Received: 27 April 2018 | Revised: 27 May 2018 | Accepted: 28 May 2018

DOI: 10.1111/pce.13359

ORIGINAL ARTICLE

WILEY Plant, Cell & Environment

Nutrient demand and fungal access to resources control the carbon allocation to the symbiotic partners in tripartite interactions of *Medicago truncatula*

Arjun Kafle[1] | Kevin Garcia[1] | Xiurong Wang[1,2] | Philip E. Pfeffer[3] | Gary D. Strahan[3] | Heike Bücking[1]

[1] South Dakota State University, Biology and Microbiology Department, Brookings, South Dakota

[2] South China Agricultural University, State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, Root Biology Center, Guangzhou, China

[3] Eastern Regional Research Center, USDA, Agricultural Research Service, Wyndmoor, Pennslyvania

Correspondence
H. Bücking, Biology and Microbiology Department, South Dakota State University, Brookings, SD 57007.
Email: heike.bucking@sdstate.edu

Funding information
USDA, Grant/Award Number: 2017-67014-26530; SD Soybean Research and Promotion Council; Agricultural Experiment Station at SDSU

Abstract

Legumes form tripartite interactions with arbuscular mycorrhizal fungi and rhizobia, and both root symbionts exchange nutrients against carbon from their host. The carbon costs of these interactions are substantial, but our current understanding of how the host controls its carbon allocation to individual root symbionts is limited. We examined nutrient uptake and carbon allocation in tripartite interactions of *Medicago truncatula* under different nutrient supply conditions, and when the fungal partner had access to nitrogen, and followed the gene expression of several plant transporters of the Sucrose Uptake Transporter (SUT) and Sugars Will Eventually be Exported Transporter (SWEET) family. Tripartite interactions led to synergistic growth responses and stimulated the phosphate and nitrogen uptake of the plant. Plant nutrient demand but also fungal access to nutrients played an important role for the carbon transport to different root symbionts, and the plant allocated more carbon to rhizobia under nitrogen demand, but more carbon to the fungal partner when nitrogen was available. These changes in carbon allocation were consistent with changes in the SUT and SWEET expression. Our study provides important insights into how the host plant controls its carbon allocation under different nutrient supply conditions and changes its carbon allocation to different root symbionts to maximize its symbiotic benefits.

KEYWORDS

arbuscular mycorrhizal symbiosis, carbon transport, *Ensifer meliloti*, legumes, nitrogen uptake, rhizobia, *Rhizophagus irregularis*, sucrose transport, sucrose uptake transporter (SUT), sugars will eventually be exported transporter (SWEET)

1 | INTRODUCTION

Legumes, such as soybean, cowpea, and *Medicago*, are among the most important crop species worldwide. They account for 27% of the world's primary crop production, for 33% of the dietary nitrogen (N) needs of humans (Vance, 2001), and play a significant role in crop rotations and in the soil N cycle. The majority of legumes form tripartite interactions and are simultaneously colonized with N-fixing bacteria and arbuscular mycorrhizal (AM) fungi. It is well known that these interactions can substantially contribute to the nutrient acquisition of legumes and increase the fitness of both the host and the different root symbionts (Afkhami & Stinchcombe, 2016; Mortimer, Pérez-Fernández, & Valentine, 2009; Ossler, Zielinski, & Heath, 2015).

N-fixing rhizobia bacteria reside within specialized root nodules that provide them with an oxygen-reduced environment for biological N_2-fixation (BNF). Within nodules, rhizobia differentiate into bacteroids that are able to convert atmospheric N_2 to NH_3 through their nitrogenase complex. NH_3 is exported together with amino acids

through the bacteroid membrane towards the host cells (Udvardi & Poole, 2013) and can contribute with up to 99% to the total N uptake of the plant under low N supply conditions (Burchill et al., 2014). AM fungi, on the other hand, form an extensive extraradical mycelium in the soil that takes up nutrients, such as phosphate (P) and N, and transfers them to the host via specialized fungal structures in root cortical cells, called arbuscules (Bücking & Kafle, 2015; Fellbaum et al., 2014; Smith & Read, 2008). In addition, AM fungi improve the resistance of their host plant against abiotic (e.g., drought and salinity) and biotic stresses (pathogens; Smith & Read, 2008).

It is well established that a synergy of benefits can occur and that the host plant can gain more from tripartite interactions than from single inoculations with either symbiont (Afkhami & Stinchcombe, 2016; Antunes, de Varennes, Zhang, & Goss, 2006; Bournaud et al., 2017; Meng et al., 2015; Yasmeen, Hameed, Tariq, & Ali, 2012). The N-fixing capability of rhizobia is often limited by the P availability, and AM fungi can stimulate root nodulation, nitrogenase activity, and BNF through their positive effect on plant P nutrition (Ding et al., 2012; Owino-Gerroh, Gascho, & Phatak, 2005; Püschel et al., 2017; Vesterager, Nielsen, & Hogh-Jensen, 2006). Plants can simultaneously benefit from N that is provided by both root symbionts, and nodulated legumes colonized by AM fungi with access to an external NH_4^+ source became less reliant on BNF (Mortimer, Perez-Fernandez, & Valentine, 2012). Similarly, Nod factors produced by rhizobia have been shown to enhance AM colonization (Xie et al., 1995; Xie, Muller, Wiemken, Broughton, & Boller, 1998), and both symbiotic interactions share parts of a common signal transduction pathway (Delaux, Séjalon-Delmas, Bécard, & Ané, 2013; Kistner et al., 2005; Zhu, Riely, Burns, & Ané, 2006). However, negative effects have also been observed, and the prior inoculation by either rhizobia or AM fungi can limit the subsequent colonization by either symbiont (Catford, Staehelin, Larose, Piché, & Vierheilig, 2006; Catford, Staehelin, Lerat, Piché, & Vierheilig, 2003; Valentine, Mortimer, Kleinert, Kang, & Benedito, 2013). It has been suggested that plants control the extent of root colonization by both symbionts by an auto-regulatory mechanism, possibly to limit the high carbon (C) costs associated with these interactions (Kassaw, Bridges Jr., & Frugoli, 2015; Mortimer, Pérez-Fernández, & Valentine, 2008; Reid, Ferguson, Hayashi, Lin, & Gresshoff, 2011). Both interactions are costly, and AM fungi can receive up to 20% (Jakobsen & Rosendahl, 1990; Snellgrove, Splittstoesser, Stribley, & Tinker, 1982; Wright, Read, & Scholes, 1998) and rhizobia up to 30% of the host photosynthates (Provorov & Tikhonovich, 2003).

In plants, sucrose is the main carbohydrate for long-distance transport and is loaded in the leaves into the phloem and then transferred to the sink tissues. In root nodules, sucrose is mainly converted to malate, which is considered to be the primary C source transferred across the symbiosome membrane to the bacteroids (Oldroyd, Murray, Poole, & Downie, 2011; Udvardi & Poole, 2013). Hexoses were long seen as the major C form that is transferred across the interface to the AM fungus (Helber et al., 2011), but recent reports revealed that fatty acids can also be exported out of the root cell and transported to the fungal symbiont (Bravo, Brands, Wewer, Dörmann, & Harrison, 2017; Jiang et al., 2017; Keymer et al., 2017; Luginbuehl et al., 2017; Rich, Nouri, Courty, & Reinhardt, 2017). This C supply plays a critical role for symbiont function (Fellbaum et al., 2014; Kiers et al., 2011; Kiers, Rousseau, West, & Denison, 2003), but how C is partitioned and directed to different symbiotic partners is still unknown. It has been suggested that Sucrose Uptake Transporters (SUT) could be involved in the regulation of beneficial C fluxes towards the fungal symbiont (Doidy et al., 2012; Garcia, Doidy, Zimmermann, Wipf, & Courty, 2016), and recently, Sugars Will Eventually be Exported Transporters (SWEET) have been identified in AM and nodulated roots (Kryvoruchko et al., 2016; Manck-Götzenberger & Requena, 2016; Sugiyama et al., 2017). The SWEET family mediates the influx and efflux of sugar molecules from cells and plays a role in phloem loading and unloading (Lemoine et al., 2013). In *Medicago*, *MtSWEET11* is specifically expressed in root nodules, but loss-of-function mutants were not compromised in BNF, indicating that this transporter could be involved in sugar distribution within root nodules but may not be a critical component for BNF (Kryvoruchko et al., 2016).

Despite their importance for nutrient uptake and crop yield, nutrient to C exchange dynamics in tripartite interactions are only poorly understood. Exploiting the full yield potential of legumes will require a better understanding of these interactions, but functional insights into these interactions are currently mainly derived from experiments with plants associated with a single symbiont. The goal of our study is to contribute to a better understanding on how host plants regulate their C allocation to their different root symbionts in tripartite interactions, because this knowledge is critical to improve the nutrient efficiency and symbiotic benefits in agriculturally important legumes.

2 | MATERIAL AND METHODS

2.1 | Plant, fungal, and bacterial material

Medicago truncatula (A17) seeds were scarified with concentrated H_2SO_4 and surface sterilized with 8% bleach for 2 min. The plants were pregerminated on moist filter paper in Petri dishes for 3 days in the dark, followed by 7 days under light. To facilitate lateral root development, we cut the primary roots of the germinated seedlings before transferring them for 20 days into a hydroponic solution containing 0.05 mM KH_2PO_4, 0.125 mM NH_4NO_3, 0.30 mM KCl, 0.5 mM $CaCl_2 \cdot 2H_2O$, 0.312 mM MgSO4 · $2H_2O$, 6.8 μM Fe-EDTA, 1.50 μM $MnCl_2 \cdot 2H_2O$, 8.08 μM H_3BO_3, 0.05 μM Zn-EDTA, 0.14 μM $CuCl_2 \cdot 2H_2O$, and 0.01 μM $Na_2MoO_4 \cdot 2H_2O$ (Ingestad, 1960). Twice daily, the solution was stirred and replaced once after 10 days.

We then transferred the seedlings into custom-made multicompartment systems (12 cm × 8 cm × 8 cm, L × H × W) with three compartments, two root compartments (RC), and one hyphal compartment (HC; Figure S1). All compartments were filled with 200 ml soil substrate consisting of 60% turface (Profile Products LLC, IL, USA), 30% sand, and 10% organic soil (13.05 mg L^{-1} nitrate, 2.28 mg L^{-1} ammonium, and 24.19 mg L^{-1} available P, Olsen's extraction; Experiment 1) or 80% sand, 10% perlite, and 10% organic soil (14.77 mg L^{-1} nitrate, 9.03 mg L^{-1} ammonium, and 20.77 mg L^{-1} available P, Olsen's extraction; Experiment 2). Both RCs were separated by a 0.1-cm-thick plastic sheet that was sealed at all sides by silicone

(Aqueon, Franklin, WI, USA) to prevent any cross-contamination between the RCs. The HC was separated from the RC by a plastic sheet with a hole (~3.12-cm diameter) that was closed on both sides with a 50-μm nylon mesh. In between the two fine mesh layers, we placed a coarse nylon mesh with a pore size of 1,000 μm to form an air gap and to prevent mass flow from the HC to the RC. The mesh layers prevented the crossover of roots from the RC to the HC but allowed in the AM colonized growth systems the crossover of the fungal mycelium into the HC. We divided the root system of the plants equally into two root halves, and each root half was transferred into an independent RC. After transplanting, the plants were grown in a controlled-environment chamber with a 25°C/20°C day and night cycle, 30% humidity, and a photosynthetic active radiation of 225 μmol m^{-2} s^{-1}.

We produced the fungal inoculum of *Rhizophagus irregularis* Schenck & Smith (DAOM 197198) in axenic Ri T-DNA transformed carrot (*Daucus carota* clone DCI) root organ cultures in Petri dishes filled with mineral medium (St-Arnaud, Hamel, Vimard, Caron, & Fortin, 1996). After approximately 8 weeks of growth, the spores were isolated by blending the medium in 10 mM citrate buffer (pH 6.0). The bacterial inoculum was produced by growing *Ensifer meliloti* Dangeard (1021; previously *Sinorhizobium meliloti*) in tryptone yeast media on a rotatory shaker at 250 rpm at 28°C for 20 hr. Before the inoculation, the bacteria were centrifuged and resuspended in autoclaved tap water. Fungal and bacterial inocula were added into a hole in the soil close to the root approximately 5 cm below the soil surface.

