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Carbon Uptake and the Metabolism and Transport of Lipids in an Arbuscular Mycorrhiza[1]

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Both the plant and the fungus benefit nutritionally in the arbuscular mycorrhizal symbiosis: The host plant enjoys enhanced mineral uptake and the fungus receives fixed carbon. In this exchange the uptake, metabolism, and translocation of carbon by the fungal partner are poorly understood. We therefore analyzed the fate of isotopically labeled substrates in an arbuscular mycorrhiza (in vitro cultures of Ri T-DNA-transformed carrot [*Daucus carota*] roots colonized by *Glomus intraradices*) using nuclear magnetic resonance spectroscopy. Labeling patterns observed in lipids and carbohydrates after substrates were supplied to the mycorrhizal roots or the extraradical mycelium indicated that: (a) ^{13}C-labeled glucose and fructose (but not mannitol or succinate) are effectively taken up by the fungus within the root and are metabolized to yield labeled carbohydrates and lipids; (b) the extraradical mycelium does not use exogenous sugars for catabolism, storage, or transfer to the host; (c) the fungus converts sugars taken up in the root compartment into lipids that are then translocated to the extraradical mycelium (there being little or no lipid synthesis in the external mycelium); and (d) hexose in fungal tissue undergoes substantially higher fluxes through an oxidative pentose phosphate pathway than does hexose in the host plant.

AM fungi are obligate symbionts that colonize the roots of the majority of crop plants. Among the benefits for the host plant that have been ascribed to this symbiosis are resistance to plant pests (Hooker et al., 1994), improved water relations (Davies et al., 1993), increased growth and yield (Mosse, 1973), and increased nutrient uptake (George et al., 1995). The extraradical fungal mycelium acts as an extension of the root system, enabling more thorough exploration of the soil for nutrients such as phosphorus, zinc, and copper; the nutrients are then transported to intraradical hyphae and transferred to the host. Inoculation with AM fungi is useful in horticultural applications such as replacing indigenous fungi when soils are fumigated to control pathogens (Timmer and Leyden, 1978). There is reason to believe that inoculation of agricultural fields may also be useful in some contexts, such as in the conversion from high- to low-input agriculture (Galvez et al., 1995, and refs. therein). However, inoculation on such a large scale is currently impractical because of the failure of attempts to culture AM fungi axenically. This failure and the desire to understand and manage the cost of the symbiosis to the host plant have inspired interest in the carbon nutrition of AM fungi (for review, see Smith and Reed, 1997).

In the symbiotic state, significant carbon flow takes place from the plant to the fungus via the internal fungal structures (Shachar-Hill et al., 1995; Pfeffer et al., 1996; Smith and Reed, 1997). Although the nutrient transfer from fungus to host has been extensively studied, transfer of carbon from host to fungus and its subsequent metabolism and transport are poorly understood.

Previous studies have shown that fungal spores formed outside of mycorrhizal roots store carbon predominantly as lipids, with relatively small amounts of carbohydrate (Amijee and Stribley, 1987; Bécard et al., 1991; Schubert et al., 1992; Bonfante et al., 1994). Recent work has indicated that hexoses such as Glc and Fru are taken up by fungal structures within the root (Shachar-Hill et al., 1995; Pfeffer and Shachar-Hill, 1996; Saito, 1997; Solaiman, 1997). These sugars were directly incorporated into trehalose and glycogen in short-term experiments (Shachar-Hill et al., 1995; Pfeffer et al., 1996), suggesting that carbon taken up in the root is exported to the extraradical mycelium and that carbohydrates are converted to lipids. However, although the extent to which external fungal structures might also take up carbon and the location and pathways by which carbohydrates are converted into storage lipids have been studied previously (Cox et al., 1975; Cooper and Losel, 1978; Gaspar et al., 1997), they have not yet been established. Other recent findings on the transfer of nitrogen and carbon between plants linked by mycorrhizal fungi (Hamel et al., 1992; Simard et al., 1997; Nasholm et al., 1998) provoke questions as to what form(s) of carbon might be transferred from fungus to host (Hamel and Smith, 1992;

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Abbreviations: AM, arbuscular mycorrhizal; OPPP, oxidative pentose phosphate pathway.

Johansen and Jensen, 1996). We have sought to address these questions using stable isotope labeling and NMR spectroscopy, which can yield detailed information on metabolism and transport in plant-microbe symbioses (for review, see Pfeffer and Shachar-Hill, 1996; Pfeffer and Shachar-Hill, 1996).

To follow the carbon fluxes between the symbionts, it was necessary to use an intact mycorrhiza that allowed substrates to be added to either the roots or the extraradical mycelium. The longer labeling periods that were required in this study also necessitated a system that was free of other microorganisms. We therefore used the in vitro dual-mycorrhizal culture system in divided Petri plates, as developed by St-Arnaud et al. (1996). Although this is an artificial system for studying AM physiology (especially the role of the host plant), it offers unique advantages for investigating fungal transport and metabolism. Specifically, it permits the selective addition of labeled substrates to either the colonized roots or the external fungal mycelium and the straightforward isolation of each of these tissues.

MATERIALS AND METHODS

Growth, Labeling, and Harvest of Cultures

Figure 1 is a schematic diagram of the culture system and our labeling scheme. Ri T-DNA-transformed roots of carrot (*Daucus carota* L.) colonized by the AM fungus *Glomus intraradices* Schenck & Smith (generously supplied by J.A. Fortin, University of Montreal) were grown at 24°C in defined simple "M" medium (Bécard and Fortin, 1988; Bécard and Piché, 1992) solidified with 0.4% (w/v) gellan gum (Phytagel, Sigma)[2]. Plugs of vesicular-AM-colonized growing roots with some external hyphae and spores were transferred to the "root compartment" of divided Petri plates, 9 cm in diameter, that contained about 1 cm of medium so that there was no direct contact between the media in each half of the plate. These cultures were inverted and incubated at 24°C for 6 to 9 weeks until hyphae of *G. intraradices* grew over to the "fungal compartment." Any roots that began to grow over the divider during this or later stages of the experiments were excised and removed. Once in the fungal compartment, hyphae proliferated and then sporulated profusely over a period of 2 to 4 months, as noted by St-Arnaud et al. (1996).

Labeling experiments were initiated by making a well (9 mm in diameter and 1 cm deep) in either the root or fungal compartment of each plate with a 0.9-cm-diameter flame-sterilized cork borer. Labeled substrates (0.5–0.7 mL of filter-sterilized aqueous solutions) were placed in these wells 1 or 2 weeks after the fungus crossed the barrier (4 weeks for ^{2}H experiments). The solutions were absorbed into the gellan within 1 d and the plates were then incubated upside down for 4 to 8 weeks before harvest (12 or 14 d for most ^{2}H experiments).

[2] Reference of brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Hexose and mannitol uptake and labeling experiments were conducted with the addition of 0.14 g of substrate in solution to the well, yielding approximately 25 mM, which was close to the original 29 mM Suc. Succinate and acetate were added, yielding 2 and 4 mM, respectively, with potassium as a counterion. For ^{2}H labeling, 2% to 4% ^2H_2O was added to the well.

