

Partitioning of Intermediary Carbon Metabolism in Vesicular-Arbuscular Mycorrhizal Leek

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Vesicular-arbuscular mycorrhizal fungi are symbionts for a large variety of crop plants; however, the form in which they take up carbon from the host is not established. To trace the course of carbon metabolism, we have used nuclear magnetic resonance spectroscopy with [¹³C]glucose labeling in vivo and in extracts to examine leek (*Allium porrum*) roots colonized by *Glomus etunicatum* (and uncolonized controls) as well as germinating spores. These studies implicate glucose as a likely substrate for vesicular-arbuscular mycorrhizal fungi in the symbiotic state. Root feeding of 0.6 mM 1-[¹³C]glucose labeled only the fungal metabolites trehalose and glycogen. The time course of this labeling was dependent on the status of the host. Incubation with 50 mM 1-[¹³C]glucose caused labeling of sucrose (in addition to fungal metabolites) with twice as much labeling in uncolonized plants. There was no detectable scrambling of the label from C1 glucose to the C6 position of glucose moieties in trehalose or glycogen. Labeling of mannitol C1,6 in the colonized root tissue was much less than in axenically germinating spores. Thus, carbohydrate metabolism of host and fungus are significantly altered in the symbiotic state.

VAM fungi form symbiotic associations with higher plants in which the fungi receive fixed carbon and in turn transport phosphate to the host (Cooper and Tinker, 1978). Numerous studies have demonstrated effects of VAM colonization on the growth, phosphate levels (Koide, 1991), carbon partitioning, and carbohydrate levels in a wide range of host species (reviewed by Cooper, 1984; Harris and Paul, 1987). Much of our current understanding of host/fungi interactions has been based on correlations among these "macroscopic" parameters and colonization levels. Yet much remains to be elucidated concerning the reciprocal effects of the symbionts on intermediary metabolism. The VAM fungi occupy small volume fractions in mycorrhizal root tissues (Harley and Smith, 1983) and neither dissection nor axenic culturing has yielded sufficient quantities of fungal material for detailed metabolic study (Bécard and Fortin, 1988). These difficulties have meant

that little is known in detail of the metabolic routes by which carbon fixed by the host is utilized by the fungus and still less of the effects (if any) of the host on fungal metabolism. Studies by Losel and Cooper (1978) and Bevege et al. (1975) using ¹⁴C radiolabeling give information on the classes of compounds labeled in VAM-colonized tissue by photosynthate and by various root-fed substrates, but do not elucidate the labeled structures or the possible pathways that may produce them.

Much more detailed metabolic information is available from studies of axenically cultured ectomycorrhizal fungi (Martin et al., 1984, 1985a, 1985b, 1988; Ramstedt et al., 1987). In these and in some nonmycorrhizal fungi (Dutsch and Rast, 1972; Jennings and Burke, 1990) mannitol becomes highly labeled when labeled Glc is supplied and plays a probably important if still disputed role(s) (Martin et al., 1988; Jennings and Burke, 1990). In these studies substantial fractions of label supplied as Glc were observed to scramble among different positions of hexose and hexitol molecules, reflecting metabolite cycling. The major carbohydrate store in VAM fungal spores has been shown to be trehalose (Bécard et al., 1991; Schubert et al., 1992), and low levels of trehalose have also been reported in extraradical mycelium (Amijee and Stribley, 1987) and in mycorrhizal roots (Schubert et al., 1992). However, the extent to which the carbohydrate metabolism of VAM fungi is otherwise similar to that of ectomycorrhizal species is not known.

In the present study we have used NMR spectroscopy in vivo and in extracts in conjunction with [¹³C]Glc labeling to study carbohydrate metabolism in leek (*Allium porrum*) roots and in *Glomus etunicatum*, both when growing separately and in mycorrhizal association. The results demonstrate significant effects on host carbohydrate metabolism that do not appear to be mediated by improved phosphate supply. Substantial differences are observed in fungal metabolism between free-living, germinating spores and the fungus in the symbiotic state. Furthermore, fungal utilization of Glc in the symbiotic state is altered when the host is treated in ways designed to reduce the supply of photosynthate.

Abbreviations: NTP, nucleotide triphosphate; VAM, vesicular-arbuscular mycorrhizae.

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MATERIALS AND METHODS

Production of Mycorrhizal Leeks

Allium porrum L. cv Musselburgh seedlings were transplanted into conical plastic pots (165 cm³, Supercell C-10, Stuewe and Sons,² Corvallis, OR) on September 11 through 14, 1992. The potting mix consisted of a 1:1 (v/v) mixture of sand and calcined clay (Turface, Applied Industrial Materials Corp., Deerfield, IL) into which was incorporated a sand:calcined clay pot culture inoculum of *Glomus etunicatum* Becker and Gerdemann (No. 8961 from Native Plant Industries, Salt Lake City, UT) to yield 2000 spores per pot. Plants were fertilized weekly with 20 mL of Hoagland solution with 0.1-strength phosphate (which contains 0.1 mM phosphate) (Hoagland and Arnon, 1938). Supplemental light was supplied to yield 14-h photoperiods during the winter months. Percentage root length colonized by *G. etunicatum* was estimated using the gridline intersect method (Newman, 1966) after clearing and staining (Phillips and Hayman, 1970). All colonized roots were more than 70% colonized by the gridline intersect method.

