The fungus does not transfer carbon to or between roots in an arbuscular mycorrhizal symbiosis

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Summary

• Carbon transfer from fungus to plant in the arbuscular mycorrhizal (AM) symbiosis has been reported, but its significance and even its existence have been called into question and the issue remains controversial. We investigated carbon movement from fungus to plant and from one mycorrhizal root system to another via a common AM fungal network in monoxenic cultures to avoid limitations of some previous studies.

• ^{13}C and ^{14}C labeled substrates were supplied to functioning *in vitro* AM mycorrhizas between Ri T-DNA transformed carrot (*Daucus carota*) roots and *Glomus intraradices* to follow carbon movement into and between host and fungal metabolite pools.

• Fungal triacylglycerol and trehalose were labeled when permeant substrates were supplied to the extraradical mycelium (ERM), but host-specific compounds in the roots did not become labeled. When labeled glucose was provided to a donor root system, label moved to recipient roots via a common AM fungal network but remained in fungal compounds.

• We conclude that carbon flow in the AM symbiosis is normally unidirectional from plant to fungus and that while carbon is translocated by the fungus from one metabolically active root system to another, it remains within the intraradical mycelium (IRM).

Key words: arbuscular mycorrhizal symbiosis, carbon translocation, GCMS, *Glomus intraradices*, NMR, Ri T-DNA carrot (*Daucus carota*) roots

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Introduction

The arbuscular mycorrhizal (AM) symbiosis is ancient (Simon *et al.*, 1993; Taylor *et al.*, 1995; Redecker *et al.*, 2000), widespread (Smith & Read, 1997) and vital to the life of plants. The majority of land plants rely on AM fungi to facilitate the uptake of mineral nutrients, particularly phosphorus, from the soil. In return the fungal partner receives around 20% of the photosynthetically fixed carbon for the formation, maintenance and function of mycorrhizal structures (Finlay & Söderström, 1992; Wright *et al.*, 1998). This exchange involves the flow of huge quantities of material worldwide. Phosphorus transfer has been better studied (Smith & Read, 1997), but in recent years progress has also been made in understanding the mechanisms of carbon movement from plant to fungus (for reviews see Jakobsen, 1995; Bago *et al.*, 2000).

There is also evidence for the movement via common AM networks of substantial amounts of carbon between the roots of plants of the same and different species (Francis & Read, 1984; Grime *et al.*, 1987; Lerat *et al.*, 2002). These reports are based on the detection in one plant of ^{14}C supplied to another plant and together with stable isotope studies they indicate that up to half the carbon moved from plant to fungus may be moved to the root system of another plant (Graves *et al.*, 1997). This has profound implications for our understanding of plant communities and competition and, since the AM symbiosis is over 400 million years old (Redecker *et al.*, 2000), it is potentially important in the evolution of land plants.

Serious doubts have been raised as to whether the detection of a carbon label in another plant (particularly in mycorrhizal roots) represents a nutritionally meaningful transfer, or indeed any transfer at all. These doubts are based on evidence that label in 'recipient' plants remains largely or entirely in the mycorrhizal roots, even under conditions where root-to-shoot carbon flow is encouraged (Fitter *et al.*, 1998), and on a critical evaluation of the results of studies in which transfer was reported (Robinson & Fitter, 1999). Even in cases where transfer of ^{14}C, albeit small, has been detected in shoots of recipient plants following $^{14}CO_2$ incubation of donor plants, the likelihood of photosynthetic recycling raises serious doubts on the validity of their interpretation (Fitter *et al.*, 1998). Another possible route for transfer of label without nutritionally meaningful net C transfer is via the fixation of labeled CO_2 released by fungal respiration in the roots and re-fixed via anapleurotic or gluconeogenic carboxylase reactions. Furthermore, if one examines the amount of carbon proposed to have been transferred in each of these studies (Grime *et al.*, 1987; Lerat *et al.*, 2002); it becomes apparent that it does not represent a nutritionally significant contribution to the recipient. A careful examination of ^{13}C transfer in which a source-sink relationship was maintained between plants and where photosynthetic recycling was eliminated, suggested that although carbon does move between plants, it remains in fungal structures, never crossing the interface into recipient root tissues (Robinson & Fitter, 1999). Furthermore, a recent study by Zabinski *et al.* (2002) using a pulse of 20 min of $^{13}CO_2$ labeling of a native grass in the presence of an invasive *Centaurea maculosa* plant found no evidence for carbon transfer although there was enhanced growth.

However, reports that non-photosynthetic parasitic plants are epiparasitic via AM fungi (Bidartondo *et al.*, 2002) and that non-photosynthetic tobacco mutants cocultured with the wild type grew better in the presence of an AM inoculum (Müller & Dulieu, 1998) indicate that nutritionally significant carbon movement from the fungus to the plant can indeed occur under certain circumstances. Thus the importance of carbon transfer from AM fungi to plants remains unsettled.

The most direct way to address this issue is to supply labeled substrates and analyze the flow of label into host and fungal carbon compounds, and to do so under conditions where complications from transfer of label via the soil or by photosynthetic capture of label released by respiration are eliminated. With this objective a monoxenic culture system with transformed carrot roots colonized by *Glomus intraradices* growing in divided Petri plates (Mugnier & Mosse, 1987, St. Arnaud *et al.*, 1996) was used, an approach which also facilitates the extraction of pure fungal tissues. In other ^{13}C labeling studies on the utilization of carbon from hexose, it was shown, that results in whole plants (Shachar-Hill *et al.*, 1995) are comparable to those obtained with the monoxenic culture system (Pfeffer *et al.*, 1999). Previous studies with the monoxenic culture system have also shown that it is possible to distinguish labeling in host and fungal metabolic pools and to label fungal storage and transport pools by supplying selectively labeled substrates to the AM roots and to the fungal ERM (Pfeffer *et al.*, 1999; Lammers *et al.*, 2001; Bago *et al.*, 2002, 2003).

The objective of the present study was to determine whether fungal carbon is metabolically available to the host and whether carbon is transferred from one AM root system to another one, growing in a separate carbon-limited compartment connected to the first by the hyphae of an arbuscular mycorrhizal fungus (a common mycorrhizal network).