Tissues were harvested by solubilizing the gel in 10 mM sodium-citrate buffer, pH 6.0, at 4°C (Doner and Bécard, 1991) in a blender at high speed for 5 s, followed by intermittent blending at low speed for 5 min. Tissue was then collected on a 38-mesh sieve, rinsed with deionized water, and either transferred to an NMR tube for in vivo analysis or frozen on dry ice for extraction. Extraradical mycelium from three replicate plates was pooled for each sample. Tissues from the root compartments of one to three plates were used for each root sample. We obtained 0.80 ± 1.0 g fresh weight of root tissue and 0.038 ± 0.003 g fresh weight of fungal tissue (hyphae and spores) from the respective compartments of each plate. Estimates made through the microscope of fungal material in the fungal compartment were at least 20 times those of the fungal material outside the roots in the root compartment. Samples of colonized roots were cleared and stained as described by Phillips and Hayman (1970); estimates made through the microscope of fungal material inside the roots indicated that fungus comprised less than 2% of the mycorrhizal root mass.

^2H_2O Equilibration Measurements

^2H_2O was added to the root compartments of three plates and to the fungal compartments of three other plates. Cores of gellan, each approximately 0.55 g, were removed 3, 6, 9, and 12 d later from the periphery of both sides of each Petri plate. Each core was incubated at 4°C in 3 mL of distilled water in a sealed tube for at least 2 d to allow full equilibration of ^{2}H. The ^{2}H levels in the medium were analyzed by ^{2}H-NMR (see below). Mycorrhizal roots and extraradical mycelia from the same plates were also harvested, extracted, and analyzed. Partitioning of ^{2}H across the divider was quantified by placing 1.2 mL of solution from the labeled side of the plate in the outer compartment of a dual-compartment NMR tube (Pfeffer et al., 1979) with 10 mM $Gd(NO_3)_3$ added as a shifting agent (Shachar-Hill et al., 1997). The same volume of solution from the unlabeled side was added to the inner compartment.

Trapping $^{13}CO_2$

The possibility of complete respiration of hexose was investigated by adding 125 mg of $U_6{}^{13}$C-Glc to the fungal sides of three plates and to the root side of another plate; 125 mg of unlabeled Glc was added to the root side of a control plate. After the Glc-containing solution had been absorbed by the gel, the lid of each plate was removed and the plate was inverted and placed over a Petri plate bottom containing 10 mL of 1 N KOH solution. The plates with their KOH traps were then wrapped with laboratory film and incubated at 25°C for 9 weeks. The KOH solutions

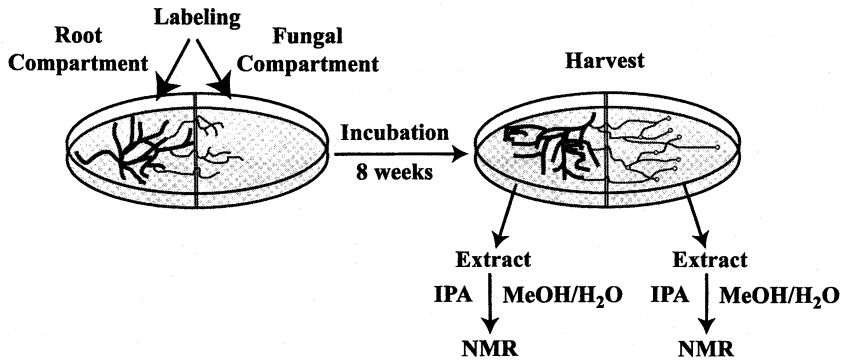

Figure 1. Method of labeling and analysis of metabolism and transport in carrot roots colonized by *G. intraradices*. IPA, Isopropyl alcohol. Root tissue was 20 times the amount of fungal tissue found on the fungal side; the fungal tissue on the root side represents an insignificant amount of fungal hyphae and spores. The hyphae within the root represent only 1% to 2% of the tissue.

were then recovered, evaporated, and examined by ^{13}C-NMR for $^{13}CO_3^{2-}$ content. The mycorrhizal roots and external fungal tissues were harvested and extracted as described above.

Methanol:Water Extraction

Frozen root or mycelium samples were lyophilized and ground at −20°C with 0.30 g of acid-washed sand in a mortar with 3 to 5 mL of methanol:water (70:30, v/v) according to the method reported by Martin et al. (1984). This extraction removes primarily monosaccharides, disaccharides, oligosaccharides, amino acids, small organic acids, and traces of phospholipids. After passing the suspension through filter paper, the solution was evaporated to remove the methanol. The remaining aqueous solution was freeze-dried, and the residue was dissolved in ^2H_2O for NMR analysis. Fine, insoluble particles were removed from NMR samples by centrifugation at 12,000*g* for 10 min or by filtration through a 0.25-μm-pore filter. The solid material remaining on the filter paper was freeze-dried and used for subsequent extraction with isopropyl alcohol.

Extraction, Methylation, and Analysis of Lipids from Residue

The freeze-dried solids were extracted in 30 to 40 mL of boiling isopropyl alcohol for 20 min according to the procedure of Chapman and Moore (1993), except that chloroform was not used in this extraction in order to restrict this extraction to the neutral lipids and fatty acids. The isopropyl alcohol solution was filtered and the filtrate was evaporated under a stream of nitrogen. This sample was dissolved in $CDCl_3$ for NMR analysis; any undissolved materials were removed as above. After NMR analysis the samples were evaporated to dryness and methylated by the procedure of Sasser (1991) for analysis by GC-MS.

NMR Spectroscopy

Most spectra were recorded with a 400-MHz spectrometer (Unity Plus, Varian Instruments, Palo Alto, CA) and a superconducting magnet (9.4T, Oxford Instruments, Oxford, UK). Several ^{1}H-extract spectra were acquired at 600 and 700 MHz on different spectrometers (Bruker Instruments, Billerica, MA). Broad-band probes (5 mm) were used for ^{13}C- and ^{2}H-extract spectra; a 5-mm ^{1}H probe with a Z gradient and broad-band decoupling coils was used for ^{1}H and ^{1}H-^{13}C correlation (heteronuclear, single-quantum coherence) spectra; and a 10-mm broad-band probe was used to obtain ^{2}H equilibration and in vivo ^{13}C spectra.

Spectra for ^{13}C extracts were accumulated with an 80° pulse angle, WALTZ ^{1}H decoupling, and a recycle time of 4.5 s (methanol:water extracts) or 13 s (isopropyl alcohol extracts). Total acquisition times were between 12 and 36 h, depending on the concentration of each sample. In vivo ^{13}C spectra were obtained as described by Bécard et al. (1991) for extraradical mycelium and as described by Shachar-Hill et al. (1995) for leek roots. ^{1}H spectra were obtained using 80° pulses and recycle times of 4.5 s or, when necessary, 12 s to prevent distortion of the relative intensities of the ^{1}H-^{12}C and ^{1}H-^{13}C signals (London, 1988). The susceptibility-shifted ^{2}H spectra of aqueous samples were obtained in an unlocked mode with a pulse angle of 90° and a recycle time of 7 s.