2.2 | Experimental design

We conducted two experiments and examined the C allocation to different root symbionts depending on whether the fungal partner had access to an exogenous N supply (Experiment 1) and depending on the nutrient demand conditions of the host (Experiment 2; Figure S1). In Experiment 1, we studied the C allocation in four different systems with (a) two noninoculated root halves (Ø/Ø), (b) one noninoculated root half and one inoculated with *R. irregularis* (Ø/AM), (c) one noninoculated root half and one inoculated with *E. meliloti* (R/Ø), and (d) two inoculated root halves, one inoculated with *E. meliloti*, and one inoculated with *R. irregularis* (R/AM; Figure S1). The AM root halves were inoculated with 500 spores at transplanting and the rhizobia root halves 3 weeks after transplanting. Because the root system of one sacrificed plant did not show clear signs of AM inoculation after 3 weeks, we repeated the AM inoculation with 100 spores 4 and 7 weeks post-transplanting. To induce nutrient demand, the plants were fertilized three times with relatively low P and N concentrations (125 μM N as NH_4NO_3 and 50 μM KH_2PO_4 in the soil) in a modified Ingestad (1960) nutrient solution. To test whether the access of N for the AM fungus has an effect on the C allocation, we added 4 mM of $^{15}NH_4Cl$ (Sigma Aldrich, St. Louis, USA; +N) in a modified nutrient solution (Ingestad, 1960; no other P or N source) to the HC of half of the systems 12 weeks after transplanting. The controls (−N) received the same nutrient solution but without ^{15}N. To control for any leakage or any mass flow from the HC to the RC, we also added $^{15}NH_4Cl$ to the control treatments (Ø/Ø) and to systems that were only inoculated with *E. meliloti* (R/Ø). Because none of these systems showed any ^{15}N enrichment, we later considered them as −N. Four weeks later (16 weeks post-transplanting), the plants were labelled with $^{13}CO_2$ as described below.

In Experiment 2, we examined the C allocation to both root symbionts in tripartite interactions under different nutrient demand conditions for the host. We inoculated one of the RCs at transplanting with ~1,000 spores of *R. irregularis* and the other RC 3 weeks later with 1 ml (O.D. of 0.28) of a bacterial suspension with *E. meliloti*. Until the final nutrient treatment, we fertilized each RC every week with a modified Ingestad (1960) nutrient solution containing 250 μM NH_4NO_3 and 50 μM KH_2PO_4. The nutrient concentrations were relatively low to induce P and N demand conditions and to stimulate the AM and rhizobial colonization of the root systems. Ten weeks after transplanting, the nutrient demand conditions of the plants were varied by adding a modified Ingestad (1960) nutrient solution with combinations of low (L) or high (H) P or N concentrations to both RCs (LPLN, LPHN, HPLN, or HPHN). The nutrient levels in the soil solution were 50 μM or 650 μM KH_2PO_4 (LP or HP) or 0.25 mM or 1.8 mM NH_4NO_3 (LN or HN), respectively. Three weeks later (13 weeks post-transplanting), the plants were labelled with $^{13}CO_2$.

For the labelling with $^{13}CO_2$, we covered the soil in the growth chambers with a transparent plastic foil and transferred all plants into an air tight chamber (76 × 61 × 15.6 cm), in which 118 μl ml^{-1} $^{13}CO_2$ was released for 2 hr. A fan ensured a homogenous distribution of $^{13}CO_2$ within the chamber during the labelling. The plants were harvested 24 hr after labelling and were analysed for their biomass characteristics, fungal and bacterial colonization rates, nutrient contents, ^{13}C-labelling, and gene expression.

2.3 | Biomass characteristics and quantification of rhizobial and AM root colonization

After harvest, each root half was weighed and divided into three aliquots; one aliquot was flash frozen in liquid N and stored at −80°C for gene expression analysis, one aliquot was stored in 50% ethanol (v:v) to determine the fungal and bacterial root colonization, and one aliquot and the plant shoots were dried in an oven at 70°C for 48 hr. Based on the fresh to dry weight ratio of this root aliquot, the total root biomass was determined. Root nodules were removed, counted, and dried in an oven at 70°C for 48 hr. To determine the AM colonization, the roots were cleared with 10% KOH solution at 80°C for 30 min, rinsed, and stained with 5% ink at 80°C for 15 min (Vierheilig, Coughlan, Wyss, & Piché, 1998). We analysed a minimum of 150 root segments to determine the percentage of AM root colonization by the gridline intersection method (McGonigle, Miller, Evans, Fairchild, & Swan, 1990).

2.4 | Measurements of nitrogenase activity

To measure the nitrogenase activity of the root nodules, we carefully removed an aliquot of the nodulated root half at plant harvest, loosely wrapped it in moist filter paper, and transferred the samples into airtight 30-ml tubes sealed with rubber cork. We injected 10% (3 ml) acetylene gas and measured the production of ethylene after 24 hr using an Agilent Technologies 7890A Gas Chromatography System

(Santa Clara, CA, USA). Sample peak areas were fitted to a calibration curve, and the ethylene production was normalized to the nodule number of the root aliquots.

2.5 | Quantification of P, ^{13}C, and ^{15}N in plant tissues

Aliquots of shoot and root tissues were first pulverized with a tissue homogenizer (Precellys 24, Cayman Chemical Company, Ann Arbor, MI, USA). We digested the plant tissues with 2 N HCl for 2 hr at 95°C and determined the P content spectrophotometrically at 436 nm after adding ammonium molybdate vanadate solution (Fisher Scientific, Pittsburgh, USA). ^{13}C in the shoot and root tissues was quantified using a Costech 4010 and Carlo Erba 1110 Elemental Analyser coupled to a Thermo Delta Plus XP IRMS at the stable isotope facility of the University of Wyoming (Laramie, WY, USA). The conversion of $\delta^{13}C$ into the C contents in plant biomass was conducted according to Ruehr et al. (2009). Percentage ^{13}C allocations were calculated based on the total $\delta^{13}C$ that was recovered from the plants after ^{13}C labelling and under consideration of the plant biomass.

For the ^{15}N analysis by quantitative nuclear magnetic resonance spectroscopy, we first digested 10–15 mg aliquots of homogenized and oven-dried root and shoot material in 750 µl concentrated H_2SO_4. Samples were then heated for 2 hr at 225°C followed by an addition of 36 drops of 30% H_2O_2 (three drops at a time every 30 s) as previously described (Fellbaum et al., 2012). The solution was then heated for an additional 3 hr at 225°C to remove any traces of water and allowed to cool. Forty microlitres of the resulting clear solution of $(NH_4)_2SO_4$ in H_2SO_4 was dissolved into 600 µl of 99.9% d_6 dimethyl sulfoxide containing 0.05% (v:v) tetramethylsilane reference (Norell Scientific, Vineland, NJ). The ^1H spectrum was obtained in a 5-mm tube placed in a *z*-axis pulsed field gradients probe on a 14.1 Tesla Agilent nuclear magnetic resonance spectrometer (Santa Clara, CA, USA) operating at 600 MHz. The spectra were acquired using ~1,400 transients with a 90° (10.8 µs) pulse width, spectral width of 12 ppm, pulse delay of 2.0 s, and acquisition time of 1.7 s at 25°C. The percentage of total N labelled with ^{15}N in the tissue was determined by dividing the integrated area of the ^1H-^{15}N doublet resonances by the sum of the integrated doublet and triplet resonance areas.

2.6 | Gene expression analysis

We determined the transcript levels in the roots of two AM-inducible plant genes, the P transporter *MtPT4* (Chiou, Liu, & Harrison, 2001; Harrison, Dewbre, & Liu, 2002; Javot, Penmetsa, Terzaghi, Cook, & Harrison, 2007), and the ammonium transporter *MtAMT2;3* (Breuillin-Sessoms et al., 2015; Straub, Ludewig, & Neuhäuser, 2014). In addition, we analysed the expression levels of three plant sucrose transporters from the SUT family, *MtSUT1-1*, *MtSUT2*, and *MtSUT4-1* (primers according to Doidy et al., 2012), and seven transporters of the SWEET family, *MtSWEET1b*, *MtSWEET6*, *MtSWEET9*, *MtSWEET11*, *MtSWEET12*, *MtSWEET15c*, and *MtSWEET15d* (primers according to Kryvoruchko et al., 2016). Because *MtSWEET9* showed only low and very inconsistent levels of expression in our experiments, the results of this transporter are not shown. All following steps concerning RNA extractions, cDNA synthesis, and qPCR amplifications were performed according to the manufacturer's instructions unless stated otherwise. Briefly, we homogenized the root samples with a mortar and pestle cooled with liquid N and extracted total RNA using the PureLink™ RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA). The extracted RNAs were treated with TURBO™ DNase (Thermo Fisher Scientific) and quantified by a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). cDNAs were synthesized from 400 to 600 ng of DNase-treated RNAs using the RNA Maxima First Strand cDNA Synthesis Kit with dsDNase (Thermo Fisher Scientific) and diluted with RNase-free water to a final concentration of 20 ng μl^{-1} if needed. qPCRs were performed using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), 1 µl of 20 ng μl^{-1} cDNAs, and 5 µM of forward and reverse primers (Table S1) for each gene in a 20-µl reaction mix using a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific). The PCR conditions were as follows: 50°C for 2 min; 95°C for 15 min; 40 cycles at 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s; dissociation at 95°C for 15 s; 60°C for 15 s; and 95°C for 15 s. We used *MtTef1α* as a reference gene (Gomez et al., 2009), and the expression coefficients were calculated using the $2^{-\Delta Ct}$ method. The results are based on three to four biological replicates and three technical replicates.

2.7 | Statistical analysis

The data of Experiment 1 are based on three to seven biological replicates (plants that showed any sign of a cross-contamination between the two RC were removed, on average five biological replicates), and the data of Experiment 2 are based on three biological replicates. We used one-way analysis of variance ($P \leq 0.05$) with colonization type or nutrient treatment as fixed factor followed by the least significance difference or the Student's *t* test when the data passed Leven's test for homogeneity of variance and the Shapiro–Wilk normality test. If the data set failed these tests, the data set was log-transformed prior to the analysis. An analysis of covariance was used to confirm the results of the analysis of variance and to account for the effects of the covariate (biomass) in Experiment 1 on the statistical evaluation of the nutritional benefits. To identify statistical significant differences between the means, the statistical software Statistix 9 Analytical Software (Tallahassee, Florida, USA) or R was used. The regression analysis of the SUT transporter expression and ^{13}C allocation was conducted by R using one standard deviation from the mean for the analysis. The results of the statistical analysis are provided in Table S2 and S3.