Extraction Procedure and HPLC Analysis of Extracts

To isolate carbohydrate fractions from colonized and noncolonized leek tissue and spores, samples (100–400 mg) were frozen on dry ice, lyophilized overnight, and then ground in a mortar with cold methanol:water (40 mL, 70:30, v/v). The resulting mixtures were further homogenized using a tissue grinder while the grinding chamber was kept in an ice bath. Extracts were centrifuged at 18,000g for 10 min. The pellets were resuspended in cold methanol:water (40 mL, 70:30, v/v) and centrifuged again. The supernatants were combined and the methanol was removed by rotary evaporation at 30°C. Water was removed from the resulting aqueous solutions by lyophilization.

The fructan polymers were separated from the oligosaccharides by size-exclusion column chromatography using a polyacrylamide gel (P-2 gel, Bio-Gel, Richmond, CA).

Deionized soluble carbohydrates were characterized on a Dionex (Sunnyvale, CA) anion-exchange chromatograph using a CarboPack PAI column (4 × 250 mm) and pulsed amperometric detection. Carbohydrates were eluted from the anion-exchange column in an aqueous eluent containing 150 mM sodium hydroxide throughout with a sodium acetate gradient from 10 to 25 mM for 1.5 min, constant (isocratic) 25 mM acetate for 65 min, and a gradient of 25 to 50 mM acetate for 12 min. Individual carbohydrates were quantified by comparing peak areas with those of known standards.

NMR Spectroscopy

In vivo ³¹P and ¹³C NMR spectra were recorded at 161.7 and 100.4 MHz, respectively, using a JEOL GX-400 spec-

² Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

trometer with an Oxford Instruments (Oxford, UK) 9.4-T superconducting magnet (55-mm bore) and a 10-mm-diameter broad-band probe. Excised root tissue was continuously perfused with oxygenated Hoagland solution (Pfeffer et al., 1986) containing 0.6 mM ¹³C₁-labeled Glc (99 atom %, Cambridge Isotope Labs, Cambridge, MA) unless otherwise specified. ¹H-decoupled in vivo ³¹P NMR spectra were obtained with a 36°C pulse angle, a recycle time of 0.163 s, low-power decoupling, and total acquisition times of 2 to 4 h. All resonances are reported relative to a signal from a capillary containing hexamethylphosphoramide and are quoted relative to the resonance at 0 ppm from 85% H₃PO₄ (Pfeffer et al., 1992).

¹H-decoupled in vivo ¹³C spectra were obtained with an 81°C pulse angle, a recycle time of 1.38 s, bi-level decoupling, and a total acquisition time of 3 h. Chemical shifts are quoted relative to the Suc C5 resonance (82.19 ppm) in an external standard relative to tetramethylsilane (Rolin et al., 1992). ¹³C NMR spectra of root tissue extracts were recorded at 50.2 MHz using a Varian (Sunnyvale, CA) Gemini-200 spectrometer with a 4.7-T superconducting magnet and a 5-mm ¹³C/¹H switchable probe. These spectra were accumulated with a 75°C pulse angle, recycle time of 2.5 s, and total acquisition between 12 and 36 h depending on the concentration of each sample. The identification of each carbohydrate component was made by comparison with authentic samples. Fructan shifts were identified by comparison with their literature values (Liu et al., 1993; Timmermans et al., 1993).

Mass Spectral Analysis of Isotopically Labeled Carbohydrates

For analysis of mono- and disaccharides by HPLC-MS, lyophilized extracts were peracetylated with equal volumes of acetic anhydride and pyridine (100 μL of each for 1–10 mg of sample) at 70°C for 20 to 30 min. Samples were dried under a stream of nitrogen and then dissolved in chloroform. Samples were introduced into the mass spectrometer (H-P 5989A) through a Hewlett-Packard 1050 high-performance liquid chromatograph (no column, methanol mobile phase) interfaced to the mass spectrometer with a Hewlett-Packard 59980B particle beam separator maintained at 70°C.

For analysis of individual monosaccharides by GLC-MS, the aldonitrile derivatives were prepared by first reacting 1 to 10 mg of lyophilized extract with 100 μL of 2.5% hydroxylamine-HCl in pyridine for 10 min at 70°C followed by the addition of acetic anhydride (100 μL) and further reaction at 70°C for 25 min. The aldonitriles were chromatographed on a 15 m SP-2330 (Supelco, Bellefonte, PA) capillary column with the column temperature increasing from 150 to 250°C at 4°C/min.

For both analyses the mass spectrometer source temperature and the GC-MS interface temperature was 250°C. Isotopic enrichment was calculated by subtracting the level of natural isotopic abundance observed for standard peracetylated saccharides. Isotopic ratios were measured at peak concentrations to avoid errors due to interfering background ions.