The identification of peaks in ^{13}C and ^{1}H spectra was made from a combination of previously reported values for lipids (Gunstone et al., 1977) and for other metabolites (Fan, 1996), by matching with spectra of authentic samples, by spiking extracts with authentic compounds, and/or by analysis of heteronuclear single quantum coherence spectra. Because the shift of the signal from the double-bond carbons (C11, 12) of the ω-5 C16 has not, to our knowledge, been reported previously, we compared it with the known C13,14 shifts of the ω-5 C18 analog (Gunstone et al., 1977). We found that they were the same; the additional two methylene carbons had no effect on the position of the double-bond carbon resonance of these molecules. The assignment of these signals is consistent with the presence of this compound as the major fatty acid in this fungal species (Graham et al., 1995; and our GC-MS data, below). Chemical shifts were referenced to the Suc C5′ resonance (82.19 ppm) (plant methanol:water extracts), the trehalose C1 resonance at 94.1 ppm (fungal and mycorrhizal methanol: water extracts), or the fatty acid methyl peak at 14.1 ppm (isopropyl alcohol extracts), and are expressed (in parts per million) downfield from tetramethylsilane.

HPLC Analysis of Lipid Extracts

HPLC analysis was performed (model 2350 pump, model 2360 gradient programmer, and model V4 UV-

visible detector, ISCO Separations, Lincoln, NE), and an evaporative light-scattering detector (Mark III, Alltech Associates, Deerfield, IL) was operated at 40°C with 1.6 L/min of nitrogen as the nebulizing gas. The lipids were analyzed using a method similar to that reported by Moreau et al. (1998). Separation of the lipid classes was made on a column (100 × 3 mm i.d., LiChrosorb 5Si60, Chrompack, Raritan, NJ) operated at a flow rate of 0.5 mL min^{-1}. The solvents were hexane (solvent A), isopropanol (solvent B), and 0.04% triethylamine in water (solvent C). The linear gradient of A:B:C was 0 min at 100:0:0 (v/v), 5 min at 19:1:0, 10 min at 85:15:0, 15 min at 8:12:0, 53 min at 40:51:9, 68 min at 40:51:9, 73 min at 8:12:0, 78 min at 100:0:0, and 100 min at 100:0:0.

MS Analysis of Fatty Acid Methyl Esters

MS analyses of the fatty acid methyl esters were performed with each sample on a gas chromatograph (series 5890, Hewlett-Packard) equipped with a splitless injector and a capillary column (25 m × 0.22 mm, cross-linked 5% phenylmethylsilicone, Ultra 2, Hewlett-Packard) with helium as the carrier gas interfaced to a mass-selective detector (model 5972, Hewlett Packard), as described by Graham et al. (1995). Identity of each fatty acid methyl ester was confirmed by comparison with authentic standards and peak matching with the MS library database.

Quantifying Isotope Levels

The ^{13}C isotopic abundance (atom % ^{13}C) at the labeled positions of various compounds was calculated by comparison with natural-abundance ^{13}C isotopic abundance (1.1%) signals at unlabeled positions of the same compounds. Whenever possible, the ^{13}C-^{1}H satellites of ^{1}H signals in proton spectra were used to obtain the ^{13}C content at one or more carbon positions of each compound (C1 of sugars, methyls of fatty acids, and glyceryl C1,3). The ^{13}C content at other positions was then determined by comparison of the intensities of the resonances in the ^{13}C spectra. Spectroscopic conditions were chosen to avoid distortion caused by different relaxation rates or by nuclear Overhouser-effect enhancements in the different signals being compared.

Signals from double-labeled molecules give split (doublet) peaks in ^{13}C-NMR spectra if the labels are at adjacent carbon positions within the molecule because of J-coupling (approximately 40 Hz) between ^{13}C nuclei. Single-labeled molecules give single peaks whose positions in the spectrum are in the middle of the doublet peaks from double-labeled molecules of the same compound. Using a double-labeled substrate (e.g. $^{13}C1,2$-Glc) can give rise to both single- and double-labeled products, resulting in three peaks (doublet plus singlet; see Fig. 7). This allows quantitation of the proportions of single- and double-labeled molecules. The proportion of single-labeled products is taken as a measure of the level of OPPP activity. To calculate the proportion of hexose that goes through the OPPP before being converted into products, it is first necessary to account for any contribution to the peaks caused by unlabeled molecules that have background natural-abundance levels of ^{13}C. Possible errors in the estimate of this latter quantity can cause miscalculations of OPPP activity; we therefore interpret these numbers only as indicators of relative fluxes in different pools.

For ^{2}H-labeling determinations, the amount of ^{2}H in each lipid sample was quantified by comparing its signal with the natural-abundance ^{2}H signal of the solvent ($CHCl_3$). The solvent was evaporated, the sample redissolved in 99% $CDCl_3$, evaporated down twice, and finally dissolved in 99% $CDCl_3$ for ^{1}H NMR analysis. The ^{1}H content was then determined by integrating the lipid signals in the ^{1}H spectrum relative to the residual ^{1}H resonance of the deuterated solvent. The ratio of the ^{1}H and ^{2}H values for each sample yielded a relative fractional ^{2}H enrichment value and thereby allowed the direct comparison of labeling in samples that contained different total amounts of lipid.

Equilibration of ^{2}H between compartments was quantified from ^{2}H spectra containing susceptibility-separated peaks (Shachar-Hill et al., 1997) of ^{2}H in water from each fungal and root compartment. The ratio of the ^{2}H resonances (reflecting the content of ^{2}H in each compartment) was then adjusted for any differences in weight among the samples.

RESULTS AND DISCUSSION

Short-Term Hexose Uptake by *G. intraradices*

In preliminary experiments, we monitored in vivo the carbon metabolism in mycorrhizal leek roots colonized by *G. intraradices* as was previously studied with leek colonized with *Glomus etunicatum* (Shachar-Hill et al., 1995). We observed that exposure of the roots colonized by *G. intraradices* to 0.5 mM $^{13}C1$-Glc resulted in labeling of trehalose and glycogen within 24 h (data not shown) in a way very similar to that seen with roots colonized by *G. etunicatum* (Shachar-Hill et al., 1995). We also observed the labeling of trehalose and glycogen in in vivo spectra of transformed carrot roots colonized with *G. intraradices*. These observations suggest that *G. intraradices* and *G. etunicatum* have similar short-term carbon metabolic processes, and that *G. intraradices* has carbohydrate stores in the transformed carrot root mycorrhiza similar to those in the roots of leek plants.