3 | RESULTS

3.1 | Tripartite interactions can act synergistically on biomass and nutrient uptake of plants

In Experiment 1, we examined plants that were colonized with different symbiotic partner combinations (Ø/Ø, R/Ø, Ø/AM, and R/AM), and in which the fungal partner of half of the systems had access to ^{15}N-NH_4Cl via the HC (+N). The plant biomass data demonstrate that the host plants were under N demand (Figure 1). The plants that were

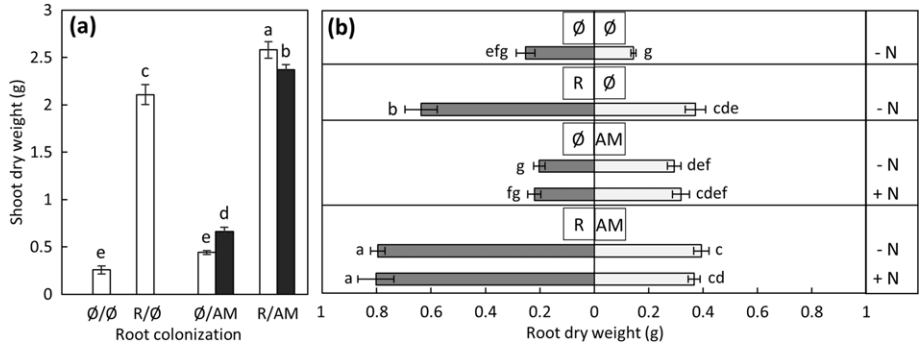

FIGURE 1 Shoot (a) and root (b) dry weights of *Medicago truncatula* depending on the colonization with different root symbionts and under different nitrogen (N) supply conditions for the fungal partner (+N, black bars in (a): addition of $^{15}NH_4Cl$ to the hyphal compartment; −N, white bars in (a): no addition of $^{15}NH_4Cl$ to the hyphal compartment; Experiment 1). Root colonization abbreviations: Ø/Ø: controls, both root halves noninoculated; R/Ø: one root half colonized by *Ensifer meliloti*, one root half noninoculated; Ø/AM: one root half colonized by *Rhizophagus irregularis*, one root half noninoculated; R/AM: one root half colonized by *R. irregularis*, one root half colonized by *E. meliloti*. Different letters on the bars (means ± standard error of the mean) indicate statistically significant differences within each graph according to the least significant difference test ($P \leq 0.05$). Analysis of variance results are shown in Table S2

inoculated with *E. meliloti* (R/Ø and R/AM) had a significantly higher shoot and root biomass than control plants (Ø/Ø), or plants that were only inoculated with *R. irregularis* (Ø/AM). However, plants that were coinoculated (R/AM) had a higher shoot and root biomass than plants that were inoculated with *E. meliloti* alone (R/Ø). Colonized root halves were larger than noncolonized root halves, and rhizobial root halves were larger than AM root halves (Figure 1b). Fungal access to N led to an increase in shoot biomass, but only in plants that were inoculated with *R. irregularis* alone (Ø/AM; Figure 1a). In contrast, plants that were coinoculated with both symbionts (R/AM) showed a slight decrease in shoot biomass when the fungus had access to N.

We compared the root colonization and the activity of N-fixing root nodules in single- (Ø/AM or R/Ø) or dual-inoculated systems (R/AM) and found that the AM colonization in dual-inoculated systems was significantly lower than in Ø/AM systems (Figure S2a). In contrast, the total nodule number per root system was not affected (not shown), but the dry weight of individual root nodules and the N-fixing activity of these nodules were significantly higher in dual-inoculated systems than in R/Ø systems (Figure S2b,c). The addition of ^{15}N to the HC did not have an effect on the root colonization patterns or the N-fixing activity of the root nodules.

Control plants (Ø/Ø) and plants that were only inoculated with *R. irregularis* (Ø/AM) showed higher levels of P in their tissue than plants that were dual-inoculated (R/AM) or inoculated with *E. meliloti* alone (R/Ø; Figure 2a,b). This is likely the result of a dilution effect caused by the higher biomass in *E. meliloti* inoculated systems and suggests that N was a more limiting factor than P for plant growth during the experiment. The AM symbiosis increased the P contents in the mycorrhizal root halves of Ø/AM systems and in the shoots of dual-inoculated plants (R/AM) compared with all the other plant systems (Figure S3a,b). Consistent with the higher N-fixing activity of the nodules in tripartite interactions, we found an increase in the N concentrations in the shoots and the N contents of shoots and rhizobial root halves of dual-inoculated plants (R/AM) compared with plants that were only inoculated with *E. meliloti* (R/Ø; Figures 2c,d and S3c,d). However, single- or dual-inoculated systems with *E. meliloti* (R/Ø or R/AM) had higher N root and shoot concentrations and contents than control plants (Ø/Ø) or systems that were only AM inoculated (Ø/AM). Fungal access to N increased the root and shoot N concentrations in single-inoculated (Ø/AM) but not in dual-inoculated systems (R/AM; Figure 2c,d).

3.2 | C allocation to root symbionts depends on the pathway for symbiotic N uptake

We added labelled ^{15}N-NH_4Cl to the HC and found an enrichment exclusively in the roots and shoots of plants that were colonized with AM fungi (Figure 3a,b). The enrichment in the shoots and in the roots with ^{15}N, however, was significantly higher in single-inoculated (Ø/AM) than in dual-inoculated (R/AM) systems. The relatively low enrichment with ^{15}N in the R/AM systems can be explained by a dilution effect caused by the strong increase in biomass and the relatively low AM root colonization of these plants (Figures 3 and S2a). The transport of ^{15}N through the extraradical mycelium to the host led also to a higher enrichment in the second root half (nonmycorrhizal root half in Ø/AM systems or nodulated root half in R/AM systems). Consistent with an ^{15}N-isotope dilution effect through the BNF activity of root nodules, the ^{15}N enrichment in the control roots of noninoculated systems (Ø/Ø) was slightly higher than in systems inoculated with *E. meliloti* (significant according to the nonparametric Wilcoxon Mann Whitney's Rank Sum test). None of the control plants (Ø/Ø), or plants that were only inoculated with rhizobia (R/Ø), showed any ^{15}N enrichment above natural abundance in roots or shoots, indicating that there was no mass flow from the HC to the RCs. Therefore, these systems are considered as −N treatments.

Plants that were inoculated with *E. meliloti* had significantly higher $\delta^{13}C$ levels in their shoots than noninoculated (Ø/Ø) or AM-inoculated (Ø/AM) systems (Figure 3c). Nodulated root halves acted as strong C sinks and showed a significantly higher $\delta^{13}C$ enrichment than the noninoculated root halves in R/Ø systems or the mycorrhizal root halves in R/AM systems (Figure 3d). When the fungus had no access to N, plants allocated more C to the nodulated root half, and the $\delta^{13}C$ enrichment in the AM root half was significantly lower. The C allocation to the nodulated root half, however, was significantly lower

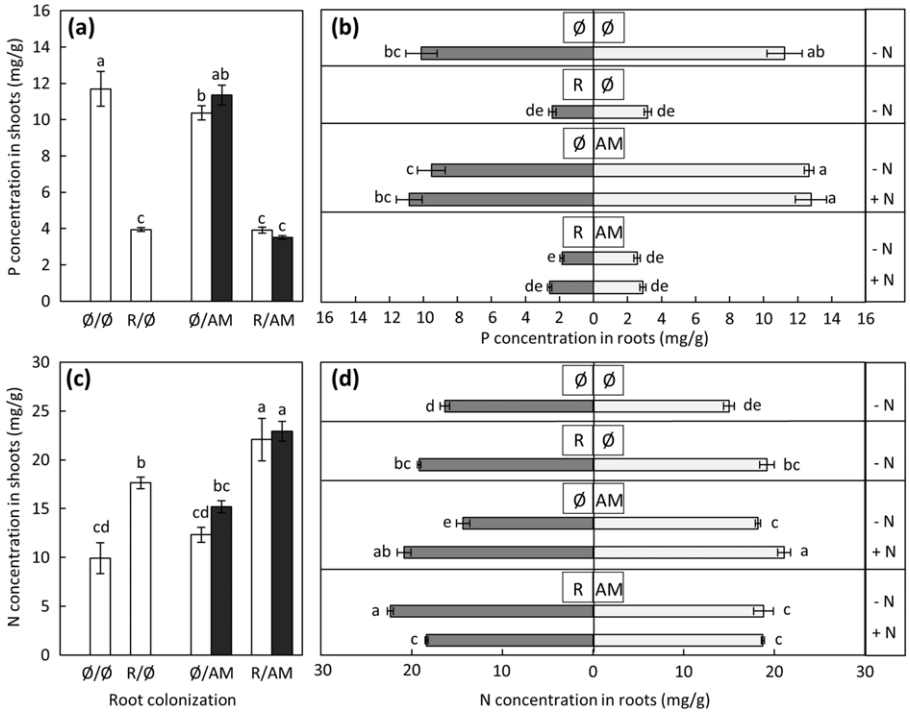

FIGURE 2 Phosphorus (P; a, b) and nitrogen (N; c, d) tissue concentrations in the shoots (a, c) or roots (b, d) of *Medicago truncatula* plants depending on the colonization with different root symbionts and under different N supply conditions for the fungal partner (+N, black bars in (a) and (c): addition of $^{15}NH_4Cl$ to the hyphal compartment; −N, white bars in (a) and (c): no addition of $^{15}NH_4Cl$ to the hyphal compartment; Experiment 1). Root colonization abbreviations: Ø/Ø: controls, both root halves noninoculated; R/Ø: one root half colonized by *Ensifer meliloti*, one root half noninoculated; Ø/AM: one root half colonized by *Rhizophagus irregularis*, one root half noninoculated; R/AM: one root half colonized by *R. irregularis*, one root half colonized by *E. meliloti*. Different letters on the bars (means ± standard error of the mean) indicate statistically significant differences within each graph according to the least significant difference test ($P \leq 0.05$). Analysis of variance results are shown in Table S2. P: phosphate

when the fungus had access to an exogenously supplied N source and did not differ from the C allocation into the AM root halves. Expressed on a percentage base, plants that were colonized with rhizobia (R/Ø; R/AM) allocated only 19.7% of the assimilated C to their root system, whereas AM plants (Ø/AM) invested 38.9% and control plants (Ø/Ø) 52.5% of their assimilated C into their root systems.

3.3 | Fungal access to N affects the expression of sucrose transporters in the roots of tripartite interactions

In order to identify the molecular mechanisms that control the C allocation to AM or nodulated roots, we evaluated the expression levels of the three sucrose transporters, *MtSUT1-1*, *MtSUT2*, and *MtSUT4-1*, and of seven SWEETs from *M. truncatula*, *MtSWEET1b*, *MtSWEET6*, *MtSWEET9*, *MtSWEET11*, *MtSWEET12*, *MtSWEET15c*, and *MtSWEET15d*. Because *MtSWEET9* showed only low and inconsistent expression levels in our experiments, we did not further consider this transporter in the analysis. With the exception of *MtSWEET11* that was exclusively expressed in rhizobial roots, all other transporters were expressed in noninoculated, mycorrhizal, and in nodulated roots, but their transcript levels were dependent on the root colonization and on the nutrient availability for the fungal partner. Compared with control roots (Ø/Ø), rhizobial roots in single-inoculated systems (R/Ø) showed higher transcript levels of *MtSUT2* and *MtSUT4-1* (Figure S4b, c). The rhizobial and AM root half of single-inoculated systems showed higher expression levels of *MtSWEET1b* and *MtSWEET6* than the noninoculated root halves (Figure S5), but in dual-inoculated systems, the transcript levels were down-regulated in the AM root halves (Figure 4).

The transcript levels of several transporters were consistent with the observed changes in C allocation to the AM or rhizobial root halves in tripartite interactions (Figure 4). *MtSUT1-1*, *MtSUT2*, *MtSUT4-1*, *MtSWEET12*, *MtSWEET15c*, and *MtSWEET15d* were significantly up-regulated in the AM roots of dual-inoculated systems when the fungus had access to N. When the fungus was unable to provide N, nodulated root halves showed higher transcript levels, but when the fungus had access to an exogenous N supply, the transcript levels of all transporters increased in the AM root halves. The transcript levels of the AM root halves were now higher than in the rhizobial root halves (*MtSUT1-1*, *MtSWEET12*, *MtSWEET15c*, and *MtSWEET15d*), comparable with the rhizobial root halves (*MtSUT4-1*), or only slightly lower than in rhizobial root halves (*MtSUT2*; Figure 4). *MtSUT2* and *MtSUT4-1* were also up-regulated in the nodulated root halves of R/Ø systems, indicating that these transporters do not only play a role in the C allocation to AM colonized roots but also to the roots colonized with the N-fixing symbiont (Figure 4).