Materials and Methods

Tissue culture

Monoxenic *in vitro* cultures of Ri T-DNA transformed roots of carrot (*Daucus carota* L.) colonized by *Glomus intraradices* (DAOM 197198 Biosystematics Research Center, Ottawa, Canada) were used for the experiments. Conditions for this monoxenic culture were as previously described (St. Arnaud *et al.*, 1996, Pfeffer *et al.*, 1999). For stable isotopic labeling experiments on one mycorrhizal host root system and the associated extraradical mycelium, transformed carrot roots were grown together with *G. intraradices* inoculum from a previous monoxenic culture on one side of divided Petri plates containing solidified M medium (Chabot *et al.*, 1992). The fungal ERM, but not the roots were permitted to grow over the dividing barrier into the other half of the plate into the fungal compartment which contained M medium without sucrose, where they proliferated and subsequently sporulated (St. Arnaud *et al.*, 1996).

For root to root transfer experiments the contents of the fungal compartments of 8–13 week-old split plate cultures of Ri T-DNA transformed carrot roots colonized by *G. intraradices* were removed and replaced with fresh medium modified to contain only 2.5 g sucrose l^{-1}. Nonmycorrhizal carrot roots were added to this distal compartment, into which the fungal extraradical mycelium grew (Douds, 2002) and colonized the newly added roots that were C limited and therefore sinks for C flow (recipient roots).

^{13}C labeling experiments

Labeling experiments were performed as previously described (Bago *et al.*, 1999; Pfeffer *et al.*, 1999). ^{13}C-labeled substrates were added to one compartment (concentrations after addition were 4 mM [2–^{13}C]acetate, or 10 mM [1,3–^{13}C] or [2–^{13}C]glycerol) 1 week after fungal crossover to the fungal compartment and plates were then incubated for 8 weeks at 24°C. After labeling, donor and recipient roots were harvested separately by blending the solidified medium in 10 mM sodium citrate buffer, pH 6.0 at 4°C (Doner & Bécard, 1991)

and recovering the root tissue by filtration. All samples collected were frozen on dry ice and stored at −80°C until extraction. Fungal and root tissues were stored at −80°C after harvest before extraction for NMR and mass spectrometric analysis.

For root to root transfer experiments 125 mg [1–^{13}C]glucose was added dropwise as a filter-sterilized solution to the first, donor root compartment 11–13 d after fungal crossover to the second, recipient root compartment. Cultures then were incubated for 8 weeks in the dark at 24°C. Any roots crossing the divider during this time were removed. During the time course of the experiments the donor roots initially grew rapidly and then slowed their growth about 4 weeks after glucose addition. The recipient roots in the carbon limited compartment which had 25% of the sucrose level grew slowly during the time course. 8–23% of the recipient roots were found to be colonized by *G. intraradices* at the end of the experiment. A third experiment was conducted to demonstrate that transformed carrot roots growing in medium containing only 2.5 g sucrose l^{-1} were capable of taking up glucose. Roots were transplanted into plates containing the low sucrose M medium and grown for 15 d ($n = 6$). [1–^{13}C]glucose (100 mg) was then added and the roots were incubated at 24°C for further 6 weeks. The roots were recovered at the end of the experiment by gentle stirring in sodium citrate buffer and frozen as described above.

^{14}C root to root transfer experiment

14 d after fungal hyphae crossed the divider of plates grown as described above, a solution containing 125 mg glucose (of which 0.0001% was [U-^{14}C]glucose, the final concentration in the medium was 27.8 mM) was filter sterilized and added drop-wise to the donor root compartment. 1, 2, 4, 6 and 8 weeks after addition of the [U-^{14}C]glucose, donor and recipient roots were taken from several plates, rinsed in water and the ^{14}C content in these root samples was determined by liquid scintillation counting. Additionally, ERM samples of both sides were collected after solubilization of the medium with 10 mM citrate buffer at pH 6 and removal of the roots using a stereomicroscope. All tissue samples were dried in an oven, weighed and solubilized by a tissue solubilizer (TS-2, RPI Corporation, Mt Prospect, IL, USA) at 60°C. The ^{14}C content of the medium and the tissue samples was analyzed by liquid scintillation counting (Beckman Coulter, LS 6500) and the counting efficiency was determined using an internal standard.

Extraction and derivatization of carbohydrates and neutral lipids

Carbohydrates were extracted from both the ERM and the mycorrhizal roots from the split Petri plates with methanol/water (70 : 30, v:v) and the residues were boiled in isopropyl alcohol to extract the neutral lipids as previously described (Pfeffer *et al.*, 1999). Methanol/water extracts were filtered, rotary film evaporated under reduced pressure to remove methanol, lyophilized and redissolved in deuterated water. Isopropyl alcohol extracts of triaclyglycerol (TAG) were evaporated and redissolved in $CDCl_3$ (C^2HCl_3). The samples were then analyzed by NMR spectroscopy (as described below) and subsequently lyophilized or evaporated in a stream of nitrogen and then derivatized for GCMS analysis (see below).

One hundred μg of the neutral carbohydrate fraction obtained from the methanol/water extraction was dissolved in 5 μL of pyridine and applied to a column containing 18 mg of Sox-XTd celite 545 (Fisher Scientific, King of Prussia, PA, USA). The pyridine was removed evaporatively by blowing N_2 through the columns. The sample was then acetylated in a stream of acetyl chloride gas for 2 h and then eluted with 300 μL of chloroform. Further elution using 100 μL aliquots of chloroform was performed until no further derivative was detected.

A melting point capillary narrowed at one end, containing a one mm glass bead to support a column, was filled to a height of approximately 2.5 cm (after settling and tapping) with finely ground NaOH and celite 545 (2 : 1). Five μL of a DMSO solution containing 0.2–0.4 μg/mL of the sugar mixture was applied to the bed with a syringe. After 5 min methyl iodide vapor was passed over the bed with suction for 1 min. The permethylated sugars were eluted with $CHCl_3$ and examined by GCMS.

The neutral lipids were transmethylated using a modification of the alkali catalyzed method of Christopherson & Glass (1970). Approximately 100 μL of a solution of the lipid in n-hexane was vortexed in a screw capped vial with a Teflon liner at full speed with 200 μL of 1.5 M sodium methoxide in methanol for 1 min. Aqueous citric acid (0.3 mL, 2 M) was immediately added and the reaction mixture was vortexed for a few seconds and then centrifuged at 3500 r.p.m. for 3 min. The upper organic layer was removed and an aliquot was analyzed by GCMS.