Addition of $^{13}C1$-Glc to the Root Compartment

Glc labeled with ^{13}C in the C1 position ($^{13}C1$-Glc) was supplied to the colonized carrot roots in vitro (see "Materials and Methods" and Fig. 1). In vivo ^{13}C-NMR spectra of labeled and unlabeled mycelium from the fungal compartments are shown in Figure 2, A and B, respectively. These spectra show only the resonances associated with the abundant fungal storage lipids. Polymers such as cell walls and DNA do not contribute to in vivo NMR spectra. Mobile carbohydrates contain only a small proportion of the ^{13}C in the mycelium, and peaks from trehalose can be seen only when these spectra are amplified. This is consistent with previous observations of spores of *G. intraradices* (Bécard et al., 1991). Resonances in the region from 14 to 38 ppm

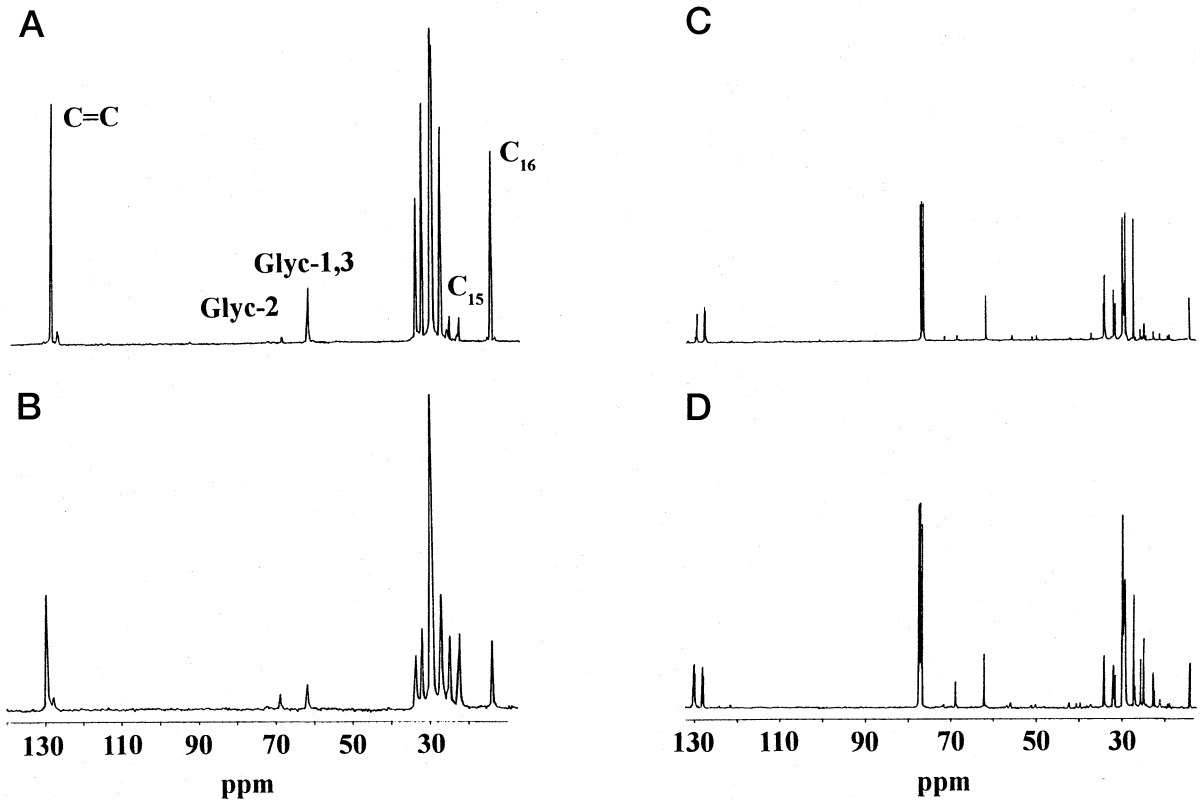

Figure 2. A, In vivo ^{13}C-NMR spectrum of extraradical mycelium of *G. intraradices* prelabeled with ^{13}C1-Glc from the root compartment. B, Unlabeled fungal tissue of the same age as that shown in A. C, ^{13}C-NMR spectrum of isopropyl alcohol extract of *G. intraradices*-colonized root tissues after labeling with ^{13}C1-Glc in the root compartment. D, ^{13}C-NMR spectrum of isopropyl alcohol extract of unlabeled *G. intraradices*-colonized root tissues.

represent saturated carbons of the fatty acid chain of 11,12-hexadecenoate triglyceride, which was the predominant mobile lipid that we observed (consistent with previous observations by Graham et al. [1995]; Fig. 2, A and B). The unsaturated carbons (C11 and C12) gave signals close to 130 ppm. C1 and C3, the glyceryl carbons, are equivalent and gave signals at the same position (62.1 ppm). The C2 carbon peak was at 68.9 ppm.

A comparison of A and B in Figure 2 shows an approximately 10-fold ^{13}C enrichment of the glyceryl C1,3 carbons and of the even-numbered fatty acid carbon positions. This can be seen most easily from the ratios of intensities from C1,3:C2 and C16:C15, assuming that no labeling had taken place in C2 or C15. However, proton-satellite spectra of the corresponding extracts indicate a minor amount of labeling (0.5%–0.7%) in the C2 and C15 positions, presumably caused by dark fixation of $^{13}CO_2$ (see below). The predominant labeling in the C1,3 and C16 positions suggests that Glc taken up by the fungus in the root compartment was metabolized via glycolysis to C-3-labeled trioses which then labeled C1,3 glycerol via dihydroxyacetone phosphate. Under this scheme, fatty acids were labeled in alternating positions from C2-labeled acetyl-CoA derived from C3-labeled pyruvate.

In Figure 2, C and D, are ^{13}C spectra of isopropyl alcohol extracts of labeled and unlabeled mycorrhizal roots, respectively. HPLC analysis of the isopropyl alcohol extracts confirmed that this material was essentially all nonpolar storage lipid (triglycerides and fatty acids with retention times of 10–24 min) and contained only 1% to 2% phospholipids (with retention times of 45–65 min; data not shown). In contrast, HPLC profiles of chloroform:methanol extracts of the same tissue showed more than 10% phospholipid. GC-MS analysis of the isopropyl alcohol extracts showed that the mixture was dominated by the 18:2 fatty acid, with 2% to 3% of 16:1 and 18:0 fatty acids and less than 1% of other unidentified polyenoic acids (data not shown). The ^{13}C spectra represent both host and fungal nonpolar lipids extracted from the mycorrhizal roots that were exposed to ^{13}C1-Glc in the root compartment. Figure 2C shows a labeling pattern similar to that observed in Figure 2A, which is consistent with the pathways for lipid synthesis described above.

Addition of ^{13}C1-Glc to the Fungal Compartment

The glyceryl portion of the ^{13}C spectrum of isopropyl alcohol extracts of mycorrhizal root and fungal tissues from plates labeled with ^{13}C1-Glc from the root or fungal side are shown in Figure 3. The C1,3 signal has twice the intensity of the C2 signal in the unlabeled control samples. When ^{13}C1-Glc was added to the root compartment, lipids from both the root and fungal compartments were enriched at the C1,3 positions relative to the C2 position. Trehalose