Significant transcript levels of the AM-specific P transporter *MtPT4* and the NH_4^+ transporter *MtAMT2;3* were only detected in the mycorrhizal root halves of the Ø/AM systems, independent on whether the fungus had access to ^{15}N or not. The low expression levels of both transporters in the mycorrhizal root halves of dual-

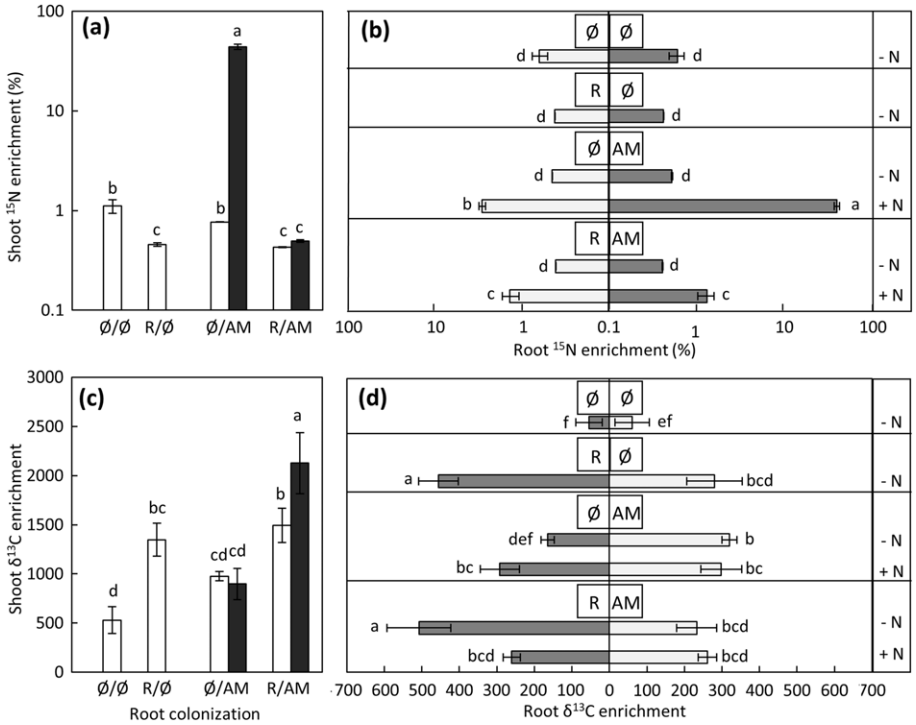

FIGURE 3 Enrichment with ^{15}N (a, b) or ^{13}C (c, d) in the shoots (a, c) or roots (b, d) of *Medicago truncatula* plants depending on the colonization with different root symbionts and under different nitrogen (N) supply conditions for the fungal partner (+N, black bars in (a) and (c): addition of $^{15}NH_4Cl$ to the hyphal compartment; −N, white bars in (a) and (c): no addition of $^{15}NH_4Cl$ to the hyphal compartment; Experiment 1). Root colonization abbreviations: Ø/Ø: controls, both root halves noninoculated; R/Ø: one root half colonized by *Ensifer meliloti*, one root half noninoculated; Ø/AM: one root half colonized by *Rhizophagus irregularis*, one root half noninoculated; R/AM: one root half colonized by *R. irregularis*, one root half colonized by *E. meliloti*. Different letters on the bars (means ± standard error of the mean) indicate statistically significant differences within each graph according to the least significant difference test ($P \leq 0.05$). Analysis of variance results are shown in Table S2

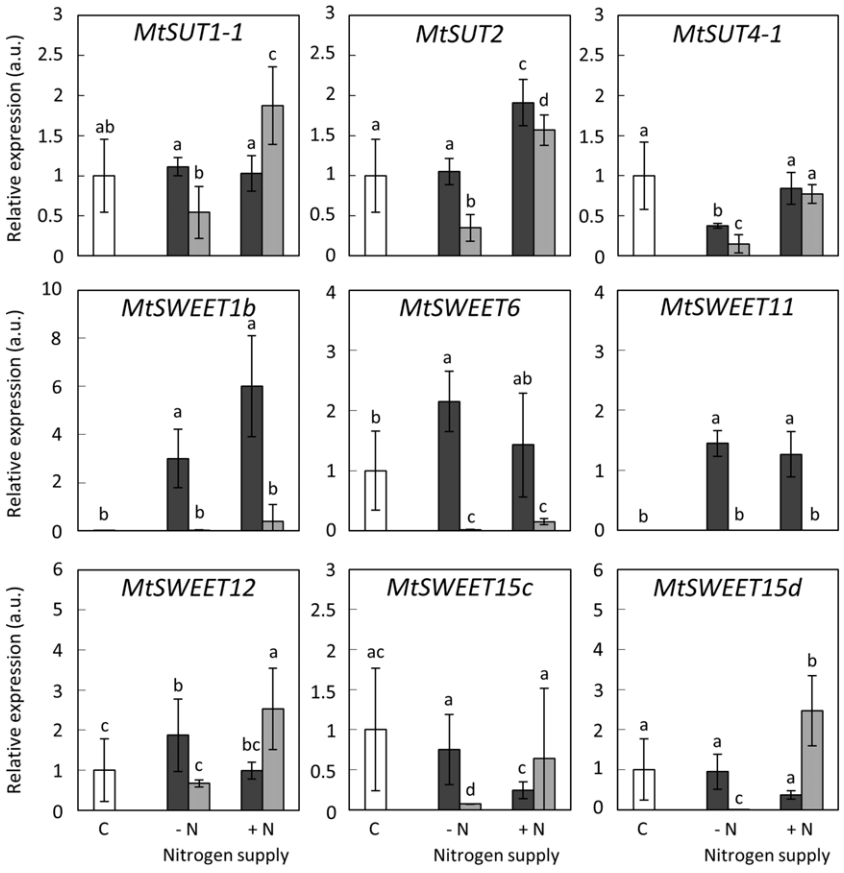

FIGURE 4 Relative expression of three sucrose transporters (*MtSUT1-1*, *MtSUT2*, and *MtSUT4-1*) and of six SWEETs (Sugars Will Eventually be Exported Transporter; *MtSWEET1b*, *MtSWEET6*, *MtSWEET11*, *MtSWEET12*, *MtSWEET15c*, and *MtSWEET15d*) in *Medicago truncatula* roots depending on the colonization with different root symbionts and under different nitrogen (N) supply conditions for the fungal partner (+N: addition of $^{15}NH_4Cl$ to the hyphal compartment; −N: no addition of $^{15}NH_4Cl$ to the hyphal compartment; Experiment 1). Shown is the expression of R/AM systems (black bars: rhizobial root halves; grey bars: arbuscular mycorrhizal root halves) compared with control roots of Ø/Ø systems (C). Data (means ± standard error of the mean) are expressed in arbitrary units (a.u.). Independent statistical analyses were performed for each split-root system compared with the control, with letters indicating statistically significant differences (least significant difference test, $P < 0.05$). Analysis of variance results are shown in Table S2

inoculated systems (R/AM) are consistent with the strong reduction of the AM colonization in these systems (Figure S6a,b).

3.4 | Plants allocate C resources to their symbiotic partners depending on their nutrient demand

We examined the effect of different nutrient demand conditions on the C allocation to different root symbionts in tripartite interactions of *M. truncatula*. Three weeks before the plants were labelled with $^{13}CO_2$, we changed the nutrient demand conditions of the host plant by adding low or high P or N concentrations to both RCs (LPLN, LPHN, HPLN, and HPHN). The nutrient treatments did not have a significant effect on shoot biomass, AM colonization (76.2 ± 4.2%, mean ± standard error of the mean), or nodule dry weights of the roots (51 ± 2.4 mg, mean ± standard error of the mean; Figure S7). We only observed that the biomass of the AM root half was smaller than the rhizobial root half under LPLN conditions (Figure S8).

The P and N concentrations of the shoots were not significantly affected by the different nutrient treatments (Figure 5a,c). There were, however, indications for an increase in the P and N shoot contents with higher nutrient availabilities (significant according to the nonparametric Wilcoxon Mann Whitney's Rank Sum test, Figure S9a,c). Root nodules acted as strong P sinks, and the tissue concentration of P and N in the root nodules was higher than in the AM roots or in the rest of the rhizobial root halves (Figure 5b,d). The AM root halves had, however, higher P concentrations and contents than the rhizobial root halves (Figures 5b and S9b). Although the P and N tissue concentrations and contents in the rhizobial root half and the root nodules were generally not affected by the nutrient treatments, the N tissue concentration and the N and P contents of the AM root halves increased when the plants were supplied with higher P and N concentrations (Figures 5d and S9b,d). We found, however, a lower N tissue concentration in the root nodules at LPLN, indicating reduced N fixation rates of the nodules at low P supply conditions (Figure 5d).

The different nutrient demand conditions had a clear effect on the C allocation in tripartite interactions of *M. truncatula*. Although under low N supply conditions (LPLN and HPLN), significantly more assimilated ^{13}C could be recovered from the rhizobial root half, the ^{13}C contents in the AM root halves increased under high N supply conditions (LPHN and HPHN) (Figure 6b). When the N supply for the plants was low, only 19.7 ± 5.0% (LPLN) or 23.3 ± 4.3% (HPLN) of the total ^{13}C that was allocated to the root system and was transferred to the AM root halves, but under high N supply conditions, this percentage increased to 29.9 ± 5.3% (LPHN) or 35.4 ± 2% (HPHN), respectively (data not shown).

3.5 | The expression of plant SUT and SWEET transporters is consistent with the observed differences in C allocation under different nutrient demand conditions for the host

The observed changes in the C allocation to both root symbionts in response to different nutrient supply conditions are consistent with changes in the plant sucrose transporter expression. The transcript levels of *MtSUT1-1* in the AM root halves were significantly higher than in the nodulated root halves under all nutrient supply conditions, indicating that this transporter may play a role for the C transport to AM roots (Figure 7a). The expression of *MtSUT1-1* in AM roots was particularly high under LPHN conditions. However, the transcript levels of *MtSUT1-1* were not correlated to the measured C allocation. By contrast, changes in the expression levels of *MtSUT2* and *MtSUT4-1* were clearly correlated to the amount of C that was allocated into the different root halves. Although nodulated roots had significantly higher transcript levels of *MtSUT2* than AM roots under low N supply conditions (LPLN and HPLN), there were no significant differences under high N supply conditions (LPHN and HPHN; Figure 7b). In contrast, the expression levels of *MtSUT4-1* were not affected by different nutrient supply conditions and also did not differ between AM and nodulated root halves (Figure 7c).

The transcript levels of *MtSWEET1b*, *MtSWEET15c*, *MtSWEET15d*, and also of the rhizobial specific transporter *MtSWEET11* were downregulated in the AM or rhizobial root halves under high nutrient supply conditions for the host (Figure 8). *MtSWEET1b* showed high expression levels in the rhizobial root halves and *MtSWEET15c* and *MtSWEET15d* in the AM root halves. In contrast, *MtSWEET6* and *MtSWEET12* showed similar transcript levels in both root halves, and

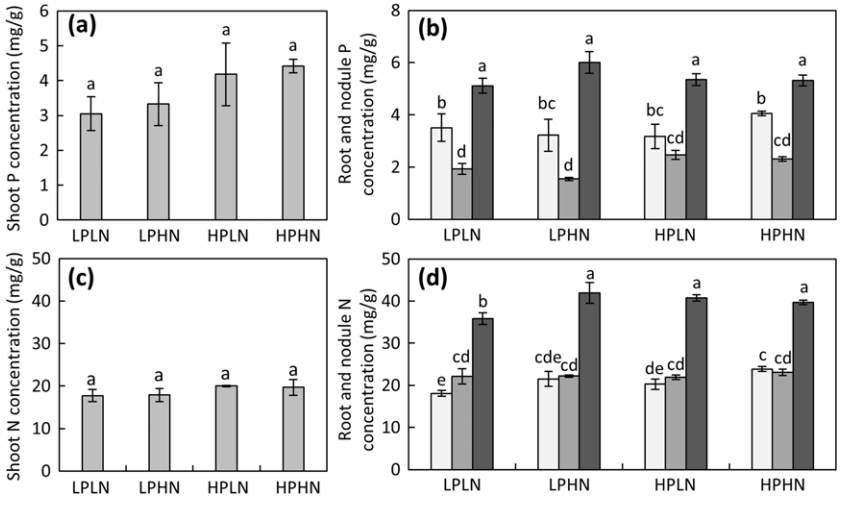

FIGURE 5 Phosphorus (a, b) and nitrogen (N) tissue concentration (c, d) in shoots (a, c) and different root fractions (b, d) of *Medicago truncatula* in symbiosis with the arbuscular mycorrhizal fungus *Rhizophagus irregularis* and the nitrogen-fixing diazotroph *Ensifer meliloti* under different nutrient supply conditions (L: low, H: high, P: phosphate; N: nitrogen; Experiment 2). Root fractions in b, d: light grey: arbuscular mycorrhizal root halves, middle grey: rhizobial root halves, and dark grey: root nodules. Different letters on the bars (means ± standard error of the mean) indicate statistically significant differences within each graph according to the least significant difference test ($P \leq 0.05$, $n = 3$). Analysis of variance results are shown in Table S3