Identification of labeling in the glyceryl portion of neutral lipids

Two hundred μL of 2 N HCl in n-propyl alcohol was added to the extracted TAG and vortexed until the lipid was completely dissolved. The latter was stored for 5 d at room temperature, at which time complete transesterification of the lipids had occurred, as shown by thin layer chromatography.

One-hundred μL of the solution was transferred to a 2 mL screw cap vial with a Teflon liner and the solvent and HCl were removed under a stream of N_2 at room temperature until no HCl could be detected by pH paper. One ml of dichloromethane and 250 μL of distilled water were then added and the mixture was vortexed for approximately 2 min. The vial was allowed to stand for 30 min to allow the

layers to completely separate. The upper layer was removed as completely as possible with a syringe and evaporated to dryness in a shell vial under N_2 and low heat. The residue was dissolved in 20 µL of pyridine and 5 µL was applied to 20 mg of celite 545 (Fisher Scientific, King of Prussia, PA, USA) supported by a 1 mm glass bead contained in a 50 µL glass replacement tube (Drummond glass replacement tube, Fisher Scientific, King of Prussia, PA, USA). The tube had been narrowed at one end in a flame to retain a 1 mm glass bead. The pyridine was evaporated from the celite under reduced pressure for 15 min at room temperature. The capillary was exposed for 20 min to the vapors of acetyl chloride (Aldrich, Milwaukee, WI, USA) drawn through under reduced pressure. The bed was eluted with two 100 µL aliquots of cyclopentane (Aldrich, Milwaukee, WI, USA). The residue was analyzed for glycerol triacetate by GCMS.

NMR analysis

Conditions and instrumentation for obtaining ^1H and ^{13}C NMR spectra were as previously described (Bago *et al.*, 1999; Pfeffer *et al.*, 1999) using a 400 MHz instrument (Varian, Palo Alto, CA, USA) for ^{13}C and a 750 MHz instrument (Bruker Instruments, Billerica, MA, USA) for ^1H spectroscopy. The total percentage ^{13}C at each carbon position of trehalose was determined by first using ^1H spectra to assign total ^{13}C content in the C_1 position (which was done by comparing the intensity of the signals from hydrogens bonded to ^{12}C atoms to the intensity of signals from hydrogens bonded to ^{13}C atoms) and then analyzing ^{13}C spectra to determine the relative ^{13}C content in the other carbon positions. Due to restrictions on the availability of 750 MHz instrument time ^1H spectra were not run on all samples and the percentage ^{13}C values quoted in the results and discussion are calculated using the absolute ^{13}C contents at C_1 from a subset of > 10 samples for which ^1H spectra were run. Confidence in this procedure was gained by noting that ^{13}C spectra showed that the relative ^{13}C contents at the different C positions of trehalose were the same to within ±10% for all replicate samples. Because of the complexity of the sucrose ^1H spectrum we only used the ^1H spectra to evaluate the ^{13}C labeling in the C_1 position. Determination of the ^{13}C content in the TAGs was made from the ^{13}C spectrum using the ratio of $C_{1,3}$ to C_2 areas for the glyceryl portion of the molecule and the Cω to Cω-1 ratio to assess labeling in the fatty acid portion of the molecules. These values were verified with the ^1H satellite measurements of the glyceryl$_{1,3}$ protons and terminal methyl protons and or C_2 fatty acid protons, respectively.

GCMS analysis

Analysis of carbohydrates, *f*atty *a*cid *m*ethyl *e*sters (FAMEs) and glycerol derived from the hydrolysis of the triacylglycerides was performed on a Trace 2000 gas chromatograph (Thermo Electron, Madison, WI, USA) equipped with a splitless injector using an open-tubular column of 0.25 µm-thick BP-15 film (0.18 mm i.d. × 0.4 mm o.d. × 30 m, Agilent corporation, Palo Alto, CA, USA) which was interfaced to a Thermo Finnigan quadrupole mass detector (Thermo Electron, Madison, WI, USA). Zone temperatures were: injector, 290°C: column held at 50°C for 1.0 min after injection, increasing to 250°C at 10°C min^{-1}, then to 280°C at 25°C min^{-1} then constant for 8 min; and detector, 350°C. For the glycerol triacetate analysis the zone temperatures were: injector, 290°C; column held at 100°C for 1.0 min after injection, increasing to 250°C at 5°C min^{-1}; and detector, 350°C. Helium carrier gas flow was 1 mL/min for all analyses. Labeling in sucrose and trehalose was determined by monitoring the mass isotopic distribution in either the m-32 ions (loss of methanol from the permethylated disaccharide units) having a m/z ratio of 422 or the m-59 ions for loss of acetate from the peracetylated hexose units having a m/z ratio of 331. The isotopic enrichment of the fatty acid portions of the TAG was determined from the isotopic ratios of the molecular ions of m/z 168, 170, 194, 196 and 198 corresponding to 16 : 1, 16 : 0, 18 : 2, 18 : 1 and 18 : 0 FAMEs. The isotopic enrichment in the glycerol portion of the molecule was determined from the GCMS of the m-73 ions (loss of acetate and a methyl group from the glycerol triacetate) having a m : z ratio of 145 for the m_0 ion.

Mass spectra of FAMEs were compared to the mass isomer distributions calculated assuming random distribution of label in the precursor pools. The average labeling in the C_1 and C_2 positions of acetyl CoA were deduced from NMR spectra of the underivatized TAG samples which yielded the average labeling in odd and even fatty acid carbon positions (which are synthesized from the C_1 and C_2 positions of acetyl CoA). The comparison of calculated and measured mass isomer distributions showed whether labeling in precursor pools was random or not. When labeling was not random, the mass spectral isotopic ratios were simulated assuming two metabolic pools, one unlabeled and one labeled, with the labeling level in the labeled pool and the proportion of lipid made in the labeled vs. the unlabeled pool being varied to optimize the fit between observed and simulated mass isomer distributions. Simulations were made using binomial distribution probabilities implemented in spreadsheet software (Excel, Microsoft).