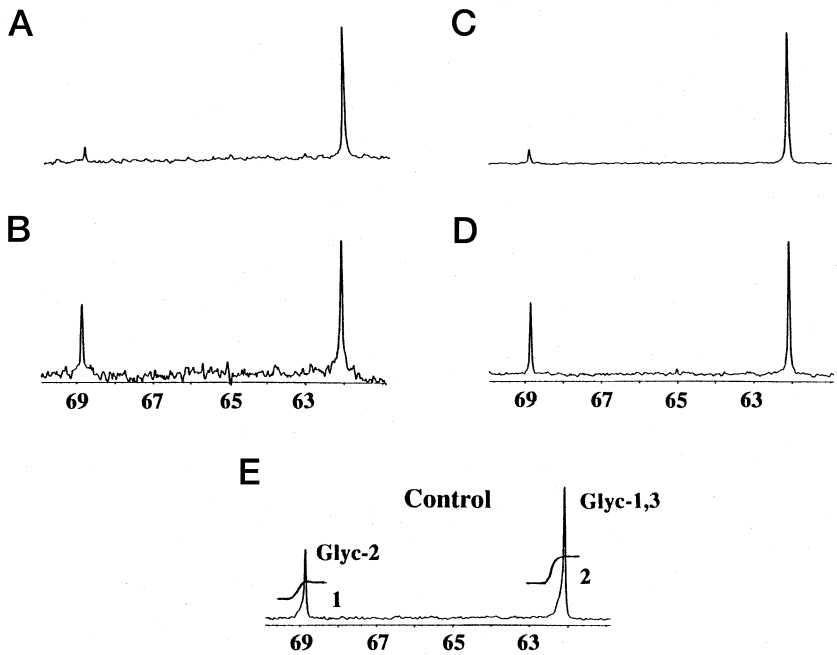

Figure 3. Glyceryl region of the ^{13}C-NMR spectra of isopropyl alcohol extracts of external mycelium after ^{13}C1-Glc labeling from the root side of the Petri plate (A), of external mycelium after ^{13}C1-Glc labeling from the fungal compartment (B), of mycorrhizal root tissue after ^{13}C1-Glc labeling from the root side (C), of mycorrhizal roots after ^{13}C1-Glc labeling from the fungal compartment (D), and of unlabeled lipids from the fungal compartment of unlabeled plates showing integrated areas (controls) (E).

and Suc were also labeled (not shown). However, when ^{13}C1-Glc was added to the fungal compartment, the spectra show that neither fungal (Fig. 3B) nor root (Fig. 3D) compartment contained labeled lipids, nor were compounds in methanol:water extracts labeled (data not shown). The GC-MS profile of fatty acid methyl esters of the isopropyl alcohol extracts showed, as previously reported (Graham et al., 1995), a predominance (80%) of the 16:1 fatty acid (ω-5 octadecenoic acid), with a small amount of a mixture of saturated C16 and polyenoic acids (data not shown).

It might be argued that ^{13}C1-Glc taken up on the fungal side did not label lipids or carbohydrates because of active respiration and/or OPPP activity, which leads to the release of labeled $^{13}CO_2$. To test this possibility, we used Glc labeled uniformly with ^{13}C (U-^{13}C-6-Glc) in CO_2-trapping experiments. No release of $^{13}CO_2$ above natural-abundance levels was detected when U-$^{13}C_6$-Glc was added to the fungal side. Natural-abundance levels of $^{13}CO_2$ were also detected from unlabeled plates, showing the sensitivity of the trapping and NMR detection. When the substrate was added to the root compartment, 18 times more $^{13}CO_2$ was detected than from both the control (natural-abundance) and the labeled U-$^{13}C_6$-Glc from the fungal compartment. There was no labeling of host or fungal isopropyl alcohol or methanol:water extracts when U-$^{13}C_6$-Glc was also supplied to the fungal compartment. These results suggest that exogenous Glc is not significantly metabolized by the external mycelium.

The analysis of spectra (together with ^{1}H spectra of the same samples) showed that when ^{13}C1-Glc was supplied to the root compartment, fungal lipids in the extraradical mycelium were labeled to 8.3% $\pm$ 1.3% in C1,3, to close to 0.7% in C2 of the glyceryl moieties, to 8.9% $\pm$ 1.1% in the C16 fatty acid carbons, and to close to 0.5% in the penultimate fatty acid carbons. The combined host and fungal lipids from the Glc-labeled root compartment showed 16.9% $\pm$ 2.1% labeling in C1,3, 2.1% in C2 glyceryl carbons, 18.9% $\pm$ 2.7% in the C16 fatty acid carbons, and 2.8% in the penultimate fatty acid carbons (n = 4). Labeling in the C2-glyceryl carbons would not be expected from the metabolism of ^{13}C1-Glc through glycolysis. The low level of enrichment observed in the odd-numbered carbons of the fatty acids and the C2 glyceryl carbon could be a consequence of dark re-fixation of $^{13}CO_2$ that was released from respiration or from OPPP.

Use of Other Substrates

Experiments in which ^{13}C1-Fru was added to root compartments gave levels of ^{13}C in host and fungal glycerol and fatty acids in that compartment that were 30% lower than the levels seen when plates were labeled with ^{13}C1-Glc. Trehalose and Suc were also labeled in these experiments (data not shown). No labeling was detected when Fru was supplied to the fungal compartment. Thus, like Glc, Fru can be taken up by the host and fungus in the root compartment, but not by the extraradical mycelium in the fungal compartment.

No labeling was detected in lipid extracts when either ^{13}C1,6-mannitol or ^{13}C2,3-succinate was added to the root compartment. These substrates resulted in no detectable labeling (detection limit, approximately 0.5% ^{13}C enrichment) in Suc, trehalose, Gln, GABA, triglycerides, or fatty acids when each was added to the fungal compartment. Thus, neither mannitol nor succinate was taken up by the fungus.

^{13}C-NMR Double-Bond Profiles for Determining the Relative Labeling of Host and Fungal Lipids

Both host and fungal lipids could be extracted from tissue in the root compartment using isopropyl alcohol; thus labeling calculated from glyceryl signals of these ex-

tracts represents labeling in both symbionts. To assess the incorporation of ^{13}C into the host and fungal lipids in such mixtures, we examined the double-bond region of the colonized roots and of the external fungal mycelium in both labeled and unlabeled states. In Figure 4, A, B, and C, are the double-bond regions of ^{13}C spectra of lipid extracts derived from nonmycorrhizal roots, mycorrhizal roots, and external fungal mycelium from unlabeled plates, respectively. In Figure 4, D, E, and F, are from the equivalent tissues from plates that had been labeled with $^{13}C1$-Glc on the root side. Figure 4A is from a spectrum of a nonmycorrhizal root showing the four double-bond carbon resonances of *cis*-9,10,12,13 octadecadienoate triglyceride (Gunstone et al., 1977), the predominant lipid extracted from carrot root tissue.

An additional natural-abundance peak at approximately 129.9 ppm was present in mycorrhizal root tissue (Fig. 4B). This resonance corresponds to the two double-bonded carbons of the *cis*-11,12 hexadecenoate triglyceride associated with *G. intraradices*. Figure 4C shows the natural-abundance signal from these carbons in an unlabeled fungal extract. The spectra of unlabeled mycorrhizal extracts allowed an estimate of the amount of fungal lipid within the root without the need for separation techniques. Unfortunately, it was not possible to obtain absolute ^{13}C content values for these carbons from the proton satellite spectra because of poor resolution of the double-bond proton resonance.

Labeling of mycorrhizal carrot root tissue with $^{13}C1$-Glc showed not only an increase in the root lipid C10 and C12 resonance intensities, but also a substantial increase (200%) in the C12 fungal lipid resonance seen at 129.9 ppm (Fig. 4E). The final spectrum (Fig. 4F) shows the increase in the C11,12 peak (with the C12 carbon having most of the labeling) of the fungal triglyceride from the fungal compartment after the introduction of $^{13}C1$-Glc to the root compartment. The labeling patterns in host and fungal fatty acids are consistent with the labeling in alternating carbon positions of fatty acid chains made from C2-labeled acetyl-CoA and demonstrate that both host and fungal lipids are labeled in the mycorrhizal root.