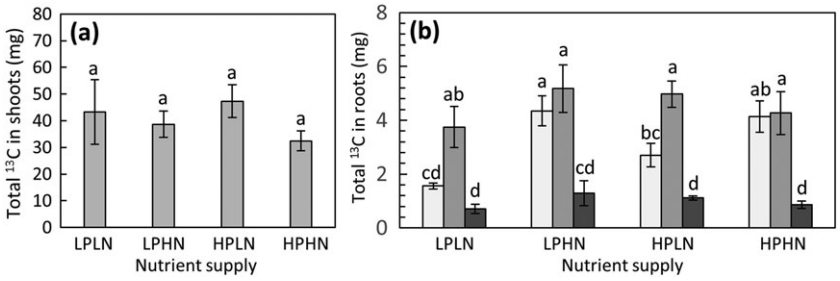

FIGURE 6 Recovered ^{13}C contents in shoots (a) and different root fractions (b) of *Medicago truncatula* in symbiosis with the arbuscular mycorrhizal fungus *Rhizophagus irregularis* and the nitrogen-fixing diazotroph *Ensifer meliloti* under different nutrient supply conditions (L: low, H: high, P: phosphate, N: nitrogen; Experiment 2). Root fractions in b: light grey: arbuscular mycorrhizal root halves, middle grey: rhizobial root halves, and dark grey: root nodules. Different letters on the bars (means ± standard error of the mean) indicate statistically significant differences within each graph according to the least significant difference test ($P \leq 0.05$, $n = 3$). Analysis of variance results are shown in Table S3

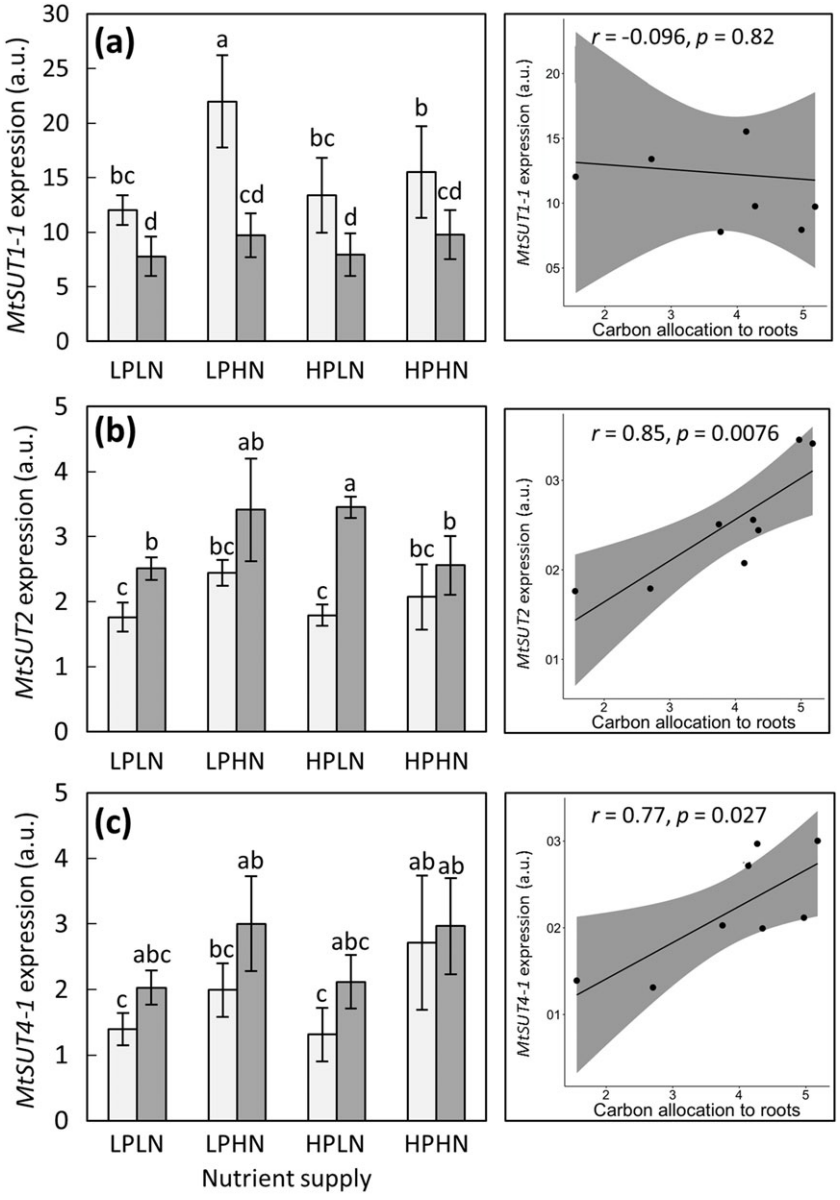

FIGURE 7 Relative expression of *MtSUT1-1* (a), *MtSUT2* (b), and *MtSUT4-1* (c) in the roots of *Medicago truncatula* in symbiosis with the arbuscular mycorrhizal fungus *Rhizophagus irregularis* (light grey bars) and the nitrogen-fixing diazotroph *Ensifer meliloti* (middle grey bars) under different nutrient supply conditions (L: low, H: high, P: phosphate, N: nitrogen; Experiment 2). Data are expressed in arbitrary units (a.u.). Figures on the right show the correlation between the means in the expression level of each transporter and the measured carbon allocation into the root halves. Different letters on the bars (means ± standard error of the mean) indicate statistically significant differences within each graph according to the least significant difference test ($P \leq 0.05$, $n = 3$). Analysis of variance results are shown in Table S3

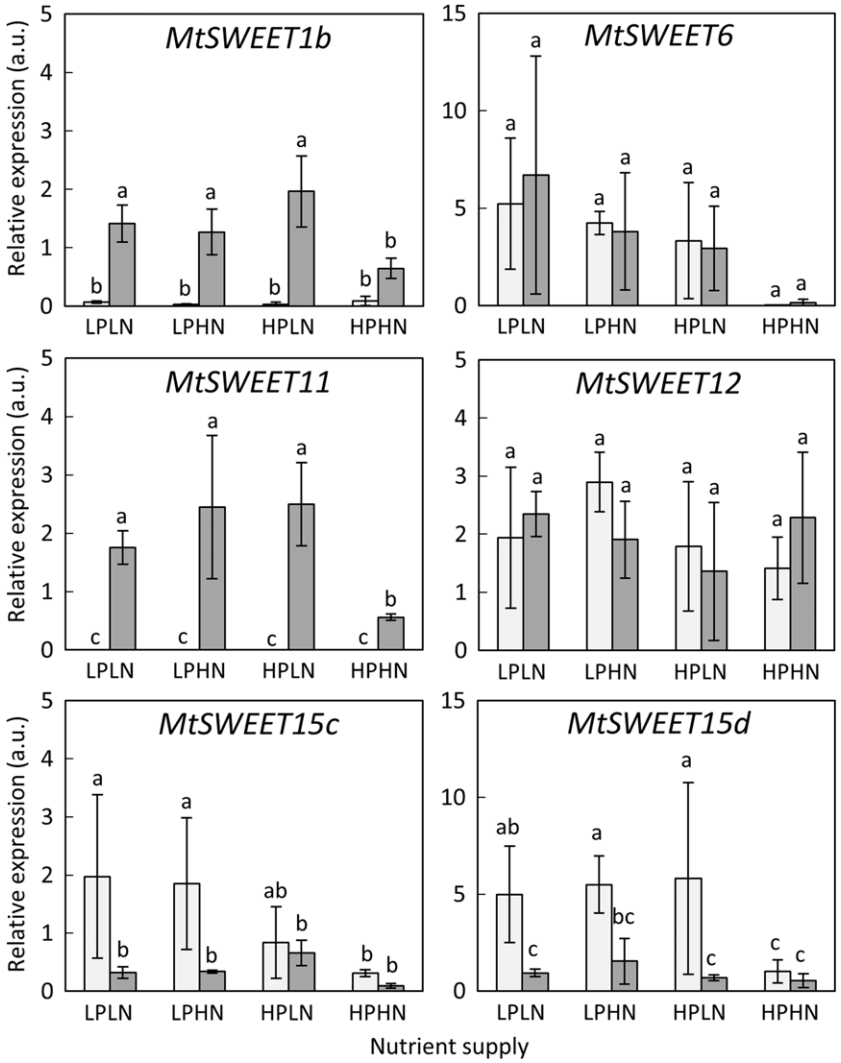

FIGURE 8 Relative expression of *MtSWEET1b*, *MtSWEET6*, *MtSWEET11*, *MtSWEET12*, *MtSWEET15c*, and *MtSWEET15d* in the roots of *Medicago truncatula* in symbiosis with the arbuscular mycorrhizal fungus *Rhizophagus irregularis* (light grey bars) and the nitrogen-fixing diazotroph *Ensifer meliloti* (dark grey bars), under different nutrient supply conditions (L: low, H: high, P: phosphate, N: nitrogen; Experiment 2). Data are expressed in arbitrary units (a.u.). Different letters on the bars (means ± standard error of the mean) indicate statistically significant differences within each graph according to the least significant difference test ($P \leq 0.05$, $n = 3$). Analysis of variance results are shown in Table S3

the expression levels were not affected by the nutrient demand conditions of the host. We also examined the expression of the AM-inducible P transporter *MtPT4* and NH_4^+ transporter *MtAMT2;3*. We found an expression of these transporters only in the roots colonized by *R. irregularis* but not in nodulated roots (Figure S10a,b). The exclusive expression of *MtPT4* and *MtAMT2;3* in the AM root halves and of *MtSWEET11* in the rhizobial root halves (Figures 4 and 8) indicates that there was no cross-contamination between both RCs.

4 | DISCUSSION

Legumes form tripartite interactions with AM fungi and rhizobia, and both symbionts play a key role for the nutrient efficiency of this agronomically important group of plants. Both symbionts affect the interactions of the plant with the other partner (Afkhami & Stinchcombe, 2016; Larimer, Clay, & Bever, 2014; Xie et al., 1998), but our functional understanding of these complex interactions is mainly based on experiments with individual symbionts, either AM fungi or rhizobia. We analysed nutrient transport, C allocation, and plant gene expression in different interactions when the fungal partner had access to an exogenous N supply, and in tripartite interactions under different nutrient demand conditions for the host, to better understand how host plants control the C costs of these interactions to maximize their symbiotic benefits.

Tripartite interactions can have a synergistic effect on plant biomass particularly under low N conditions. We found that plants in tripartite interactions had a significantly higher root and shoot biomass, N tissue concentrations and contents, and P contents than plants that were only colonized by rhizobia or AM fungi (Figures 1a, b; 2c,d; and S3a,c). Synergistic responses in tripartite interactions have also been described by other authors especially under low P and N supply conditions (Bournaud et al., 2017; Larimer et al., 2014). The dual inoculation with rhizobia and AM fungi can lead to higher photosynthetic rates and improves the harvest index (proportion of seed yields in relation to the total plant biomass) of legumes (Afkhami & Stinchcombe, 2016; Kaschuk, Kuyper, Leffelaar, Hungria, & Giller, 2009). In our study, the positive impact of tripartite interactions on plant growth was mainly the result of a higher BNF activity of the nodules and the improved plant N nutrition (Figures 2c and S2b,c). Higher BNF rates in tripartite interactions have mainly been attributed to an improved P supply by the colonization with AM fungi (Kucey & Paul, 1982; Mortimer et al., 2009; Püschel et al., 2017). Root nodules act as very strong P sinks (Figure 5b), and P deficiency can cause lower BNF rates of root nodules and inhibit nodule growth (Kleinert, Venter, Kossmann, & Valentine, 2014).

