Organic farms tend to have much higher abundances of AM fungi and may be better poised to benefit from their presence, as mycorrhizae can improve soil aggregation (Rillig and Mummey 2006), decrease leaching (Asghari et al. 2005), increase the efficiency of nutrient cycling (Conyers and Moody 2009), and improve the nutritional quality of crops under certain conditions (Cavagnaro et al. 2008). Our study of the AM root transcriptome identified key N uptake and metabolism processes that were influenced by the AM symbiosis

in lower N soil conditions, highlighting the importance of an active AM symbiosis to plant nutrient uptake in lower input agriculture. More broadly, our results suggest that future field AM-root transcriptome studies may be useful to identify mechanisms by which mycorrhizae improve the efficiency of nutrient cycling, and may ultimately guide farmers to reduce their inputs and reduce nutrient losses to the environment (Fitter et al. 2011). As agriculture moves toward ecological intensification practices that include crop rotations, cover crops, and reduced pesticide, chemical fertilizer, and water inputs, mycorrhizae will almost certainly play a larger and more important role on the farmscape and in sustainable agriculture.

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