Preparation and Labeling of Leek Roots

Leek plants (1–1.5 years old) were harvested and the roots were rinsed free of soil and debris. [$^{13}\text{C}_1$]- or [$^{13}\text{C}_6$]Glc labeling of roots of intact plants was carried out with the roots fully submerged in approximately 50 mL of Hoagland solution (pH 7.0) containing 50 mM ^{13}C -labeled Glc (99 atom %), which was bubbled with oxygen during the 20 h of incubation. After this the roots were rinsed thoroughly with water to remove any remaining labeled Glc and kept in ice-cold water during cleaning and excision. Next, brown necrotic roots and any algae on the healthy roots were removed. The midsection of the full-root-length tissue (approximately 55 mm) was excised and either placed in the NMR tube for *in vivo* experiments or frozen on dry ice for extraction. During *in vivo* NMR experiments the tissue was perfused at 45 mL/min with room-temperature, oxygen-saturated Hoagland solution containing 0.6 mM $^{13}\text{C}_1$ -labeled Glc (99 atom % ^{13}C) (Pfeffer et al., 1986). Note that for some NMR experiments there was no preincubation of the intact plant and roots were cleaned and excised immediately after they were rinsed free of soil.

Germination and Labeling of Fungal Spores

Spores of *G. etunicatum* were isolated from pot culture soil by wet sieving (Gerdemann and Nicolson, 1963) and centrifugation (Jenkins, 1964). Purified spores were surface sterilized with chloramine-T, streptomycin sulfate, and gentamicin sulfate according to Bécard and Fortin (1988). After obtaining initial ^{13}C spectra (in chilled Hoagland solution without perfusion) the hydrated spores were transferred axenically into 50 mL of filter-sterilized Hoagland solution containing 50 mM $^{13}\text{C}_1$ -labeled Glc in a Petri plate. The plate was then incubated for approximately 70 h in air supplemented with 2% CO_2 at 34°C. The germinated spores were then rinsed free of incubating solution and returned to the NMR tube to obtain spectra.

RESULTS AND DISCUSSION

In Vivo ^{31}P Spectra of Colonized and Uncolonized Leek Roots

The ^{31}P NMR spectra in Figure 1 show the major, mobile, phosphate-containing metabolites in the roots of colonized and uncolonized *A. porrum* plants. The largest difference observed is the presence in the colonized root spectrum (Fig. 1B) of a substantial peak at -22 ppm representing the central phosphate moieties of polyphosphates (Loughman and Ratcliffe, 1984; Martin et al., 1985a). The fungal cytoplasm occupies at most a few percent of the total volume (as estimated by optical microscopy) and therefore the intensity of the polyphosphate resonance represents the equivalent of at least 10 mM Pi in the fungal tissue. (Estimated by comparison with NTP resonances, assuming that fungal cytoplasm occupies one-tenth as much volume as the host cytoplasm and that the concentration of NTP in the cytoplasm is approximately 1 mM. This may overestimate the polyphosphate content if there is relative saturation of the NTP signals under the fast acquisition conditions used

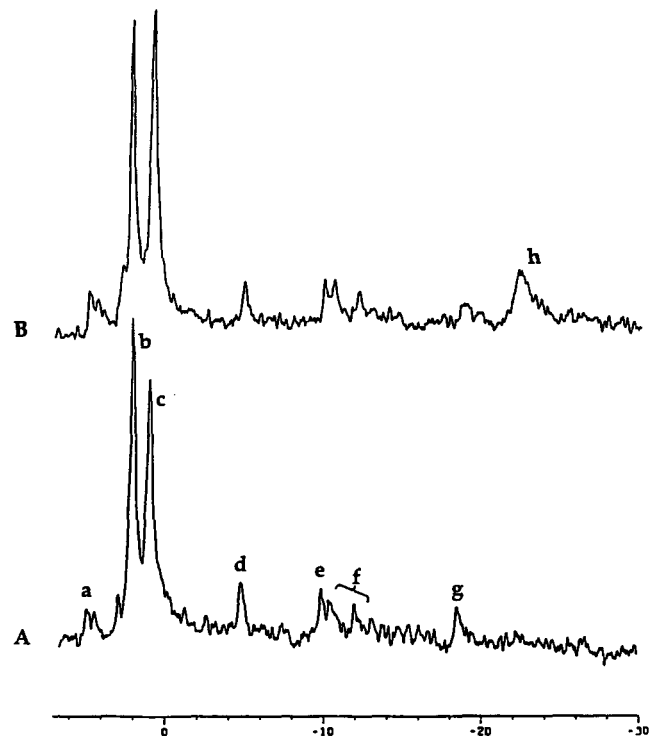


Figure 1. *In vivo* ^{31}P spectrum of excised leek roots (1 year old). A, Uncolonized; and B, colonized with *G. etunicatum*. Shift assignments: a, phosphomonoesters; b, cytoplasmic Pi; c, vacuolar Pi; d, γ -NTP; e, α -NTP; f, uridine diphosphoglucose; g, β -NTP; h, polyphosphate.

here, although we believe any such effect to be minimal (see Martin et al., 1985b). This is in the range reported (Martin et al., 1985b) for axenically grown ectomycorrhizal fungi. Although the levels of vacuolar Pi relative to other phosphate resonances are not the same in the two spectra, no consistent differences were observed between spectra of replicate plants with colonized and uncolonized roots in this resonance or those of other mobile, phosphate-containing metabolites.