Although the positive effect of the AM symbiosis on P nutrition is long known, the contribution of AM fungi to N nutrition of their host plant is still under debate (Smith & Smith, 2011). However, there is increasing evidence that AM fungi can deliver substantial amounts of N to their host plant, even if the percentage contribution to total N nutrition of the host can vary considerably and is context dependent (Ngwene, Gabriel, & George, 2013). We found that when the fungus had access to an exogenous ^{15}N source, ^{15}N was delivered to the host, and the shoot biomass and N concentrations in the roots increased (Figures 1a, 2d and 3a,b). The capability of some AM fungi to deliver N can even lead in legumes, such as *Medicago sativa*, to strong growth responses (Mensah et al., 2015). There is evidence suggesting that fungal N uptake and transport to the host make legumes less reliant on BNF and can inhibit the development of nodules (Mortimer et al., 2008; Mortimer et al., 2009). Interestingly, the competition with rhizobia can lead to an upregulation of a fungal nitrate transporter that is involved in the uptake of nitrate by the fungal mycelium (Afkhami & Stinchcombe, 2016).

In our experiments, we found no evidence for a suppression of root nodulation or nodule growth in the presence of AM fungi. However, we found a suppression of AM colonization in the dual-inoculated systems of Experiment 1 (Figure S2a). This reduced AM colonization was likely the reason why the ^{15}N transport in these systems was much lower than in the Ø/AM systems (Figure 3a,b). The reduced AM root colonization likely caused a reduced exploration of the HC to which the ^{15}N was supplied, and the low expression of *MtPT4 and MtAMT2;3* (that are specifically expressed in arbusculated cells) suggest that less arbuscules were formed in the R/AM systems (Figure S6). However, the C allocation to different root halves and gene expression data indicate that N was transferred across the AM interface in R/AM systems and that the ^{15}N enrichment is partly hidden by a dilution effect due to the strong increase in biomass (Figure 1a,b). The transport of ^{15}N labelled ammonium across the AM interface, despite the low expression of *MtAMT2;3* in the roots of R/AM systems, could be due to the functional redundancy of mycorrhiza inducible AMT transporters in mycorrhizal roots. Although only in knock out mutants of *MtAMT2;3*, a premature degeneration of arbuscules was observed, *MtAMT2;4* and *MtAMT2;5* were also up-regulated in mycorrhizal roots, and *MtAMT2;4* was able to complement NH_4^+ uptake of yeast mutants (in contrast to *MtAMT2;3*; Breuillin-Sessoms et al., 2015).

A suppression of the other root symbiont by a prior colonization of the root system by AM fungi or rhizobia has also been reported by other authors (Catford et al., 2003; Catford et al., 2006; Mortimer et al., 2013; Sakamoto, Ogiwara, & Kaji, 2013). It is well established that a prior exposure to rhizobia can limit the subsequent formation of root nodules on the root system (Ferguson et al., 2010; Foo, Heynen, & Reid, 2016). This process is known as autoregulation of nodulation and involves a root-derived signal that is perceived by a CLAVATA1-like leucine rich repeat receptor kinase (*MtSUNN* in *Medicago*) and triggers the production of a shoot-derived inhibitor that suppresses further nodule development (Reid, Ferguson, & Gresshoff, 2011; Reid, Ferguson, Hayashi, et al., 2011). Loss-of-function mutations in these genes lead to a "supernodulation" phenotype with increased nodulation, and their overexpression prevents nodulation (Reid, Ferguson, & Gresshoff, 2011). However, because this supernodulation phenotype is dependent on the nitrate supply levels, it has been suggested that an additional regulatory pathway exists in *M. truncatula* and that the transport of N or of a N derivative or changes in C partitioning could also be involved in autoregulation of nodulation (Kassaw et al., 2015; Schnabel et al., 2011). Whether this autoregulatory pathway is also active in the regulation of AM colonization in tripartite interactions is not well understood, but mutants defective in elements of this pathway also showed elevated levels of AM root colonization (Staehelin, Xie, Illana, & Vierheilig, 2011). We observed a suppression of the AM colonization in nodulated root systems only in Experiment 1 but not in Experiment 2. This discrepancy could be due to different time points of colonization by both root symbionts or could be caused by differences in the P demand conditions of the plants. There is reason to believe that in Experiment 1, the AM colonization of the plant was delayed, and the earlier colonization with N-fixing bacteria could have suppressed the subsequent colonization with AM fungi more strongly (Catford et al., 2003; Catford et al., 2006). However, the high P tissue concentrations of the noninoculated control plants compared with the nodulated plants also indicate that plant growth in Experiment 1 was primarily limited by the N supply (Figure 2a,b). By contrast, the increase of the P contents of the dual-inoculated plants under high nutrient supply conditions suggests that the plants in Experiment 2 were also limited by the P supply (Figure S9a,b).

Root symbionts compete with their nutrient resources for host plant C. Our results demonstrate that the nutrient demand of the host plays a significant role in the C allocation to AM fungi or rhizobia in tripartite interactions. Plants under N demand preferentially allocated C to their nodulated root system, whereas plants that were supplied with N allocated proportionally more C to their AM root system (Figure 6d). Given the large C investment entailed in symbiotic associations, with estimates of up to 20% of the assimilated C for the AM symbiosis (Snellgrove et al., 1982; Jakobsen & Rosendahl, 1990; Wright et al., 1998), and up to 30% for N-fixing root nodules (Provorov & Tikhonovich, 2003), plants have to strictly control the extent of microbial colonization to limit their C investment into these interactions. Our current understanding of how the host plant controls its C supply in tripartite interactions is limited. It has, however, been shown that N demand is a driver for C partitioning in plants. Legumes preferentially expand root nodules of efficient N-fixing rhizobia and selectively transfer more C to active than to inactive root nodules (Laguerre et al., 2012; Singleton & van Kessel, 1987). Host plants penalize rhizobia that fail to fix N_2 inside their root nodules (Kiers et al., 2003), and arbuscules of AM fungi that are unable to provide P for the host plant are prematurely degenerated (Javot et al., 2007; Javot et al., 2011). C acts as an important trigger for symbiotic functioning, and a reduction in the C supply reduces BNF by rhizobia (Kleinert et al., 2014), and P and N uptake and transport by AM fungi (Fellbaum et al., 2012; Fellbaum et al., 2014; Konvalinková & Jansa, 2016). It has been shown that resource exchange between host and AM fungi is controlled by a reciprocal reward mechanisms that is driven by biological market dynamics (Kiers et al., 2011). Our results demonstrate that similar mechanisms may also control the resource to C exchange in tripartite interactions. In agreement, we observed

that the fungus became a stronger competitor for host plant C when the fungus had access to an exogenous N source (Figure 3d). This is consistent with a biological market model, because N derived from AM symbionts is less costly for the host than N from BNF (Mortimer et al., 2009).

To unravel the molecular mechanisms by which the C transport to different symbiotic partners is controlled, we analysed the expression of three sucrose transporters of *M. truncatula* and found that the expression levels of *MtSUT2* and *MtSUT4-1* were positively correlated to the C allocation to different symbiotic partners (Figure 7). These transporters are not symbiosis-specific transporters and are expressed in noninoculated roots and in AM and nodulated roots. *MtSUT1-1* encodes a H^+-sucrose symporter and is putatively involved in phloem loading and unloading (Doidy et al., 2012). The high transcript levels of *MtSUT1-1* particularly in AM roots (Figure 7a) and its upregulation in AM roots when the fungus had access to an exogenous N source (Figure 4) support a possible role of this transporter in phloem unloading towards AM-colonized sink roots (Doidy et al., 2012). *MtSUT4-1* shows similarities with the sucrose transporter of *Lotus japonicus LjSUT4* that is involved in the transport of glucosides from the vacuole into the cytoplasm (Reinders, Sivitz, Starker, Gantt, & Ward, 2008). Therefore, this transporter could play a role in the release of stored C sources from the vacuole towards symbiotic root sinks (Doidy et al., 2012). *MtSUT4-1* shows a higher expression in cortical cells adjacent to arbusculated cells (Gaude, Bortfeld, Duensing, Lohse, & Krajinski, 2012), and the high correlation of its transcript levels with the observed C allocation pattern (Figure 7c) clearly suggests a role of this transporter in symbiotic C flux to both root symbionts. Consistent with a role of *MtSUT4-1* in the remobilization of C from vacuolar C storage pools, we found that *MtSUT4-1* was down-regulated in AM roots, when the fungus was unable to provide N (Figure 4c). The functional role of *MtSUT2* on the other hand has not yet been deciphered (Doidy et al., 2012). In our experiments, *MtSUT2* shows a higher expression in nodulated roots under N stress, as well as in AM roots in response to an exogenous supply of N (Figures 4 and 7b). This suggests that also this transporter might play a role in the C transport towards both beneficial root symbionts.

We also determined the expression of six SWEETs in the roots of *M. truncatula* after colonization with different root symbionts. SWEETs can not only catalyse the efflux of carbohydrates but also their uptake (Chen, 2014), and it has recently been suggested that members of the SWEET family could be involved in the symbiotic C flux (Kryvoruchko et al., 2016; Manck-Götzenberger & Requena, 2016; Sugiyama et al., 2017). In contrast to *MtSWEET11* that is specifically expressed in root nodules, none of the other *SWEETs* we tested showed a mycorrhiza-restricted induction, but three of the SWEETs, *MtSWEET1b*, *MtSWEET6*, and *MtSWEET15d*, were upregulated in AM roots compared with control roots (Figure S5). According to the *M. truncatula* gene expression atlas (http://mtgea.noble.org/v3/), *MtSWEET1b* and *MtSWEET6* are highly expressed in arbusculated cells, and their putative orthologues *StSWEET1a*, *StSWEET1b*, and *StSWEET 7a* from potato also show high transcript levels in mycorrhizal roots (Manck-Götzenberger & Requena, 2016). Although *MtSWEET1b* and *MtSWEET6* are also highly expressed in rhizobial roots, the downregulation of both transporters in the tripartite interactions of Experiment 1 (Figure 4), in which a suppression of the AM colonization by rhizobia was observed (Figure S2a), is an agreement with a potential role of both transporters for the sugar transport in arbusculated cells. Both *MtSWEET1b* and *MtSWEET6* belong to the SWEET Clade I and II, and preferentially transport hexoses, mainly glucose (Chen, 2014), what is consistent with an induction of the monosaccharide transporter MST2 with a high affinity for glucose in the fungal membrane of arbuscules (Helber et al., 2011). The significance of hexoses for C transport to the AM fungus has recently been questioned by reports revealing that fatty acids can also be exported out of the root cell and transported to the fungal symbiont. However, the transport of hexoses to the arbusculated cells will also provide the host cells with the precursor for the biosynthesis of fatty acyl groups that can be translocated to the fungal partner (Bravo et al., 2017; Keymer et al., 2017; Luginbuehl et al., 2017).

MtSWEET11, *MtSWEET12*, *MtSWEET15c*, and *MtSWEET15d* are clustered in Clade III, which presumably encodes primarily sucrose transporters, and play an important role for sucrose translocation from source to sink tissues. *MtSWEET11* is exclusively expressed in nodulated roots, and its expression is down-regulated in response to high nutrient supply conditions (Figure 8), which is consistent with a reduced C transport from the host to the root nodules under high nutrient supply conditions. Similar to the changes in the gene expression patterns that were observed for the SUTs, *MtSWEET12*, *MtSWEET15c*, and *MtSWEET15d* were upregulated in the mycorrhizal roots of tripartite interactions when the fungus had access to N (Figure 4) or were down-regulated in AM roots when the host plant itself had access to nutrients (Figure 8). This is in agreement with the observed changes in the C allocation to AM or rhizobial roots (Figures 3d and 6b) and suggests that these transporters play an important role for the sucrose transport to symbiotic sink tissues. The fact, however, that *MtSWEET12*, *MtSWEET15c*, and *MtSWEET15d* show similar changes in their expression patterns also indicates some level of redundancy in the function of these transporters. This redundancy in the SWEET family has also been discussed as the reason, why loss of function mutants of *MtSWEET11* and *LjSWEET3* that are highly expressed in the nodules of wild-type roots, did not show an impairment in nodular function (Kryvoruchko et al., 2016; Sugiyama et al., 2017).