Results

Labeling experiments on one mycorrhizal host root system and the associated extraradical mycelium

Figure 1 shows ^{13}C NMR spectra of extracts from mycorrhizal roots of cultures that had been exposed to [2–^{13}C]glycerol either in the mycorrhizal root or the fungal compartment. When [2–^{13}C]glycerol was provided to the root compartment, the glyceryl moieties of TAG and the fatty acids of both

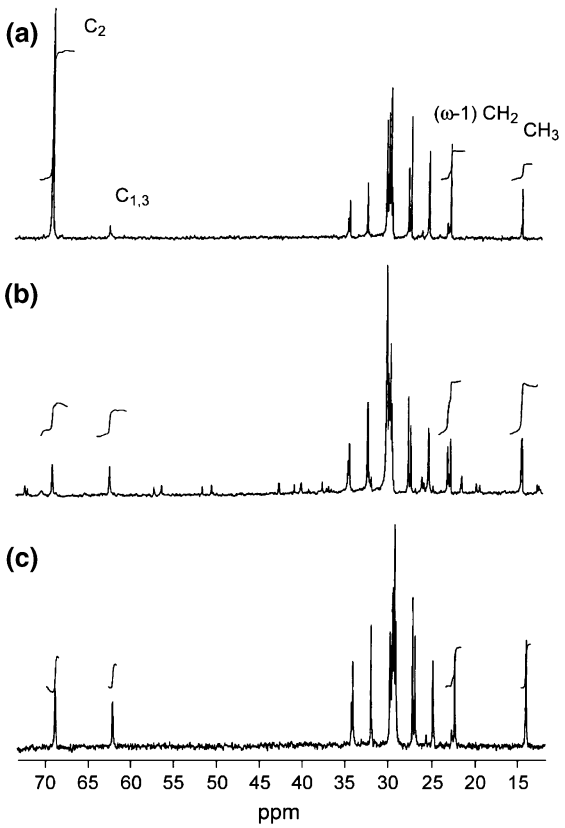

Fig. 1 ^{13}C NMR spectra of extracted TAG showing the ratios of the glycerol $C_{1,3}$ to C_2 and terminal methyl to ω-1 CH_2 carbon resonances in (a) extraradical mycelium (ERM) tissue labeled by the introduction of [2–^{13}C]glycerol to the root compartment; (b) mycorrhizal root tissue labeled by the introduction of [2–^{13}C]glycerol to the fungal compartment and (c) ERM tissue labeled by the introduction of [2–^{13}C]glycerol to the fungal compartment. In the unlabeled state the $C_{1,3}$ to C_2 resonance ratio should be 2 : 1 and the terminal methyl to ω-1 CH_2 carbon resonance ratio should be 1 : 1. Integral bars represent the relative area of each resonance in the spectrum.

plant and fungal lipids in the AM roots were labeled. Label was also observed in both glyceryl and fatty acid portions of the TAG in the fungal compartment (Fig. 1a). When [2–^{13}C]glycerol was supplied to the ERM, the glyceryl moiety but not the fatty acids of the TAG extracted from mycorrhizal roots (Fig. 1b) and ERM (Fig. 1c) became labeled. The sensitivity of the ^{13}C NMR analysis is only sufficient to detect with confidence labeling levels of 0.5% or more, so GCMS spectra of the FAMEs and glycerol triacetates from the extracted TAG's were used to ascertain whether there was a labeling of host specific fatty acids and glycerols at levels below this threshold. No labeling was detected by this more sensitive method in which the detection threshold was found to be 0.1% based on the variability in spectra of unlabeled samples (mass spectra not shown).

^{13}C and ^{1}H NMR and GCMS spectra were used to determine isotopic labeling in carbohydrates and lipids of mycorrhizal roots and ERM after supplying [1,3–^{13}C] or [2–^{13}C]glycerol or [2–^{13}C]acetate to root or fungal compartment (Table 1). Trehalose and sucrose in the mycorrhizal roots and trehalose in the ERM became significantly labeled when labeled glycerol was supplied to mycorrhizal roots, which indicates that if significant carbon transfer from fungus to host were taking place at the triose level this would have resulted in labeling of sucrose when ^{13}C glycerol was supplied to the ERM, but no labeling of sugars in the mycorrhizal roots was detected by NMR in those experiments. This was also confirmed by GCMS of the peracetylated sugars (spectra not shown).

Labeling of host and fungal fatty acids was also observed after labeling the root compartment with [2–^{13}C]acetate but not when this substrate was supplied to the ERM (Table 1). In the mycorrhizal roots the 16 : 1 fungal fatty acid was found by mass spectrometric analysis to be non-randomly labeled (see Materials and Methods section). Simulations of the mass isomer distributions indicate that it consists of an unlabeled pool comprising 82 ± 2% of the molecules and a labeled pool accounting for the remaining 18% ± 2% of the molecules whose precursor acetyl CoA was labeled to 8.1% ± 0.3% in the C_2 position. The 18 : 2 fatty acid of TAG, which is predominantly of plant origin was also non-randomly labeled, being composed of 91% ± 2.0% unlabeled molecules and 9% ± 2.0% labeled molecules whose precursor acetyl CoA was labeled to 22% ± 2.0%. The 16 : 1 fatty acid in TAG from the ERM in this experiment consisted of 78% ± 3.1% unlabeled and 22% ± 3.1% whose precursor acetyl CoA was labeled to 6.0% ± 0.5%. This suggests that when [2–^{13}C]acetate is the substrate in the root compartment containing unlabeled sucrose media, the acetate enters biosynthetic metabolic pools in only a minority of the tissue. By contrast, no labeling of host or fungal TAG fatty acids was observed by GCMS analysis in either compartment when [2–^{13}C]acetate was supplied to the ERM.

Supplying [1–^{13}C]glucose to the mycorrhizal roots results in a labeling of 18.2% ± 0.8% (n = 3) in the C_1 position of sucrose as well as 15.7% ± 1.8% (n = 3) in the C_1 and C_1' positions of trehalose as measured by NMR. This shows that any significant transfer of hexose from fungus to the host would result in labeling of sucrose when fungal carbohydrates within the root are labeled. When [1,3–^{13}C] or [2–^{13}C]glycerol was supplied to the ERM, trehalose in the ERM was significantly labeled (Table 1), and when [2–^{13}C]acetate was supplied the labeling in trehalose was even higher. However, no labeling of either TAG or sucrose was observed in the mycorrhizal roots by either NMR (Table 1) or GCMS analysis in those experiments.