Although normally one could assess the degree of VAM fungal colonization of roots with light microscopy (Newman, 1966; Phillips and Hayman, 1970), the direct detection of their "chemical presence" has not previously been demonstrated *in vivo*. Spectroscopic evidence for the presence of ectomycorrhizae was first shown by Loughman and Ratcliffe (1984) in ^{31}P NMR spectra of colonized beech roots in which the polyphosphate associated with the fungus was evident. More recent ^{31}P spectra of red pine roots colonized by *Hebeloma arenosa* (MacFall et al., 1992) and of mycorrhizal beech and pine (Gerlitz and Werk, 1994) have also shown substantial polyphosphate resonances.

^{13}C Metabolism in Colonized and Uncolonized Leek Roots

The *in vivo* ^{13}C spectra shown in Figure 2 illustrate the effects of colonization (here, as elsewhere, colonization levels were high, with over 70% colonization by the grid-line intercept method in all mycorrhizal plants assayed) on

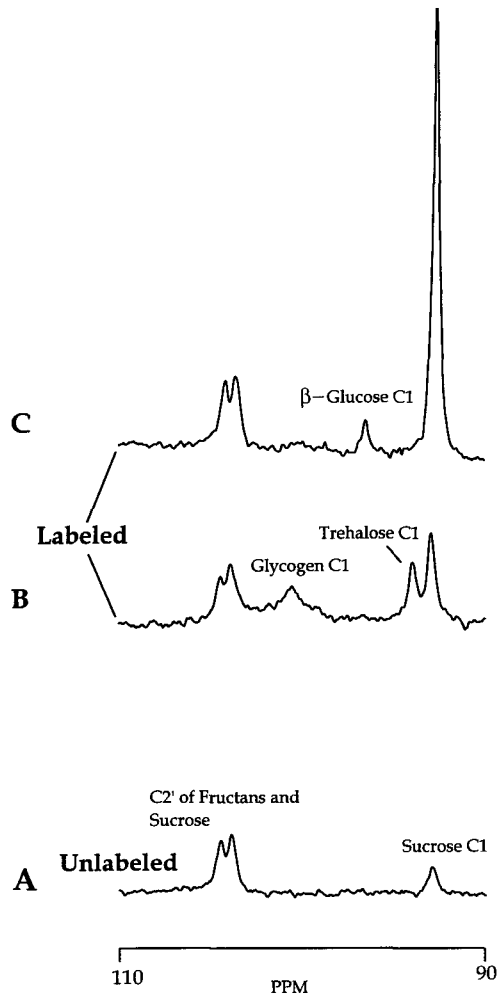


Figure 2. In vivo ^{13}C spectrum of excised leek roots colonized with *G. etunicatum* with no labeling (A); colonized with *G. etunicatum* and labeled for 20 h with 50 mM $^{13}\text{C}_1$ Glc (B); and uncolonized and labeled for 20 h with 50 mM $^{13}\text{C}_1$ Glc (C).

Glc metabolism in leek roots. Figure 2A is part of the spectrum of roots taken from a colonized plant that were perfused with oxygenated Hoagland solution during acquisition of the spectrum. The spectrum shows resonances from Suc and fructans (C2 and C2' centered around 104 ppm and C1 at 93 ppm), which arise from the ^{13}C present at natural abundance (1.1%) in the root metabolites. Spectra of uncolonized roots (not shown) show the same relative intensities of the natural abundance signals. Figure 2C is from uncolonized roots that were excised after the intact plant had been incubated in Hoagland solution containing 50 mM $^{13}\text{C}_1$ Glc for 20 h and were perfused with oxygenated, Glc-free Hoagland solution during acquisition of the spectrum. The Suc C1 resonance is significantly larger in this spectrum, showing that there was incorporation of labeled Glc into Suc during the incubation period. A spectrum of colonized roots that had, like those in Figure 2C, been incubated with labeled Glc, is shown in Figure 2B. This spectrum shows signals from label incorporated into the C1 positions of glycogen and trehalose as well as Suc.

Trehalose has been previously identified in VAM associations of *Glomus mossae* (Schubert et al., 1992) although to our knowledge the presence of glycogen has not been previously demonstrated in VAM roots. Thus, the fungus is able to incorporate exogenously supplied Glc into compounds that are separate from the metabolism of the host.

Other regions of these in vivo spectra do not show significant differences between colonized and uncolonized roots nor between plants that had been incubated with labeled Glc and those that had not. In the ^{31}P spectra, the appearance of substantial signals from fungal polyphosphates despite the low fraction of the sample occupied by fungal tissue indicates high effective concentrations. In the ^{13}C spectra, however, the fractional enrichment of ^{13}C as well as the concentration determines which metabolites are observed. For trehalose and glycogen no natural abundance signals were observed, with C1 signals being observed only upon labeling. Thus, these pools were of lower overall concentration in the tissue than other saccharides observed at natural abundance in extract spectra (see below).