We used in this study recovered ^{13}C from roots as an indicator for the C allocation to different symbiotic partners but did not consider the ^{13}C that was integrated into the fungal or rhizobial biomass or respired by the symbiotic partners from the soil (Kucey & Paul, 1982). To exactly measure the fungal or bacterial biomass in colonized roots is challenging, but the alignment of the recovered ^{13}C from individual root halves with the observed shifts in plant gene expression suggests that the recovered ^{13}C in the root halves was a sufficient indicator for the C allocation to different symbiotic partners. Further biochemical, spatial, molecular, and physiological analyses will be required to profile the role of all transporters for symbiotic functioning and to identify the shared and specific mechanisms for C allocation towards AM fungi and N-fixing bacteria. A better understanding of these processes may prove critical in maximizing the benefits of symbionts for agricultural legumes.

ACKNOWLEDGMENTS

We wish to acknowledge funding from the USDA (2017-67014-26530), the SD Soybean Research and Promotion Council, and the Agricultural Experiment Station at SDSU. We also thank Vincent Peta and Tyrel Deutscher for assistance with the measurements of the nitrogenase activity, Lindsay McKeever for performing the Kjeldahl degradations for the $^{15}N/^{14}N$ analyses, and Sierra Ash for her assistance in RNA extractions.

AUTHOR CONTRIBUTIONS

H. B. and X. W. designed the research; A. K., X. W., K. G., P. E. P., and G. D. S. performed the experiments; A. K., K. G., and H. B. collected, analysed, and interpreted the data; and H. B., K. G., and A. K. wrote the manuscript.

ORCID

Kevin Garcia http://orcid.org/0000-0003-0821-1024
Xiurong Wang http://orcid.org/0000-0002-0699-8233
Heike Bücking http://orcid.org/0000-0002-4040-0944

REFERENCES

Afkhami, M. E., & Stinchcombe, J. R. (2016). Multiple mutualist effects on genomewide expression in the tripartite association between *Medicago truncatula*, nitrogen-fixing bacteria and mycorrhizal fungi. *Molecular Ecology*, *25*, 4946–4962.

Antunes, P. M., de Varennes, A., Zhang, T., & Goss, M. J. (2006). The tripartite symbiosis formed by indigenous arbuscular mycorrhizal fungi, *Bradyrhizobium japonicum* and soya bean under field conditions. *Journal of Agronomy and Crop Science*, *192*, 373–378.

Bournaud, C., James, E. K., Faria, S. M., Lebrun, M., Melkonian, R., Duponnois, R., … Prin, Y. (2017). Interdependency of efficient nodulation and arbuscular mycorrhization in *Piptadenia gonoacantha*, a Brazilian legume tree. *Plant, Cell and Environment*. https://doi.org/10.1111/pce. 13095

Bravo, A., Brands, M., Wewer, V., Dörmann, P., & Harrison, M. J. (2017). Arbuscular mycorrhiza-specific enzymes *FatM* and *RAM2* fine-tune lipid biosynthesis to promote development of arbuscular mycorrhiza. *New Phytologist*, *214*, 1631–1645.

Breuillin-Sessoms, F., Floss, D. S., Gomez, S. K., Pumplin, N., Ding, Y., Levesque-Tremblay, V., … Harrison, M. J. (2015). Suppression of arbuscule degeneration in *Medicago truncatula* phosphate transporter 4 mutants is dependent on the ammonium transporter 2 family protein AMT2;3. *The Plant Cell*, *27*, 1352–1366.

Bücking, H., & Kafle, A. (2015). Role of arbuscular mycorrhizal fungi in the nitrogen uptake of plants: Current knowledge and research gaps. *Agronomy*, *5*, 587–612.

Burchill, W., James, E. K., Li, D., Lanigan, G. J., Williams, M., Iannetta, P. P. M., & Humphreys, J. (2014). Comparisons of biological nitrogen fixation in association with white clover (*Trifolium repens* L.) under four fertiliser nitrogen inputs as measured using two ^{15}N techniques. *Plant and Soil*, *385*, 287–302.

Catford, J. G., Staehelin, C., Larose, G., Piché, Y., & Vierheilig, H. (2006). Systemically suppressed isoflavonoids and their stimulating effects on nodulation and mycorrhization in alfalfa split-root systems. *Plant and Soil*, *285*, 257–266.

Catford, J. G., Staehelin, C., Lerat, S., Piché, Y., & Vierheilig, H. (2003). Suppression of arbuscular mycorrhizal colonization and nodulation in split-root systems of alfalfa after pre-inoculation and treatment with nod factors. *Journal of Experimental Botany*, *54*, 1481–1487.

Chen, L. Q. (2014). SWEET sugar transporters for phloem transport and pathogen nutrition. *New Phytologist*, *201*, 1150–1155.

Chiou, T. J., Liu, H., & Harrison, M. J. (2001). The spatial expression patterns of a phosphate transporter (*MtPT1*) from *Medicago truncatula* indicate a role in phosphate transport at the root/soil interface. *The Plant Journal*, *25*, 281–293.

Delaux, P.-M., Séjalon-Delmas, N., Bécard, G., & Ané, J.-M. (2013). Evolution of the plant—microbe symbiotic 'toolkit'. *Trends in Plant Science*, *18*, 298–304.

Ding, X., Sui, X., Wang, F., Gao, J., He, X., Zhang, F., … Feng, G. (2012). Synergistic interactions between *Glomus mosseae* and *Bradyrhizobium japonicum* in enhancing proton release from nodules and hyphae. *Mycorrhiza*, *22*, 51–58.

Doidy, J., van Tuinen, D., Lamotte, O., Corneillat, M., Alcaraz, G., & Wipf, D. (2012). The *Medicago truncatula* sucrose transporter family: Characterization and implication of key members in carbon partitioning towards arbuscular mycorrhizal fungi. *Molecular Plant*, *5*, 1346–1358.

Fellbaum, C. R., Gachomo, E. W., Beesetty, Y., Choudhari, S., Strahan, G. D., Pfeffer, P. E., … Bücking, H. (2012). Carbon availability triggers fungal nitrogen uptake and transport in the arbuscular mycorrhizal symbiosis. *Proceedings of the National Academy of Sciences of the United States of America*, *109*, 2666–2671.

Fellbaum, C. R., Mensah, J. A., Cloos, A. J., Strahan, G. D., Pfeffer, P. E., Kiers, E. T., & Bücking, H. (2014). Fungal nutrient allocation in common mycelia networks is regulated by the carbon source strength of individual host plants. *New Phytologist*, *203*, 645–656.

Ferguson, B. J., Indrasumunar, A., Hayashi, S., Lin, M.-H., Lin, Y.-H., Reid, D. E., & Gresshoff, P. M. (2010). Molecular analysis of legume nodule development and autoregulation. *Journal of Integrative Plant Biology*, *52*, 61–76.

Foo, E., Heynen, E. M. H., & Reid, J. B. (2016). Common and divergent shoot–root signalling in legume symbioses. *New Phytologist*, *210*, 643–656.

Garcia, K., Doidy, J., Zimmermann, S. D., Wipf, D., & Courty, P.-E. (2016). Take a trip through the plant and fungal transportome of mycorrhiza. *Trends in Plant Science*, *21*, 937–950.

Gaude, N., Bortfeld, S., Duensing, N., Lohse, M., & Krajinski, F. (2012). Arbuscule-containing and non-colonized cortical cells of mycorrhizal roots undergo extensive and specific reprogramming during arbuscular mycorrhizal development. *Plant Journal*, *69*, 510–528.

Gomez, S. K., Javot, H., Deewalthanawong, P., Torres-Jerez, I., Blancaflor, E. B., Udvardi, M. K., & Harrison, M. J. (2009). *Medicago truncatula and Glomus intradices* gene expression in cortical cells harboring arbuscules in the arbuscular mycorrhizal symbiosis. *BMC Plant Biology*, *9*, 10. https://doi: 10.1186/1471-2229-9-10

Harrison, M. J., Dewbre, G. R., & Liu, J. (2002). A phosphate transporter from *Medicago truncatula* involved in the acquisition of phosphate released by arbuscular mycorrhizal fungi. *The Plant Cell*, *14*, 2413–2429.

Helber, N., Wippel, K., Sauer, N., Schaarschmidt, S., Hause, B., & Requena, N. (2011). A versatile monosaccharide transporter that operates in the arbuscular mycorrhizal fungus *Glomus* sp. is crucial for the symbiotic relationship with plants. *The Plant Cell*, *23*, 3812–3823.

Ingestad, T. (1960). Studies on the nutrition of forest tree seedlings. III Mineral nutrition of pine. *Physiologia Plantarum*, *13*, 513–533.

Jakobsen, I., & Rosendahl, L. (1990). Carbon flow into soil and external hyphae from roots of mycorrhizal cucumber plants. *New Phytologist*, *115*, 77–83.

Javot, H., Penmetsa, R. V., Breuillin, F., Bhattarai, K. K., Noar, R. D., Gomez, S. K., … Harrison, M. J. (2011). Medicago truncatula Mtpt4 mutants reveal a role for nitrogen in the regulation of arbuscule degeneration in arbuscular mycorrhizal symbiosis. *Plant Journal*, *68*, 954–965.

Javot, H., Penmetsa, R. V., Terzaghi, N., Cook, D. R., & Harrison, M. J. (2007). A *Medicago truncatula* phosphate transporter indispensable for the arbuscular mycorrhizal symbiosis. *Proceedings of the National Academy of Sciences of the United States of America*, *104*, 1720–1725.

Jiang, Y., Wang, W., Xie, Q., Liu, N., Liu, L., Wang, D., … Wang, E. (2017). Plants transfer lipids to sustain colonization by mutualistic mycorrhizal and parasitic fungi. *Science*, *356*, 1172–1175.

Kaschuk, G., Kuyper, T. W., Leffelaar, P. A., Hungria, M., & Giller, K. E. (2009). Are the rates of photosynthesis stimulated by the carbon sink strength of rhizobial and arbuscular mycorrhizal symbioses? *Soil Biology and Biochemistry*, *41*, 1233–1244.

Kassaw, T., Bridges, W. Jr., & Frugoli, J. (2015). Multiple autoregulation of nodulation (AON) signals identified through split root analysis of *Medicago truncatula sunn* and *rdn1* mutants. *Plants*, *4*, 209–224.

Keymer, A., Pimprikar, P., Wewer, V., Huber, C., Brands, M., Bucerius, S. L., … Gutjahr, C. (2017). Lipid transfer from plants to arbuscular mycorrhiza fungi. *eLife*, *6*, e29107.

Kiers, E. T., Duhamel, M., Beesetty, Y., Mensah, J. A., Franken, O., Verbruggen, E., … Bücking, H. (2011). Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. *Science*, *333*, 880–882.

Kiers, E. T., Rousseau, R. A., West, S. A., & Denison, R. F. (2003). Host sanctions and the legume—Rhizobium mutualism. *Nature*, *425*, 78–81.

Kistner, C., Winzer, T., Pitzschke, A., Mulder, L., Sato, S., Kaneko, T., … Parniske, M. (2005). Seven *Lotus japonicus* genes required for transcriptional reprogramming of the root during fungal and bacterial symbiosis. *The Plant Cell*, *17*, 2217–2229.

Kleinert, A., Venter, M., Kossmann, J., & Valentine, A. (2014). The reallocation of carbon in P deficient lupins affects biological nitrogen fixation. *Journal of Plant Physiology*, *171*, 1619–1624.