Labeling experiments on two mycorrhizal root systems connected by a common mycorrhizal network

Labeling the mycorrhizal donor root compartment with [U-^{14}C]glucose or [1–^{13}C]glucose allowed us to examine the

Table 1 Labeling levels in the lipids and carbohydrates of mycorrhizal roots and extraradical mycelium (ERM) after ^{13}C labeled substrates were supplied (mean of $n = 3 \pm$ SEM)

Label supplied	Labeled compartment	Tissue analyzed	% labeling in lipid moieties[1]		% labeling in carbohydrates[1]	
			Glyceryl	Fatty acids	Trehalose	Sucrose
[1,3–^{13}C] Glycerol	Root	Root	48.3 ± 12	1.8 ± 0.5	5.1 ± 0.2	3.4 ± 1.5
[1,3–^{13}C] Glycerol	Root	ERM	47.0 ± 3.0	2.0 ± 0.3	9.0 ± 2.3	
[1,3–^{13}C] Glycerol	ERM	Root	1.9 ± 0.5	0	0	0
[1,3–^{13}C] Glycerol	ERM	ERM	2.9 ± 2	0	9.1 ± 0.7	
[2–^{13}C] Glycerol	Root	Root	33.9 ± 3.9	2.0 ± 0.5	ND[4]	3.1
[2–^{13}C] Glycerol	Root	ERM	40.4 ± 5.5	2.0 ± 0.2	13.8, 10.3[5]	
[2–^{13}C] Glycerol	ERM	Root	1.4 ± 0.3	0	0	0
[2–^{13}C] Glycerol	ERM	ERM	2.8 ± 0.3	0	30.7 ± 2.7, 16.1 ± 1.7[5]	
[2–^{13}C] Acetate	Root	Root	0	2.2 ± 0.5[2]	ND[4]	0
[2–^{13}C] Acetate	Root	ERM	0	0.6 ± 0.03[3]	0.9 ± 0.5	
[2–^{13}C] Acetate	ERM	Root	0	0	0	0
[2–^{13}C] Acetate	ERM	ERM	0	0	37.4 ± 5.5	

[1]Based on ^{13}C and ^{1}H NMR measurements of ^{13}C labeling in the C_1, C_2, C_5 carbon of trehalose and sucrose, the $C_{1,3}$ and C_2 glyceryl carbons of TAG and the terminal CH_3 fatty acids of TAG. [2]Analysis of the FAME mass spectrum showed this to be a mixture of 91% unlabeled and 9% labeled 18 : 2 fatty acid, 95% unlabeled. 5% labeled ω5–16 : 1 fatty acid, [3]78% unlabeled, 22% labeled ω5–16 : 1 FAME. [4]Not determined because it was at too low a concentration to measure [5]Represents C_5 and C_2 labeling, respectively.

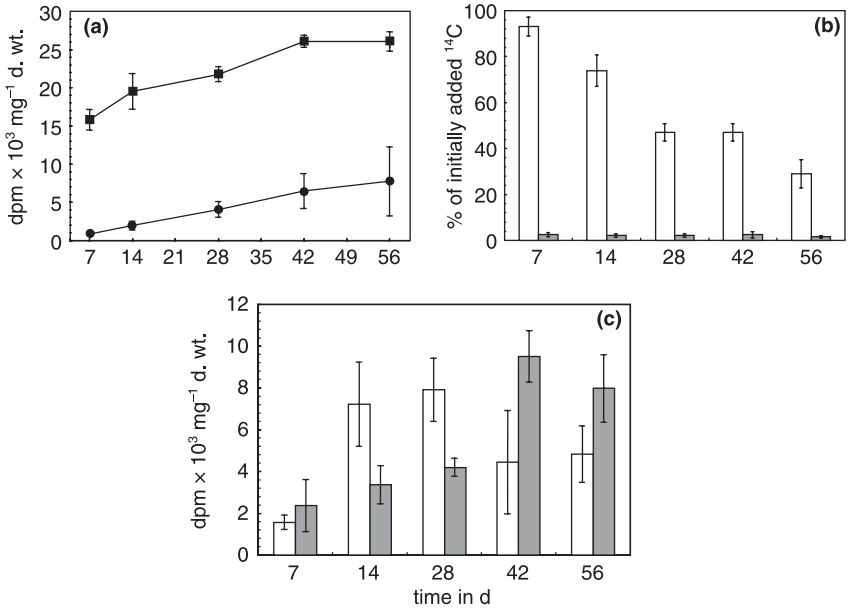

Fig. 2 (a) ^{14}C content in donor (squares) and recipient roots (circles) after supply of [U-^{14}C]glucose to the donor root compartment; (b) percent of the total radioactivity added recovered from the medium of donor (open bars) and recipient compartment (closed bars); (c) ^{14}C content of the extraradical (ERM) from donor root (open bars) and recipient root compartment (closed bars) (mean ± SEM, $n = 7$).

transfer of carbon to recipient roots, colonized by the ERM which extended from the donor root compartment into the recipient root compartment. The recipient roots were established as a carbon limited sink by using 25% of the amount of sucrose normally present in the medium, which caused a marked inhibition of root growth, but which was sufficient to prevent the complete cessation of growth and rapid senescence that was observed at lower sucrose levels (data not shown). Roots grown at this reduced carbon level remained viable and produced 26% ^{13}C (C_1) labeling of root sucrose from [1–^{13}C]glucose in the medium (NMR data not shown), so that if hexose were transferred from the fungus it would be detectable in sucrose in the recipient roots.

After supplying 27.7 mM [U-^{14}C]glucose, the ^{14}C contents of donor roots increased rapidly during the first week whereas little carbon was detected in the recipient roots at that time (Fig. 2a). The ^{14}C contents of donor and recipient roots increased during the remainder of the 8-week time course. The ^{14}C content of the donor roots continued to increase up to at least 6 weeks after glucose addition (Fig. 2a) and the uptake from the medium continued throughout the time course (Fig. 2b). The level of radioactivity detected in the