Analysis of ^{13}C Spectra of Colonized and Uncolonized Leek Roots after $^{13}\text{C}_1$ Glc Labeling

Spectra were taken of tissue extracts to identify the compounds represented in in vivo spectra of roots and to allow quantitation of fractional labeling of different compounds. Portions of the ^{13}C spectra of extracts of colonized and uncolonized roots that had been exposed to 50 mM labeled Glc for 20 h are shown in Figure 3, A and B, respectively. Some of the peaks that are unresolved in the in vivo spectra (Figs. 2 and 4) are seen in Figure 3 to be composite (see, for example, the Suc/fructan C2'/C2 peaks at 104 ppm). Such spectra show that the majority of labeling from $^{13}\text{C}_1$ Glc is incorporated into C1 of Suc in the uncolonized roots (with less incorporation into the C1 of the fructosyl moiety of the Suc and little or none into fructans). The C1 of trehalose is the predominantly labeled fungal metabolite in extracts of colonized roots (Fig. 3A), with a much smaller proportion of label being incorporated into mannitol C1,6 (64.08 ppm). A small mannitol resonance was observed in spectra of all colonized plants ($n = 5$). Spectra of uncolonized plants show only a trace of trehalose (Fig. 3B) labeling (probably due to contamination with small amounts of nonmycorrhizal fungal material) and no evidence of mannitol labeling.

^{13}C spectra of leeks (in vivo as well as in extracts) display many broad peaks corresponding to inulin oligosaccharides such as nystose, kestose, and isokestose (Liu et al., 1993; Timmermans et al., 1993) with 3 to 12 hexose subunits. The presence of these was confirmed in extracts of colonized and uncolonized roots by MS and Dionex HPLC (data not shown). To estimate the contribution of fructan resonances in these spectra to the regions of interest, extracts of the root tissue were separated into high and low mol wt fractions by column chromatography (see "Materials and Methods"). The spectrum of the separated fructan fraction shown in Figure 3C displays broad "families" of resonances whose shifts and intensities are consistent with published spectra of isolated fructans (Liu et al., 1993;

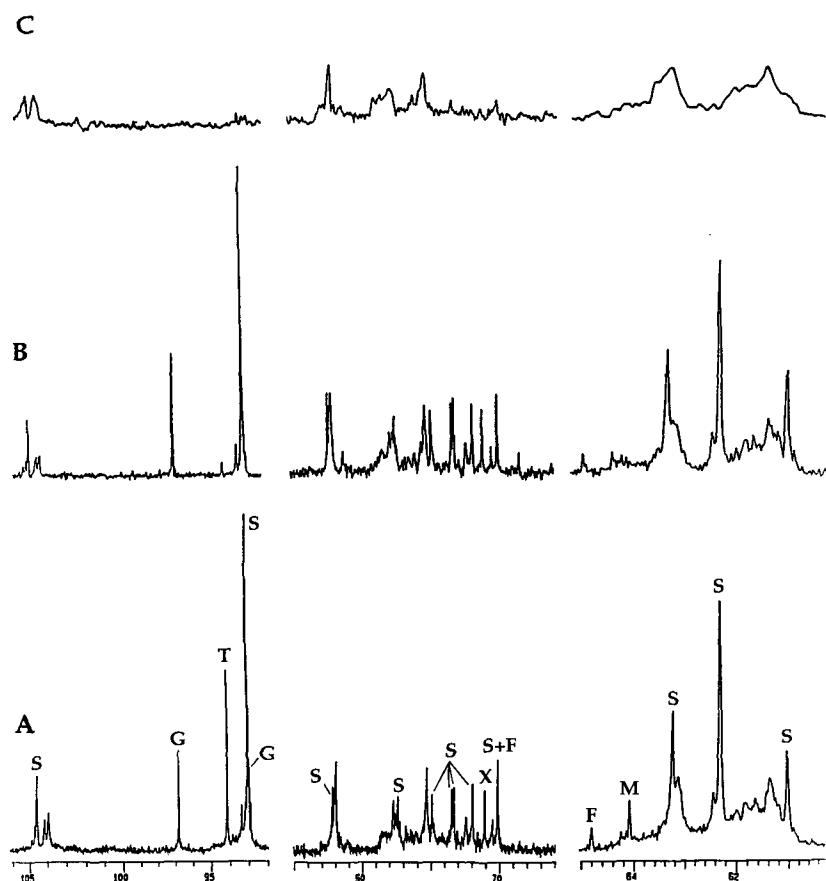


Figure 3. Regions of interest from ^{13}C spectra of extract of *G. etunicatum*-colonized *A. porrum* roots that were incubated (plant intact) with 50 mM $[^{13}\text{C}_1]\text{Glc}$ for 20 h (A); same as A except roots were uncolonized (B); and higher mol wt fructan fraction (degree of polymerization 4–12) separated from roots used for the spectrum in B on a P-2 chromatography column (C). See text for assignment of resonances. For clarity of presentation the three sections of each spectrum are not plotted on the same vertical scale. S, Suc; G, Glc; T, trehalose; M, mannitol; F, Fru; X, unknown compound.

Timmermans et al., 1993). Comparison of extracts of plants given labeled or unlabeled Glc indicated that there was no significant labeling of fructans during the 20-h incubations in 50 mM $[^{13}\text{C}_1]\text{Glc}$. Comparison of whole extract spectra such as those shown in Figure 3, A and B, with spectra of pure compounds and with spectra of separated fructans such as those shown in Figure 3C allows assignment of all the major carbohydrate resonances. The predominant resonances seen in the middle sections of Figure 3, A and B, are due to seven natural-abundance signals from Suc and to the fructan resonances seen in Figure 3C. Also present are contributions from small natural-abundance signals of Glc and Fru. The right-hand portions of Figure 3, A and B, are dominated by three Suc resonances (C6, C1', and C6' at 61.4, 62.3, and 63.2 ppm, respectively) and fructan resonances. It may be seen that the C1' resonance is larger than the adjacent natural-abundance Suc signals showing ^{13}C labeling in this position.