Konvalinková, T., & Jansa, J. (2016). Lights off for arbuscular mycorrhiza: On its symbiotic functioning under light deprivation. *Frontiers in Plant Science*, *7*. https://doi.org/10.3389/fpls.2016.00782

Kryvoruchko, I. S., Sinharoy, S., Torres-Jerez, I., Sosso, D., Pislariu, C. I., Guan, D., … Udvardi, M. K. (2016). MtSWEET11, a nodule-specific sucrose transporter of *Medicago truncatula*. *Plant Physiology*, *171*, 554–565.

Kucey, R. M. N., & Paul, E. A. (1982). Carbon flow, photosynthesis, and N_2 fixation in mycorrhizal and nodulated faba beans (*Vicia faba* L.). *Soil Biology and Biochemistry*, *14*, 407–412.

Laguerre, G., Heulin-Gotty, K., Brunel, B., Klonowska, A., Le Quéré, A., Tillard, P., … Lepetit, M. (2012). Local and systemic N signaling are involved in *Medicago truncatula* preference for the most efficient *Sinorhizobium* symbiotic partners. *New Phytologist*, *195*, 437–449.

Larimer, A. L., Clay, K., & Bever, J. D. (2014). Synergism and context dependency of interactions between arbuscular mycorrhizal fungi and rhizobia with a prairie legume. *Ecology*, *95*, 1045–1054.

Lemoine, R., Camera, S. L., Atanassova, R., Dédaldéchamp, F., Allario, T., Pourtau, N., … Durand, M. (2013). Source-to-sink transport of sugar and regulation by environmental factors. *Frontiers in Plant Science*, *4*, 272.

Luginbuehl, L. H., Menard, G. N., Kurup, S., Van Erp, H., Radhakrishnan, G. V., Breakspear, A., … Eastmond, P. J. (2017). Fatty acids in arbuscular mycorrhizal fungi are synthesized by the host plant. *Science*, *356*, 1175–1178.

Manck-Götzenberger, J., & Requena, N. (2016). Arbuscular mycorrhiza symbiosis induces a major transcriptional reprogramming of the potato SWEET sugar transporter family. *Frontiers in Plant Science*, *7*, 487.

McGonigle, T. P., Miller, M. H., Evans, D. G., Fairchild, G. L., & Swan, J. A. (1990). A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. *New Phytologist*, *115*, 495–501.

Meng, L., Zhang, A., Wang, F., Han, X., Wang, D., & Li, S. (2015). Arbuscular mycorrhizal fungi and rhizobium facilitate nitrogen uptake and transfer in soybean/maize intercropping system. *Frontiers in Plant Science*, *6*, 339.

Mensah, J. A., Koch, A. M., Antunes, P. M., Hart, M. M., Kiers, E. T., & Bücking, H. (2015). High functional diversity within arbuscular mycorrhizal fungal species is associated with differences in phosphate and nitrogen uptake and fungal phosphate metabolism. *Mycorrhiza*, *25*, 533–546.

Mortimer, P. E., Le Roux, M. R., Pérez-Fernández, M. A., Benedito, V. A., Kleinert, A., Xu, J., & Valentine, A. J. (2013). The dual symbiosis between arbuscular mycorrhiza and nitrogen fixing bacteria benefits the growth and nutrition of the woody invasive legume *Acacia cyclops* under nutrient limiting conditions. *Plant and Soil*, *366*, 229–241.

Mortimer, P. E., Perez-Fernandez, M. A., & Valentine, A. (2012). Arbuscular mycorrhiza maintains nodule function during external NH_4^+ supply in *Phaseolus vulgaris (L.)*. *Mycorrhiza*, *22*, 237–245.

Mortimer, P. E., Pérez-Fernández, M. A., & Valentine, A. J. (2008). The role of arbuscular mycorrhizal colonization in the carbon and nutrient economy of the tripartite symbiosis with nodulated *Phaseolus vulgaris*. *Soil Biology and Biochemistry*, *40*, 1019–1027.

Mortimer, P. E., Pérez-Fernández, M. A., & Valentine, A. J. (2009). Arbuscular mycorrhizae affect the N and C economy of nodulated *Phaseolus vulgaris (L.)* during NH_4^+ nutrition. *Soil Biology & Biochemistry*, *41*, 2115–2121.

Ngwene, B., Gabriel, E., & George, E. (2013). Influence of different mineral nitrogen sources (NO_3^- -N vs. NH_4^+ -N) on arbuscular mycorrhiza development and N transfer in a *Glomus intraradices*–cowpea symbiosis. *Mycorrhiza*, *23*, 107–117.

Oldroyd, G. E. D., Murray, J. D., Poole, P. S., & Downie, J. A. (2011). The rules of engagement in the legume-rhizobial symbiosis. *Annual Review of Genetics*, *45*, 119–144.

Ossler, J. N., Zielinski, C. A., & Heath, K. D. (2015). Tripartite mutualism: Facilitation or trade-offs between rhizobial and mycorrhizal symbionts of legume hosts. *American Journal of Botany*, *102*, 1332–1341.

Owino-Gerroh, C., Gascho, G. J., & Phatak, S. C. (2005). Pigeonpea response to silicon, phosphorus, and Rhizobium inoculation in an acid coastal plain soil. *Journal of Plant Nutrition*, *28*, 797–804.

Provorov, N. A., & Tikhonovich, I. A. (2003). Genetic resources for improving nitrogen fixation in legume-rhizobia symbiosis. *Genetic Resources and Crop Evolution*, *50*, 89–99.

Püschel, D., Janoušková, M., Voříšková, A., Gryndlerová, H., Vosátka, M., & Jansa, J. (2017). Arbuscular mycorrhiza stimulates biological nitrogen fixation in two *Medicago* spp. through improved phosphorus acquisition. *Frontiers in Plant Science*, *8*, 390.

Reid, D. E., Ferguson, B. J., & Gresshoff, P. M. (2011). Inoculation- and nitrate-induced CLE peptides of soybean control NARK-dependent nodule formation. *Molecular Plant-Microbe Interactions*, *24*, 606–618.

Reid, D. E., Ferguson, B. J., Hayashi, S., Lin, Y.-H., & Gresshoff, P. M. (2011). Molecular mechanisms controlling legume autoregulation of nodulation. *Annals of Botany*, *108*, 789–795.

Reinders, A., Sivitz, A. B., Starker, C. G., Gantt, J. S., & Ward, J. M. (2008). Functional analysis of *LjSUT4*, a vacuolar sucrose transporter from *Lotus japonicus*. *Plant Molecular Biology*, *68*, 289–299.

Rich, M. K., Nouri, E., Courty, P.-E., & Reinhardt, D. (2017). Diet of arbuscular mycorrhizal fungi: Bread and butter? *Trends in Plant Science*, *22*, 652–660.

Ruehr, N. K., Offermann, C. A., Gessler, A., Winkler, J. B., Ferrio, J. P., Buchmann, N., & Barnard, R. L. (2009). Drought effects on allocation of recent carbon: From beech leaves to soil CO_2 efflux. *New Phytologist*, *184*, 950–961.

Sakamoto, K., Ogiwara, N., & Kaji, T. (2013). Involvement of autoregulation in the interaction between rhizobial nodulation and AM fungal colonization in soybean roots. *Biology and Fertility of Soils*, *49*, 1141–1152.

Schnabel, E. L., Kassaw, T. K., Smith, L. S., Marsh, J. F., Oldroyd, G. E., Long, S. R., & Frugoli, J. A. (2011). The ROOT DETERMINED NODULATION1 gene regulates nodule number in roots of *Medicago truncatula* and defines a highly conserved, uncharacterized plant gene family. *Plant Physiology*, *157*, 328–340.

Singleton, P. W., & van Kessel, C. (1987). Effect of localized nitrogen availability to soybean half-root systems on photosynthate partitioning to roots and nodules. *Plant Physiology*, *83*, 552–556.

Smith, S. E., & Read, D. J. (2008). *Mycorrhizal symbiosis* (Third edition ed.). New York: Academic Press.

Smith, S. E., & Smith, F. A. (2011). Roles of arbuscular mycorrhizas in plant nutrition and growth: New paradigms from cellular to ecosystem scales. *Annual Review of Plant Biology*, *62*, 227–250.

Snellgrove, R. C., Splittstoesser, W. E., Stribley, D. P., & Tinker, P. B. (1982). The distribution of carbon and the demand of the fungal symbiont in leek plants with vesicular-arbuscular mycorrhizas. *New Phytologist*, *92*, 75–87.

Staehelin, C., Xie, Z.-P., Illana, A., & Vierheilig, H. (2011). Long-distance transport of signals during symbiosis: Are nodule formation and mycorrhization autoregulated in a similar way? *Plant Signaling & Behavior*, *6*, 372–377.

St-Arnaud, M., Hamel, C., Vimard, B., Caron, M., & Fortin, J. A. (1996). Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus *Glomus intraradices* in an in vitro system in the absence of host roots. *Mycological Research*, *100*, 328–332.

Straub, D., Ludewig, U., & Neuhäuser, B. (2014). A nitrogen-dependent switch in the high affinity ammonium transport in *Medicago truncatula*. *Plant Molecular Biology*, *86*, 485–494.

Sugiyama, A., Saida, Y., Yoshimizu, M., Takanashi, K., Sosso, D., Frommer, W. B., & Yazaki, K. (2017). Molecular characterization of LjSWEET3, a sugar transporter in nodules of *Lotus japonicus*. *Plant and Cell Physiology*, *58*, 298–306.

Udvardi, M., & Poole, P. S. (2013). Transport and metabolism in legume-rhizobia symbioses. *Annual Review of Plant Biology*, *64*, 781–805.

Valentine, A. J., Mortimer, P. E., Kleinert, A., Kang, Y., & Benedito, V. A. (2013). Carbon metabolism and costs of arbuscular mycorrhizal associations to host roots. In R. Aroca (Ed.), *Symbiotic Endophytes* (pp. 233–252). Berlin: Springer.

Vance, C. P. (2001). Symbiotic nitrogen fixation and phosphorus acquisition. Plant nutrition in a world of declining renewable resources. *Plant Physiology*, *127*, 390–397.

Vesterager, J. M., Nielsen, N. E., & Hogh-Jensen, H. (2006). Variation in phosphorus uptake and use efficiencies between pigeonpea genotypes and cowpea. *Journal of Plant Nutrition*, *29*, 1869–1888.

Vierheilig, H., Coughlan, A. P., Wyss, U., & Piché, Y. (1998). Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. *Applied and Environmental Microbiology*, *64*, 5004–5007.

Wright, D. P., Read, D. J., & Scholes, J. D. (1998). Mycorrhizal sink strength influences whole plant carbon balance of *Trifolium repens* L. *Plant, Cell and Environment*, *21*, 881–891.

Xie, Z. P., Muller, J., Wiemken, A., Broughton, W. J., & Boller, T. (1998). Nod factors and tri-iodobenzoic acid stimulate mycorrhizal colonization and affect carbohydrate partitioning in mycorrhizal roots of *Lablab purpureus*. *New Phytologist*, *139*, 361–366.

Xie, Z. P., Staehelin, C., Vierheilig, H., Wiemken, A., Jabbouri, S., Broughton, W. J., … Boller, T. (1995). Rhizobial nodulation factors stimulate mycorrhizal colonization of nodulating and nonnodulating soybeans. *Plant Physiology*, *108*, 1519–1525.

Yasmeen, T., Hameed, S., Tariq, M., & Ali, S. (2012). Significance of arbuscular mycorrhizal and bacterial symbionts in a tripartite association with *Vigna radiata*. *Acta Physiologiae Plantarum*, *34*, 1519–1528.

Zhu, H. Y., Riely, B. K., Burns, N. J., & Ané, J. M. (2006). Tracing nonlegume orthologs of legume genes required for nodulation and arbuscular mycorrhizal symbioses. *Genetics*, *172*, 2491–2499.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Kafle A, Garcia K, Wang X, Pfeffer PE, Strahan GD, Bücking H. Nutrient demand and fungal access to resources control the carbon allocation to the symbiotic partners in tripartite interactions of *Medicago truncatula*. *Plant Cell Environ*. 2018;1–15. https://doi.org/10.1111/pce.13359