Location of Lipid Synthesis

The results above support the idea that fungal lipids present in the external mycelium derive from carbon taken up as hexose by the fungal tissues within the host root. However, the conversion of carbohydrates into lipids by the fungus could take place either within the root or in the extraradical mycelium after export. We used acetate (4 mM) labeled in C1 or C2 as a substrate to ascertain where fungal lipids are made. When supplied to the root compartment, we observed low levels (<5% in all cases) of labeling in the fatty acids (but none in the glycerol moiety) extracted from the root compartment (data not shown). The double-bond regions of the ^{13}C spectra of these extracts indicate that both host and fungal lipids were labeled (data not shown). Methanol:water extracts of mycorrhizal roots labeled in the root compartment showed no evidence of labeling in either Suc or trehalose. Resonances at 70.9 and 42.7 ppm in these spectra indicated labeling in the C2 and C3, respectively, of malate; these were confirmed by two-dimensional ^{1}H-^{13}C spectra (not shown).

Significant increases in signal were observed in the carbonyl region of the ^{13}C spectrum, suggesting that carboxylic acid groups were being labeled in the water-soluble metabolites. These findings would be expected if label supplied as acetate to the roots were absorbed and incorporated into host and fungal acetyl-CoA, and suggest that fungal fatty acids extracted from the root compartment are synthesized there. Fungal lipids extracted from the fungal compartment of plates labeled on the root side showed very little or no labeling of fatty acids and no labeling of glycerol (not shown). No labeling was observed in fungal or host lipids in either compartment when acetate was added to the fungal compartment. Labeling of trehalose

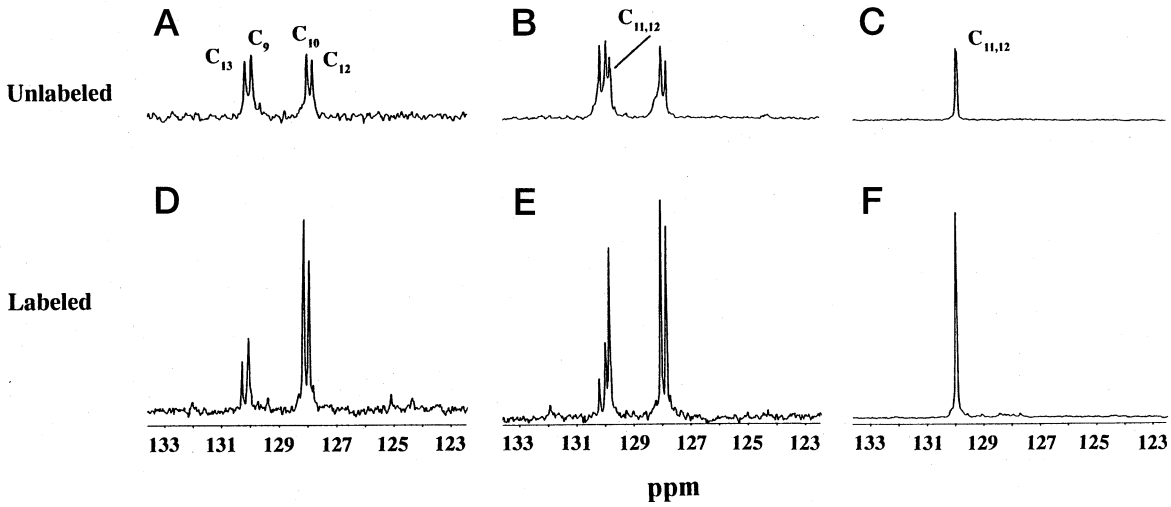

Figure 4. Double-bond regions of ^{13}C-NMR spectra of isopropyl alcohol extracts of unlabeled nonmycorrhizal (NM) root tissue (A); unlabeled mycorrhizal (M) root tissue (B); unlabeled extraradical mycelium (C); ^{13}C-labeled nonmycorrhizal root tissue (D); ^{13}C-labeled mycorrhizal root tissue (E); and ^{13}C-labeled extraradical mycelia (F).

from the fungal compartment when ^{13}C2-acetate was added to that compartment (not shown) suggests that acetate does enter the extraradical mycelium and that this tissue has gluconeogenic capacity.

The low levels of labeling in fungal lipids in the fungal compartment, irrespective of which compartment received the labeled acetate, did not allow us to determine where most of these lipids are synthesized. We therefore sought a precursor to lipid synthesis that would be more permeable than acetate in all tissues.

The enzymatic production of lipid from hexose entails the incorporation of nonexchangable hydrogen atoms, both during glycolysis and during the reduction of the growing fatty acid chains (Lehninger, 1984). Therefore, labeled water supplied to one compartment as ^2H_2O should label the fatty acids synthesized in that compartment. We supplied low levels of ^2H_2O to either root or fungal compartments for periods of 12 to 16 d, and then analyzed ^{2}H levels in the lipids in each compartment. The resulting labeling in lipids from the fungal compartment are shown in Figure 5. The large peak seen in each spectrum at 7.24 ppm is a reference signal from the low natural-abundance levels of ^{2}H in the chloroform solvent. The signals from labeling in fungal fatty acids (0.8–2.4 ppm) are indicated in Figure 5. A spectrum of the same quantity of fungal lipids from an unlabeled sample showed no ^{2}H signal, because the natural-abundance levels (0.0157%) of ^{2}H from lipids were too weak to be detected (data not shown).

From these spectra it is obvious that there was much more labeling in the lipids extracted from the fungal compartment when the label was supplied to the root compartment (calculations of ^{2}H incorporation were based on the quantities of lipid in each sample, which was determined from the respective proton spectra). This suggests that most of the fatty acid was synthesized in the root compartment and then transported in that form to the external mycelium, rather than being transported as carbohydrates and converted to lipids in the external mycelium. The relative level of labeling in fungal lipids when ^2H_2O was supplied for 2 weeks to the root compartment instead of the fungal compartment suggested that about 90% (88% and 93%; $n = 2$) of lipid synthesis occurs on the root side.

^{2}H labeling in the terminal methyl positions of fatty acid chains is expected to occur during glycolysis, whereas labeling in other positions is expected to occur during reduction of the growing fatty acid chain. Because ^{2}H signals were much greater in both cases when ^2H_2O had been supplied to the root compartment, we concluded that the fatty acids were completely synthesized from hexose in the root compartment before being exported to the extraradical mycelium.