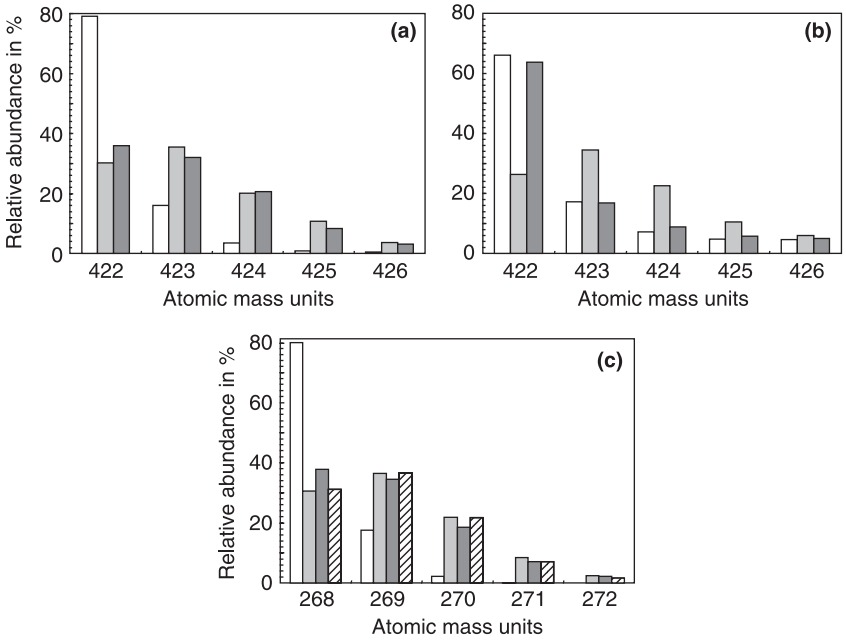

Fig. 3 Mass spectral isotopic ratios for the molecular ions 422–426 for permethylated trehalose (a) and sucrose (b), and for the molecular ions 268–272 for C16 : 1 FAME from transmethylated TAG (c); unlabeled (open), donor roots (light grey) and recipient roots (dark grey); values calculated assuming 12.5% labeling in even numbered positions and natural abundance in the odd positions (hatched bars in c).

medium from the recipient root compartment was extremely low and did not increase during the time course (Fig. 2b).

The ^{14}C content of the ERM in each compartment was also measured (Fig. 2c) and showed that carbon uptake and translocation to the recipient root compartment is significant within a week. From the second to the fourth week of the time course, labeling of the ERM in the donor root compartment was substantially higher than in the recipient root compartment. Thereafter the content of the ERM of the recipient root compartment rose and that in the donor root compartment fell. This decrease is not due to an exhaustion of glucose in the donor compartment (Fig. 2b). After 8 weeks nearly 30% of the initially added glucose was still detectable in the medium from the donor root compartment.

^{13}C labeling in trehalose and sucrose in donor and recipient roots was measured using ^{1}H NMR spectra of extracts. After 8 weeks of incubation labeling was 19.0% ± 0.8% and 12.5% ± 0.6% in the C_1 positions of sucrose and trehalose, respectively, in donor roots and was 11.6% ± 0.3% and 0.3% (n = 3) in C_1 positions of the trehalose and sucrose of recipient roots. Similar results were obtained from spectra of dual mycorrhizal plates whose donor root compartment was provided with [1–^{13}C]glucose for 4 weeks, with high levels of labeling in the C_1 positions of sucrose and trehalose from the donor roots and in trehalose from the recipient roots but little or no labeling (0.3%) in the sucrose from the donor roots. Because of limited signal-to-noise ratios and the possibility of interference from other extracted metabolites in the ^{1}H NMR spectra, GCMS measurements were carried out to exclude low labeling in sucrose of the recipient roots that was not detectable with NMR. Figure 3(a),(b) show the relative abundances of mass isomers from mass spectra of permethylated sucrose and trehalose extracted from mycorrhizal donor and recipient roots after labeling the donor root compartment with [1–^{13}C]glucose for 8 weeks. The proportion of labeled trehalose molecules in donor and recipient roots was 66.8% ± 3.3% and 53.6% ± 0.7%, respectively. The sucrose mass spectra show that 59.1% ± 4.5% of the molecules in the donor roots were labeled, but mass spectra of sucrose in the recipient roots were comparable to spectra of unlabeled sucrose samples. Simulations of mass spectra of sucrose extracted from the recipient roots showed a ^{13}C content of 1.2% ± 0.7%, in the recipient roots, which is the same as the natural abundance level of 1.1%.

Figure 3(c) shows the mass isomer distribution for a representative FAME of the fungal specific ω-5 16 : 1 fatty acid measured from donor and recipient roots, together with distributions obtained from an unlabeled sample and from a simulated mass spectrum that was calculated by assuming that the precursor pool of acetyl CoA was homogenous and labeled to 13% in the C_2 position. Such comparisons indicate that the dominant fungal storage fatty acid is made from acetyl CoA with a $^{13}C_2$ content of 11.7% ± 0.6%. Thus after 8 weeks of labeling with glucose there is one population of randomly labeled fungal fatty acid molecules in both donor and recipient roots. These observations are consistent with an analysis of the distribution of mass isomers seen in GCMS spectra of FAMEs from experiments with one set of AM roots with an fungal compartment, which revealed homogenous labeling of TAG fatty acids in both AM roots and ERM after supplying [1–^{13}C]glucose to the root compartment (data not shown).

TAG extracted from fungal tissue from the fungal compartment a small proportion (2–5%) of 18 : 2 fatty acid. Following

the addition of ^{13}C glucose to the root compartment analysis of the mass isomer distribution of this fatty acid indicates that it was made from a single precursor acetyl CoA pool that contained 11–12% ^{13}C (data not shown). By contrast, TAG extracted from mycorrhizal recipient roots in the transfer experiment contained 18 : 2 fatty acid whose labeling shows the presence of two pools: one pool accounting for 75% of the 18 : 2 fatty acid is unlabeled, and another pool accounting for 25% of the total 18 : 2 fatty acid is enriched to 12.5% with ^{13}C. The larger pool is assignable to the plant on the basis of the higher 18 : 2 fatty acid levels in roots, and the smaller pool has a very similar labeling level to the fungal 16 : 1 fatty acid. Thus both fungus and plant tissues contain the 18 : 2 fatty acid TAG in the recipient roots, however, only the fungal portion is ^{13}C labeled. The labeling in the 18 : 2 fatty acid of TAG is usually due to labeling in plant lipids (Pfeffer *et al.*, 1999). However, we measured the level of the 18 : 2 fatty acid in the TAG of colonized recipient roots and found that it is greatly diminished following their 8 weeks of growth on limited carbon, being < 20% of its normal content (data not shown).