There is no detectable scrambling of label supplied as C1 of Glc into other ring positions of Glc or other saccharides after exposure of either colonized or uncolonized roots to Glc for 20 h. This was the case both for roots of intact plants incubated in 50 mM labeled Glc and for excised roots perfused with 0.6 mM labeled Glc before extraction. This contrasts with observations by others (Ramstedt et al., 1987; Martin et al., 1988) of scrambling into the C6 positions of Glc and trehalose in free-living ectomycorrhizal fungi.

The percentage labeling of trehalose and glycogen can be determined as being at least $1.1 \times N$ where N is the signal-to-noise ratio of the resonance in question. (The least fractional enrichment level consistent with observing only the signal from the enriched position is deduced by assuming that the natural-abundance signals from unenriched positions are just below detectable levels, i.e. have signal-to-noise ratios of 1. Since natural-abundance signals arise from 1.1% of the molecules, the labeled position represents at least N times as much as the postulated undetected signal, i.e. $1.1 \times N$ percent.) The highest signal-to-noise ratio recorded in extract spectra for the trehalose C1 signal was 65 and for glycogen it was 8 (not shown), giving fractional enrichments of over 72% and over 9%, respectively. The level of glycogen observed in extracts was low (or was not observed, as in the spectrum shown in Figure 3A) compared to the levels seen *in vivo* (see Figs. 2 and 4). We postulate that the extraction of glycogen is less effective than that of trehalose, perhaps because of the lower solubility of oligo- and polysaccharides in the 70:30 methanol: water used here and elsewhere for carbohydrate extraction. Martin et al. (1984, 1988), using ^{13}C *in vivo* NMR, have observed glycogen labeling in a free-living ectomycorrhizal species, although not apparently in extracts. MS of the same extract after invertase treatment (to remove Suc) and acetylation confirmed that over 50% of the Glc moieties of trehalose in infected roots were ^{13}C labeled when the roots

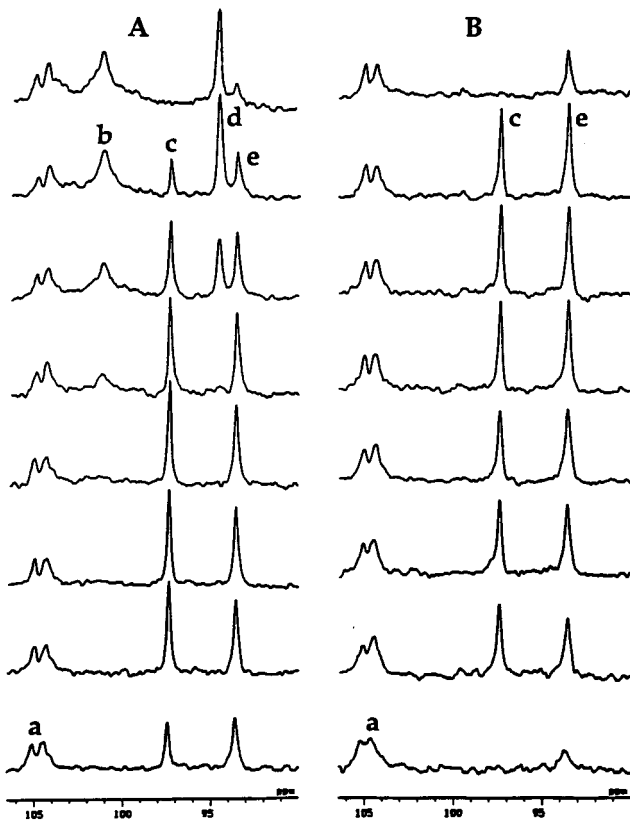


Figure 4. ^{13}C NMR in vivo time-course spectra of colonized (A) and uncolonized (B) leek roots excised and perfused with 0.6 mM [$^{13}\text{C}_1$]Glc in Hoagland solution. Each spectrum represents the accumulation of 8000 transients (3 h). The first spectrum of A (at bottom of figure) was started at 2800 scans before the addition of the [$^{13}\text{C}_1$]Glc to the perfusion medium. a, Suc C2', fructans C2; b, glycogen C1; c, β -Glc C1; d, trehalose C1,1'; e, α -Glc C1, Suc C1. The last spectrum in both A and B was in Glc-free solution after 24 h of exposure to Glc.

of the intact plant had been incubated with labeled Glc for 20 h before extraction. The high fractional labeling of the trehalose indicates that intracellular fungal Glc (its metabolic precursor) is highly labeled when exogenous Glc is supplied. Thus, there is little dilution of the label by host photosynthate under these conditions (and also when a lower level of labeled Glc was supplied to excised perfused roots; see below). This lack of isotope dilution is consistent with Glc as the major form of fixed carbon taken up by the fungus.