We observed more uniform labeling in experiments that used longer incubations because ^{2}H levels equalized in the media across the divider (presumably by evaporation and condensation). This could mean that all of the fungal lipids are synthesized on the root side, and that the diffusion of labeled water across the divider caused the low levels of labeling in the fatty acids on the fungal side when labeled water was supplied to the fungal compartment. To confirm this, the diffusion of ^2H_2O from one compartment to the

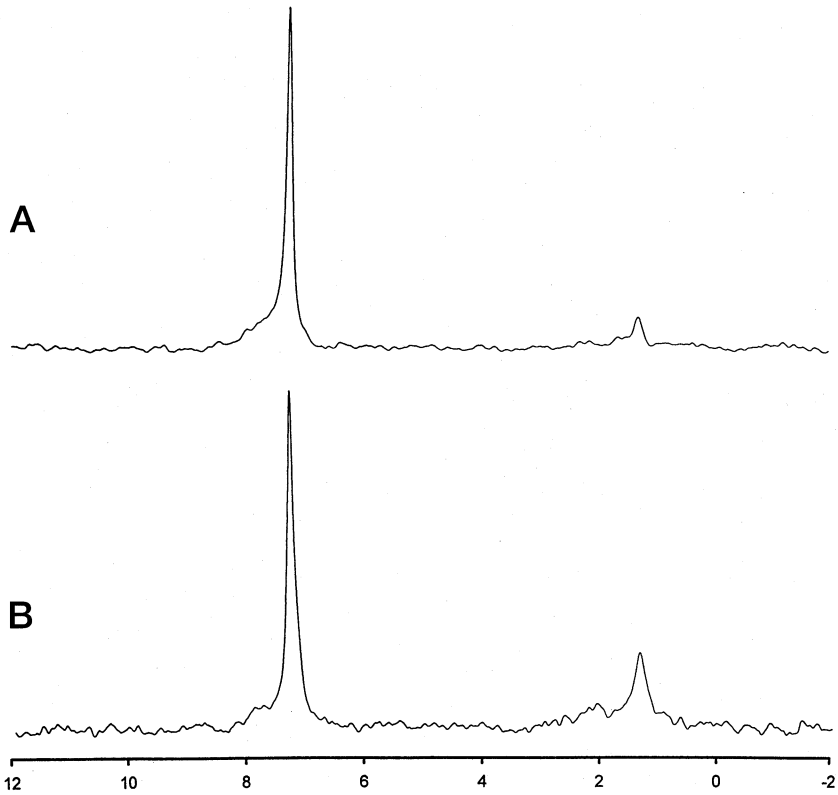

Figure 5. ^{2}H-NMR spectra of isopropyl alcohol extracted lipids from fungal tissue 2 weeks after the introduction of ^2H_2O into the fungal (A) or the root (B) compartment. Resonance at 7.24 ppm is the internal reference of natural-abundance ^{2}H in the solvent ($CHCl_3$). The ^{2}H lipid resonances were observed at 0.8 to 2.4 ppm.

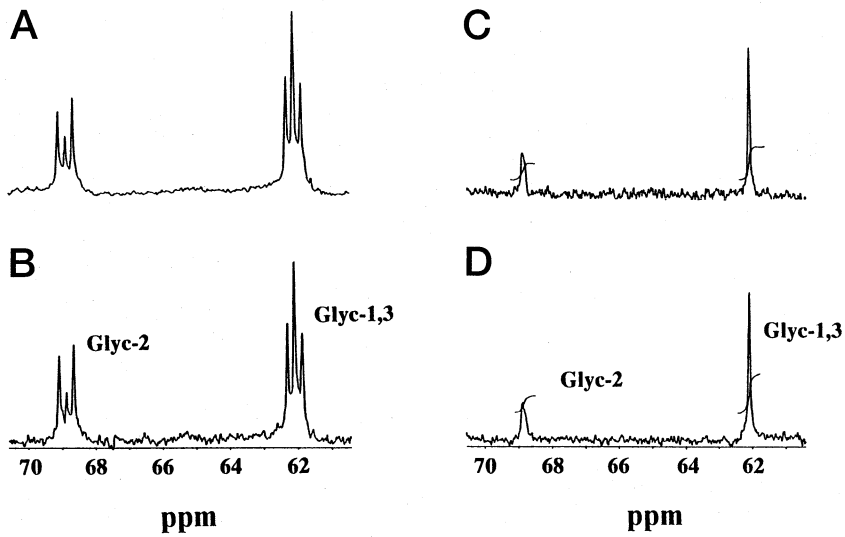

Figure 6. Glyceryl portion of the ^{13}C-NMR spectra of isopropyl alcohol extracts of extraradical mycelia labeled on the root side of the plate with ^{13}C1,2-Glc (A); mycorrhizal roots labeled on the root side of the plate with ^{13}C1,2-Glc (B); mycorrhizal roots labeled on the fungal side of the plate with ^{13}C1,2-Glc (C); and extraradical mycelia labeled on the fungal side of the plate with ^{13}C1,2-Glc (D).

other was measured by periodic sampling of the medium in the unlabeled compartment. Diffusion from root to fungal side and vice versa fit well to a simple model of exchange with a half-time for equilibration of approximately 5 d. Thus, substantial equilibration of ^2H_2O did take place during the 2-week incubation period, suggesting that all of the lipid extracted from the fungal compartment was indeed synthesized in the root compartment, and that the low labeling seen when ^2H_2O was added to the fungal side was caused by ^2H_2O that had diffused to the root compartment.

If all of the fungal lipids extracted from the fungal compartment are synthesized in the root, the level of labeling in the fungal lipids should closely reflect the level of ^2H_2O in the root compartment during the time that the fungal fatty acids were synthesized. In fact, the labeling in fungal lipids when labeled water was added to the fungal compartment was substantially lower than might be expected from the relatively rapid equilibration of labeled water between compartments. During the 2-week incubation period, the mean level of ^{2}H in the medium in the compartment that had the added ^2H_2O was only about 1.5 times higher than the level in the other compartment. This was approximately 6 times less than the observed ratio of labeling in the fungal lipids shown in Figure 5 and in other similar pairs of samples.

The relatively low level of labeling in fungal lipids when ^2H_2O was added to the fungal compartment is suggestive of a significant time lag between the synthesis of fungal lipids in the root and the export of these lipids to the external mycelium. According to this scheme, the lipids extracted from the fungal compartment after 2 weeks of incubation do not include the lipids synthesized most recently. In plates labeled on the fungal side, the lipids that did reach the fungal compartment during the incubation time were those made on the root side in the earlier part of the incubation period, when little ^2H_2O had diffused over to the root compartment. Therefore, they were less labeled than would be expected from the average ^2H_2O levels during the whole incubation time. More detailed time-course experiments using radiotracers for greater sensitivity will be needed to verify and measure this postulated lag.

Labeling with ^{13}C1,2-Glc

Label in the C1 position of hexose can be lost through decarboxylation of 6-phosphogluconate during the OPPP. Accordingly, the labeling of products when C1-labeled Glc was supplied was often less than when ^{13}C2-Glc was used. This difference was used by others to quantify the activity of the pentose phosphate pathway (Dieuaide-Noubhani et al., 1995). In the present study we used ^{13}C-1,2 double-labeled Glc as a substrate to analyze OPPP activity, taking advantage of the ability of NMR to distinguish single- and double-labeled molecules. Because hexose that was subject to OPPP would yield single-labeled products, whereas hexose converted directly to products would produce double-labeled compounds, we considered the proportion of single-labeled products as the proportion of hexose that had passed through the OPPP. Although single-labeled products could be generated by other pathways (in particular, the TCA cycle or the glyoxylate cycle in conjunction with gluconeogenesis), these pathways would result in scrambling to the C6 and other positions of hexose. Because this was not observed, we attribute all of the single labeling to the OPPP.