Discussion

The *in vitro* mycorrhizal system and its suitability

The *in vitro* AM split plate culture system developed by St. Arnaud *et al.* (1996) was used for these experiments because of the absence of soil or other microorganisms and of either photosynthetic CO_2 fixation or a diffusive pathway for label movement from one compartment to another, the presence of any of which would greatly complicate the interpretation of any observed label transfer (Fitter *et al.*, 1998). This system has also been proven to be suitable for the study of both nitrogen and phosphorus movement from fungus to host plant (Bago *et al.*, 1996; Nielsen *et al.*, 2002). Those studies and results of ^{33}P labeling (H. Bücking, unpublished) showed that there is active uptake of P by ERM and transfer to the mycorrhizal host roots. Furthermore, comparison of results from ^{13}C labeling experiments on mycorrhizal roots of whole plants (Shachar-Hill *et al.*, 1995) with findings in the present system (Pfeffer *et al.*, 1999) indicate that the mechanisms of carbon uptake and metabolism by the fungus are similar. These findings indicate that the *in vitro* symbiosis is functioning normally in nutrient transfer and this is important to the interpretation of labeling results, especially negative ones that indicate the absence of carbon transfer (see below).

Transformed roots in culture probably differ hormonally and phenologically from the roots of whole plants, so the carbon sink strength of the roots may differ from those in some whole-plant symbioses (as indeed they do from one whole plant/fungus combination to another). However providing recipient roots with only 25% of the normal sucrose level, limits their growth and should create a strong sink in the root-to-root transfer experiments. Thus we would expect to have detected at least some carbon transfer to the plant tissues of recipient roots if this process can occur.

Carbon moves from the extraradical mycelium into the intraradical mycelium but is not transferred to the host

In previous studies we have explored the routes by which carbon moves in the AM symbiosis, and reported that the fungus takes up hexose inside the root (Shachar-Hill *et al.*, 1995) and there synthesizes triacylglycerides (TAGs) from it (Pfeffer *et al.*, 1999). Some of this lipid is translocated to the ERM (Bago *et al.*, 2002). TAG synthesis in the ERM is very much less than in the IRM (Pfeffer *et al.*, 1999; Bago *et al.*, 2002) although probably not zero (Fontaine *et al.*, 2001). Therefore the observation that [2–^{13}C] or [1,3–^{13}C]glycerol provided to the ERM labeled the glyceryl portion of the TAG molecules to a significant degree (confirming the results of Bago *et al.*, 2002), is most easily explained as arising through turnover of the glyceryl moiety of TAG by transacylation in the ERM (Fig. 1, Table 1). This apparent turnover of glyceryl moieties of TAG in the ERM is consistent with the finding by Gaspar *et al.* (2001) that free palmitic (16 : 0) fatty acid is incorporated into TAG by the ERM through the action of an acetyl CoA ligase. The finding that glyceryl moieties of TAG in the mycorrhizal roots become labeled when [2–^{13}C] or [1,3–^{13}C]glycerol is provided to the ERM is consistent with the report of a significant recirculation of lipid from the ERM to the IRM by Bago *et al.* (2002).

When labeled glycerol was provided to the root compartment, the glyceryl moieties of TAG and the fatty acids of both plant and fungal lipids in the AM roots were labeled. This is consistent with the synthesis of TAG by both host and fungus in AM roots (Pfeffer *et al.*, 1999) and indicates that triose precursors transferred in significant quantities from fungus to the host would result in detectable labeling of host TAG. This indicates that TAG in the IRM is not transferred to the host, otherwise labeling of host fatty acids would be expected when labeled glycerol was supplied to the ERM. This finding is also supported by the lack of labeling in sucrose in those experiments since glycerol was readily utilized by the root to produce sucrose, when it was supplied to the root compartment (Table 1).

^{13}C enrichment of host and fungal fatty acids was also observed after labeling the root compartment with [2–^{13}C] acetate (Table 1). This shows that if acetate or other compounds entering metabolism at the level of acetyl CoA were being transferred from the fungus to the host in significant quantities, it would have been observed when fungal pools were labeled in the ERM. However, when [2–^{13}C]acetate was supplied to the ERM no evidence of lipid or carbohydrate labeling was observed in the mycorrhizal root tissues. Supplying labeled acetate or glycerol to the ERM labeled trehalose in that compartment (confirming previous observations, Lammers *et al.*, 2001) but not in the root compartment. This

indicates that carbohydrate does not move from the ERM to the IRM from which it originated, consistent with the report by Bago *et al.* (2003), that carbohydrates are transferred from IRM to ERM, probably in the form of glycogen, but not in the reverse direction. The absence of labeling in sucrose after labeling of carbohydrates in the ERM indicates that the precursor pools from which carbohydrates in the ERM are made are not available to the host; probably like carbohydrates, they do not return to the IRM in significant amounts and are not transferred to the host.

As part of a study of nitrogen movement in this *in vitro* AM system Govindarajulu *et al.* (M. Govindarajulu *et al.*, unpublished) used GCMS to analyze labeling in the amino acids of the ERM and of extracted hydrolyzed proteins of mycorrhizal roots after supplying [2–^{13}C]acetate to the ERM. Amino acids in the ERM were substantially labeled but no labeling was detected in the AM root proteins. Adding this to the results of the present study we conclude that there is no evidence for transfer of carbon from fungus to root cells. Two comments may be made regarding the negative results of this part of the study. The first is that the labeling of plant compounds detected after directly supplying labeled glucose, glycerol or acetate to the root compartment indicate that the absence of label in those compounds after supplying label to the fungal compartment is due to the absence of significant carbon transfer from fungus to root, since any substantial transfer of hexose, triose, amino acids, acetate, glycerol or other closely related metabolite would have been detected after its incorporation into host sucrose, TAG fatty acids or protein. The second is that although negative findings on fungus-to-plant carbon transfer in one or more systems cannot exclude the possibility that it does occur in others, one can at least deduce from these findings that unidirectional carbon flow from plant to fungus is sufficient and that nutritionally significant carbon flow from fungus to host is not necessary for an actively functioning mycorrhiza.

Carbon is translocated from one mycorrhizal root system to another where it remains in fungal compounds

Labeling mycorrhizal roots with [1–^{13}C]glucose results in specific labeling in fungal lipids and carbohydrates in the ERM (Pfeffer *et al.*, 1999; Lammers *et al.*, 2001) and therefore provides a way to examine the movement of carbon to roots in another compartment connected to the first (donor) roots via a common mycorrhizal network. To increase the likelihood of transfer to the second (recipient) roots, the latter were grown in medium containing 25% of the usual sucrose, which limits but does not prevent growth and under which conditions sucrose becomes substantially labeled if labeled glucose is provided to them.