It is possible to deduce the percentage labeling of Suc C1 in colonized and uncolonized roots exposed to 50 mM labeled Glc from comparison of natural-abundance Suc resonances to the C1 intensity. Colonized plants showed $3.3 \pm 0.4\%$ ($n = 4$) labeling at C1 of Suc, whereas uncolonized plants showed $12.5 \pm 4.7\%$ ($n = 3$), i.e. some 3.8 times as high as colonized plants. There is also labeling of Suc in the C1' position (the C1 of the fructosyl moiety) in both colonized ($1.3 \pm 0.4\%$, $n = 4$) and uncolonized plants ($4.7 \pm 1.7\%$, $n = 3$). The ratio of labeling in C1 to that in the C1' position was very similar for colonized and uncolonized

plants, 2.6 in each case. Thus, the C1 of the Fru in host cells (the precursor of the C1' of Suc) is labeled less than C1 of Glc in the host. Extract spectra do not show the presence of significant levels of pre-existing Fru; therefore, label entering the Fru pool in host cells from the exogenously supplied Glc is being diluted by another source. We suggest that hydrolysis of fructans may be this source.

The finding that colonization reduces the levels of labeling of Suc by the host root cells even at high Glc levels (50 mM) suggests that regulation of host metabolism rather than competition is at work. One possible mechanism of regulation is via improved phosphate supply to colonized plants (Koide, 1991). Such a mechanism is consistent with the observation (see above) of significant polyphosphate signals in colonized roots. Indeed, colonized plants were generally larger than uncolonized plants of the same age. Levels of phosphate washed out of the growth containers when plants were watered with dilute acid (0.05 M HCl and 0.025 M H_2SO_4) were significantly higher in uncolonized than in colonized plants, suggesting that phosphate uptake was higher in colonized plants. Therefore, additional uncolonized plants were watered with full-strength Hoagland solution (1 mM phosphate rather than 0.1 mM) three times each week for 25 d and then exposed to 50 mM labeled Glc for 20 h. ^{13}C spectra of extracts of the roots showed $8.5 \pm 0.6\%$ ($n = 3$) labeling at the C1 and $4 \pm 0.9\%$ ($n = 3$) at the C1' position of Suc. Thus, there are no significant changes in Suc labeling induced in uncolonized plants by this phosphate supplement.

Time Course of Glc Metabolism in Colonized and Uncolonized Roots

In vivo NMR time-course experiments were conducted to explore further the regulation of Glc metabolism in colonized and uncolonized roots. Excised roots were perfused with low levels (0.6 mM) of exogenously supplied labeled Glc to more closely mimic the levels of extracellular Glc in the apoplast.

Figure 4, A and B, are typical spectroscopic time courses of Glc metabolism by colonized and uncolonized excised roots, respectively. The resonances observed in Figure 4 are as seen in Figure 2, with the addition of signals from C1 of the two anomers of Glc. Although in vivo spectra do not allow separation of signals from C1 of Suc and C1 of α Glc, the intensity of Suc C1 may be calculated by subtracting one-half of the intensity of the C1 resonance of β Glc, assuming that the Glc anomers are in constant proportion to one another. (These signals are mainly from Glc in the perfusing medium, which is in anomeric equilibrium.) This calculation shows that in the colonized roots Suc C1 is not significantly labeled during the exposure to Glc, whereas uncolonized roots show continuous incorporation of label in successive spectra and is significant in the first 3-h spectrum after Glc addition (see increase in peak e of Fig. 4B versus Fig. 4A). Spectra acquired upon perfusion with Glc-free medium at the end of the time course (topmost spectrum in each case) confirm that colonized roots do not show significant labeling of C1 of Suc when exposed to 0.6

mM C1-labeled Glc, whereas the uncolonized roots have over 3% labeling of C1 Suc. Spectra of root extracts made after such *in vivo* time courses (not shown) confirm that there is no labeling in colonized roots at either the C1 or C1' positions of Suc.

Time-course experiments also demonstrate that in colonized roots there is a lag period of 12 to 15 h before significant amounts of label are incorporated into glycogen and trehalose. After the lag both glycogen and trehalose become labeled rapidly—the signals of each increased some 10-fold in the last 9 h of labeling. Since there is appreciable overlap of the fructan resonances in the trehalose/glycogen $^{13}\text{C}_6$ region of the spectrum (around 61.5 ppm), it was not possible to determine unambiguously whether $^{13}\text{C}_1$ -to- $^{13}\text{C}_6$ scrambling occurred during *in vivo* labeling experiments. To ascertain whether this was occurring, $^{13}\text{C}_6$ Glc was used (data not shown). (Neither the trehalose nor the glycogen C1 resonances overlap with other peaks, so any scrambling from C6 to C1 could be detected.) Only labeling of trehalose C6 and glycogen C6 was observed after a 12-h lag, with no evidence of labeling at the C1 of trehalose or glycogen. The spectra of the $^{13}\text{C}_1$ - and $^{13}\text{C}_6$ labeling experiments with colonized roots show

either high or low levels did not induce changes in the time course of utilization of exogenous Glc.