Single- and double-labeled molecules were distinguishable in the NMR spectrum, as described in "Materials and Methods." Double-labeled molecules gave split (doublet) signals and single-labeled molecules gave single peaks. Mixtures of single- and double-labeled molecules of the same compound gave triple peaks; the proportion of the signal in the central, single peak represents the OPPP activity. When ^{13}C1,2-Glc was supplied to the roots, both host and fungal lipids were labeled (Fig. 6, A and B). Addition of ^{13}C1,2-Glc to the fungal compartment did not cause labeling in host or fungal lipids (Fig. 6, C and D) or in other metabolites (data not shown). This is consistent

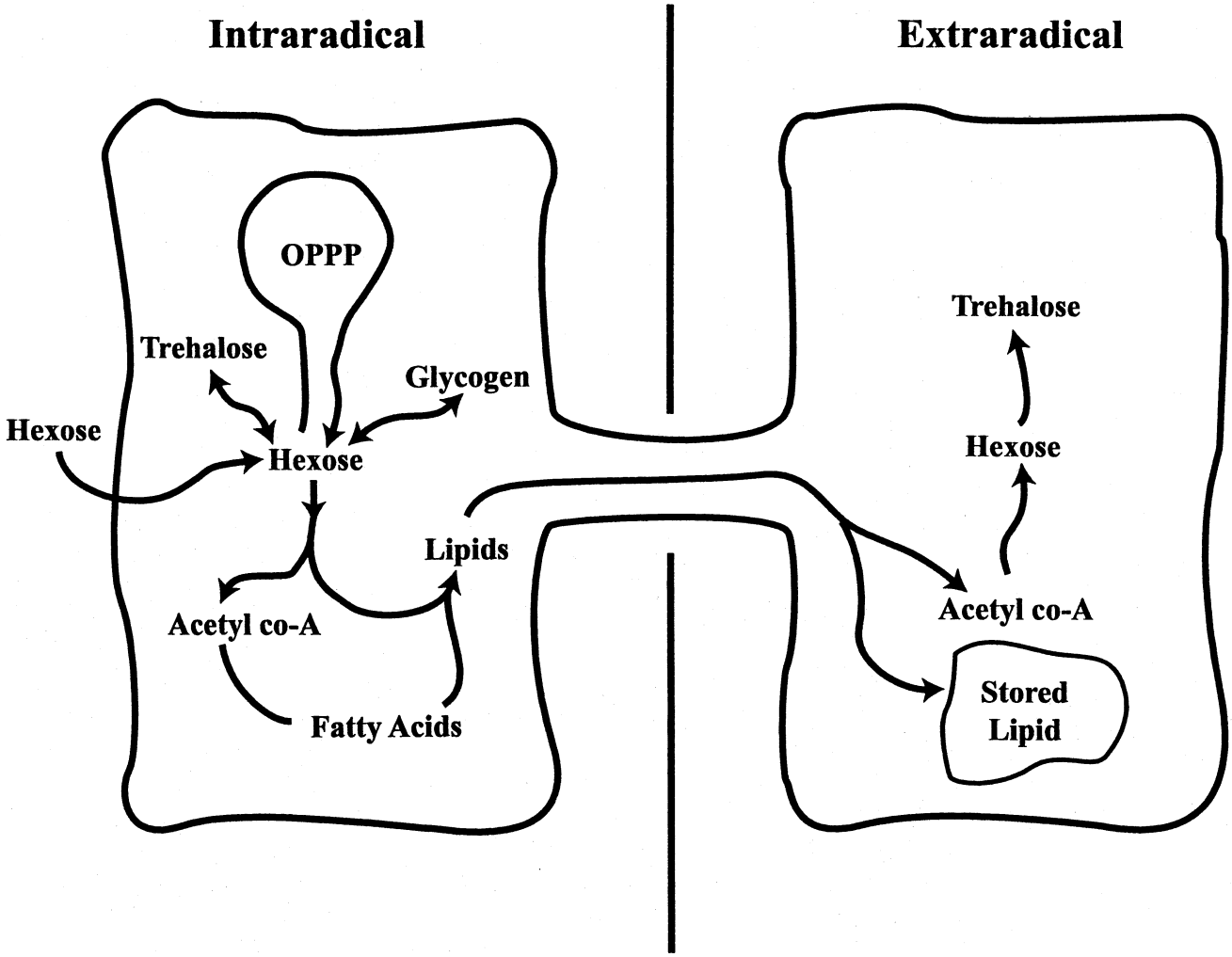

Figure 7. Proposed model for major fluxes of carbon in the fungus in the symbiotic state of AM mycorrhizae.

with our results (above) from C1 and uniformly labeled Glc experiments.

Analyses of the lipids from extraradical mycelium from plates labeled in the root compartment indicated that 26% (n = 2) of the hexose from which fungal glycerol and fatty acids are made had passed through OPPP. ^{13}C1,2-Glc supplied to the root compartment also resulted in both single- and double-labeled Suc and trehalose in the root methanol: water extracts. These spectra (not shown) indicate that approximately 25% to 30% (n = 2) of the C1 in the fungal hexose from which trehalose in the mycorrhizal root is made passed through the OPPP. Spectra of Suc and Glc extracted from mycorrhizal roots indicate that the proportion of hexose that passed through OPPP is 3 times less in the host than in the fungus (data not shown). These results also suggest that the fungal trehalose extracted from mycorrhizal roots is synthesized from the same hexose pool as the fungal lipid extracted from the external mycelium (25%–30%, compared with 26%, respectively).

These data strongly suggest direct uptake of exogenously added hexose by the fungus within the root without equilibration with host sugar pools. This is consistent with our previous short-term study (Shachar-Hill et al., 1995), in which we argued that labeling levels in intraradical fungal trehalose were too high for significant dilution by host sugars to have occurred.

CONCLUSIONS

From the observations in this study and from those in an earlier study on mycorrhizal leeks (Shachar-Hill et al., 1995), we propose a model for major fluxes of carbon in AM fungi in the mycorrhizal state (Fig. 7). According to this model, hexose is taken up by the intraradical fungal tissue but not by the external mycelium. This hexose is then converted to trehalose and glycogen that can be rapidly synthesized and degraded in the short term. Thus, trehalose and glycogen may serve to buffer the intracellular concentration of Glc (Thevelein, 1984; Hottiger et al., 1987; Jennings and Burke, 1990; Muller et al., 1995). The intraradical fungal hexose pool is subject to significant OPPP activity for the production of pentose for nucleic acid synthesis and/or to produce reducing equivalents for anabolism. Lipids are synthesized by the fungus within the root

and are stored or exported in this form to the extraradical mycelium, where they are stored or metabolized. Gluconeogenesis occurs in the extraradical mycelium by using lipids to produce hexose, the precursor of trehalose. We do not yet know whether some carbon is also exported to the external mycelium in other forms, e.g. glycogen (Bonfante et al., 1994), but we do believe that the main stored form of carbon found in the extraradical mycelium as lipids is made by intraradical hyphae.

This model is subject to a number of significant limitations and has major omissions that we hope to address in forthcoming work: (a) it is based on experiments using particular symbiotic stages of the fungal life cycle and says nothing about, e.g. asymbiotic, germinating spores; and (b) it does not take into account the potentially large fluxes of carbon to respiration and to biosynthesis of polymers (e.g. cell wall polysaccharides, nucleic acids, and proteins).

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