^{14}C labeling experiments allowed the total carbon uptake to be quantified and its partitioning among donor and recipient roots and the ERM in each compartment to be determined over an 8-week time course. The increase in ^{14}C contents by the donor roots up to at least 6 weeks after glucose addition (Fig. 2a) and the uptake from the medium throughout the time course (Fig. 2b) are consistent with continued active metabolism by the donor roots, even after they have stopped proliferating vigorously (which happens after approx. 4 weeks). The level of ^{14}C in the ERM in the recipient compartment continued to rise and on a per-tissue basis becomes much higher than the level in the donor root compartment, which is consistent with active carbon export by the fungus from the donor root compartment to the recipient root compartment. The transfer of carbon to the recipient root system is substantial and is consistent with previous reports of substantial transfer of carbon from one plant to the roots of another via a common mycorrhizal network in AM as well as in the ectomycorrhizal symbiosis (Francis & Read, 1984; Finlay *et al.*, 1986).

The level of radioactivity detected in the medium of the recipient root compartment was extremely low (Fig. 2b) and is probably due to a release of ^{14}C from the roots and the ERM during harvest with some contribution from $^{14}CO_2$ released by respiration dissolving in the medium. Exudation of ^{14}C by ERM or recipient roots does not seem to be significant since if that were the case, the levels of ^{14}C in the medium of the recipient root compartment would be expected to rise during the time course. Thus transfer of carbon from donor to recipient roots can be attributed to translocation by the fungus. A key question is whether carbon translocated by the fungus from one AM root system to another is available to the recipient plant, and this was addressed using ^{13}C labeling to identify which compounds were labeled in the recipient roots.

After labeling donor mycorrhizal roots with [1–^{13}C]glucose, both fungal TAG and trehalose became highly labeled in both donor and recipient roots. GCMS spectra showed one homogeneous pool of each of these in the recipient roots. The absence of significant labeling of sucrose in the recipient roots indicates that neither hexose nor triose is transferred to the recipient roots by the fungus. The high degree of labeling of fungal compounds in the recipient roots shows that the IRM of carbon-limited recipient roots is able to acquire most or all of its carbon from the IRM of donor roots via the ERM. The labeling of trehalose in the recipient IRM in this experiment very probably resulted either from the translocation of carbohydrate from the ERM or from synthesis of carbohydrate in the IRM from lipid translocated from the ERM. Neither of these processes has been observed in the IRM previously (Pfeffer *et al.*, 1999; Bago *et al.*, 2003; data above) and suggests that when the host does not supply carbon the IRM imports and/or metabolizes carbon more like the ERM.

The proportion of the 18 : 2 fatty acid molecules in the recipient roots that are from fungal TAG is much higher than usual, because the carbon limitation imposed on the recipient roots greatly reduces the levels of plant TAG. In addition most

of this lipid (75%) is unlabeled due to the contribution of the recipient root (pure labeled fungal mycelium only contains randomly labeled 18 : 2 fatty acid TAG). Because of these observations and because the labeling level in the labeled 18 : 2 fatty acid pool is the same as the level in the fungal 16 : 1 fatty acid (12%) we interpret the presence of a pool of labeled 18 : 2 fatty acid in the recipient roots as being due to fungal TAG in the IRM.

Our findings are consistent with the idea that carbon taken up by the AM fungus in association with one mycorrhizal root system is transferred in significant quantities to other roots connected to the same fungal mycelium but that it remains in the fungus and does not become nutritionally available to either the original host roots or to other roots. An exception to this appears to be when achlorophyllous plants cheat the symbiosis under normal (Bidartondo *et al.*, 2002) and perhaps also artificial conditions (Müller & Dulieu, 1998). This conclusion contrasts with that reached in a number of studies where the observation of ^{14}C in recipient plant roots after supplying $^{14}CO_2$ to donor plants was interpreted as being due to plant-to-plant transfer of carbon via a common mycorrhizal network (reviewed by Fitter *et al.*, 1998). We believe that several factors make the present findings more reliable. First is the use of a system where photosynthetic assimilation of 'secondary' respired $^{14}CO_2$ cannot occur and where movement of label from one root system to another by diffusion is eliminated. The second is the quantification of positional labeling in host vs. fungal compounds after providing several different positionally labeled substrates that are incorporated into both of the main forms of carbon translocated within the fungus, carbohydrates and TAG.

The source-sink dynamics for carbon transfer as well as other nutritional and physiological parameters are likely to differ from other plant-fungal combinations and this may affect the quantities of carbon transferred to the fungus and its subsequent availability for transport from the fungus to the recipient roots. Lerat *et al.* (2003a,b) reported that the carbon sink strength depends on the fungal and plant species, and even the fungal strain used. *G. intraradices* showed a strong carbon sink strength with barley, but not in association with sugar maple (Lerat *et al.*, 2003a) and Douds *et al.* (1988) found that only between 3% and 4% of the assimilated carbon of two host plant species was allocated to this AM fungus. However, the present investigation showed that even after 8 weeks the AM fungus was still supplied by the donor roots with carbon and that in spite of increasing carbon levels in the ERM and in the recipient roots the carbon pools of the recipient host root were not labeled. Recipient host plants may benefit from carbon transported from donor plants, even if it remains in the fungus, since such movement would reduce the demand of the fungus for carbon from the recipient plant while allowing it to provide soil nutrients such as phosphorus (Newman, 1988; Zabinski *et al.*, 2002). This would in effect represent a subsidy by one plant of the costs of acquiring mineral nutrition by another. Evidence has been presented for a more extreme form of cheating of the arbuscular mycorrhizal relationship in which there is net transfer of carbon from the fungus to achlorophyllous plants (Bidartondo *et al.*, 2002). The approach taken here may allow the route of carbon transfer to be elucidated in that and other epiparasitic plant-fungal situations. Experiments to compare the findings of this study to other plant-fungal combinations will be valuable, especially if such experiments can be performed using *in vitro* monoxenic whole plants in split plates – or in another system that offers the level of experimental control which is currently only offered by the system of St. Arnaud *et al.* (1996) that was used here.

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