Alternative explanations for the observed lag are: (a) the metabolic status (perhaps carbohydrate levels) of the roots determines Glc utilization or (b) excision has a detrimental effect on the roots, which then require 12 to 15 h before they are able to incorporate significant amounts of the supplied label. To distinguish between these alternatives we conducted *in vivo* time-course experiments on excised, colonized roots from plants that either had all their leaves cut off 30 h before the time course or had been kept in the dark continuously for the previous 7 d. In both these cases (data not shown) labeling of trehalose and glycogen was observed within 6 h of exposure to labeled Glc. Thus, the time course of utilization of exogenously supplied Glc by the fungus appears to be dependent on the carbohydrate status of the roots. It is clear that the carbohydrate metabolism of both host cells and fungus are regulated by the presence of (and, in the case of the fungus, also the metabolic status of) the other symbiont.

In Vivo ^{31}P and ^{13}C NMR Examination of Germinating Spores

fungi (Martin et al., 1985b, 1994; MacFall et al., 1992; Gerlitz and Werk, 1994) contain small or no significant resonances from penultimate or terminal residues. The resonance in Figure 5 representing terminal residues (labeled "b") is larger than that from penultimate residues, which suggests either that there is additional contribution to this resonance either from another compound or from polyphosphates whose penultimate resonances are not visible under high-resolution conditions.

Figure 6 shows the ^{13}C spectrum of an extract of germinating *G. etunicatum* made after 70 h of incubation with 50 mM $^{13}\text{C}_1$ -labeled Glc (see "Materials and Methods"). Incorporation of label into mannitol was considerably greater than incorporation into trehalose, which contrasts with observations made with mycorrhizal roots (see Fig. 3) with shorter labeling times. Colonized roots incubated (plant intact) for 70 h in 50 mM labeled Glc were extracted and showed low levels of mannitol labeling (not shown). Small, natural-abundance signals from C2 to C6 of trehalose and of mannitol C3,4 and C2,5 are also seen in Figure 6. These indicate that the concentration of trehalose is of the same order as the concentration of mannitol, but the percentage labeling of mannitol was higher. This is consistent with the observation (Bécard et al., 1991) that the spores contain high levels of trehalose but not mannitol before germination and that trehalose is utilized during germination. There is no evidence of scrambling of label in the germinating spores, since the intensity of the signal from C6 of trehalose is not significantly different than those from the C2 to C5 ring positions. Cycling between mannitol and Glc can be substantial in the free-living state of ectomycorrhizal and other fungal species (Dutsch and Rast, 1972; Martin et al., 1985a; Ramstedt et al., 1987). It has been suggested (Dutsch and Rast, 1972; Ramstedt et al., 1987; Martin et al., 1988) that this cycling or the net synthesis rate of mannitol regulates growth by affecting the NADP/NADPH redox

couple. Our findings suggest that the role of mannitol in rapidly growing, free-living fungi may be different than that in the symbiotic state.

CONCLUSIONS

G. etunicatum and *A. porrum* roots can utilize exogenously supplied Glc while growing either alone or in association with VAM. ^{13}C NMR spectra of mycorrhizal roots in vivo and of extracts show the incorporation of label from Glc into trehalose, glycogen, and mannitol by the fungus and into Suc by the host. Incorporation of labeled Glc into Suc by the host is markedly reduced in colonized roots, but Glc consumption is markedly increased relative to uncolonized controls. This regulation of host metabolism does not appear to be mediated by improved phosphate supply to the host despite the presence of substantial levels of polyphosphate and evidence of improved uptake of phosphate by colonized roots. Time-course experiments indicate that fungal carbohydrate metabolism is regulated by the metabolic status of the host. Unlike free-living ectomycorrhizal fungi or axenically germinating VAM fungus spores, symbiotically associated *G. etunicatum* incorporates only traces of label into mannitol. High fractional labeling of fungal metabolites from labeled Glc and the observations of mutual regulation of Glc metabolism in host and fungus support a central role for Glc in host-fungus relations. Further studies using other labeled substrates will be aimed at further elucidating carbon metabolism in vesicular-arbuscular mycorrhizal symbiosis.

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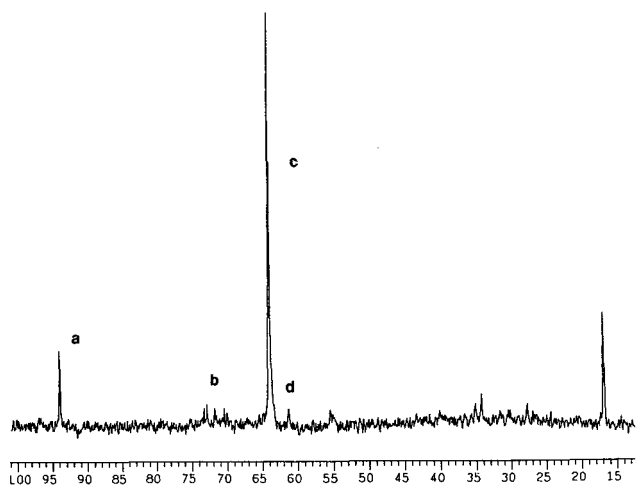


Figure 6. ^{13}C NMR spectrum of the extract of germinated *G. etunicatum* spores after 70 h of labeling with 50 mM $^{13}\text{C}_1$ Glc. Peak assignments: a, trehalose C1; b, natural-abundance resonances of trehalose C2, C3, C4, and C5 and mannitol C2,5 and C3,4; c, mannitol C1,6; d, trehalose C6. The resonances at 17.0 ppm and at approximately 55 and 35 were not identified.

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