

Regulation of Arbuscular Mycorrhization by Carbon. The Symbiotic Interaction Cannot Be Improved by Increased Carbon Availability Accomplished by Root-Specifically Enhanced Invertase Activity^[W]

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The mutualistic interaction in arbuscular mycorrhiza (AM) is characterized by an exchange of mineral nutrients and carbon. The major benefit of AM, which is the supply of phosphate to the plant, and the stimulation of mycorrhization by low phosphate fertilization has been well studied. However, less is known about the regulatory function of carbon availability on AM formation. Here the effect of enhanced levels of hexoses in the root, the main form of carbohydrate used by the fungus, on AM formation was analyzed. Modulation of the root carbohydrate status was performed by expressing genes encoding a yeast (*Saccharomyces cerevisiae*)-derived invertase, which was directed to different subcellular locations. Using tobacco (*Nicotiana tabacum*) *alc::cwlNV* plants, the yeast invertase was induced in the whole root system or in root parts. Despite increased hexose levels in these roots, we did not detect any effect on the colonization with *Glomus intraradices* analyzed by assessment of fungal structures and the level of fungus-specific palmitic acid, indicative for the fungal carbon supply, or the plant phosphate content. Roots of *Medicago truncatula*, transformed to express genes encoding an apoplast-, cytosol-, or vacuolar-located yeast-derived invertase, had increased hexose-to-sucrose ratios compared to β -glucuronidase-transformed roots. However, transformations with the invertase genes did not affect mycorrhization. These data suggest the carbohydrate supply in AM cannot be improved by root-specifically increased hexose levels, implying that under normal conditions sufficient carbon is available in mycorrhizal roots. In contrast, tobacco *rolC::ppa* plants with defective phloem loading and tobacco *pyk10::InvInh* plants with decreased acid invertase activity in roots exhibited a diminished mycorrhization.

Arbuscular mycorrhiza (AM) represents a widespread mutualistic association between soil-born fungi of the phylum Glomeromycota and most land plants. The AM interaction enables the plant to improve its supply of water and mineral nutrients, mainly phosphate. In return, the obligate biotrophic AM fungi are provided with carbon. In the Arum-type interaction, as analyzed here between *Glomus* sp. and tobacco (*Nicotiana tabacum*) or *Medicago truncatula*, the AM fungus colonizes the cortical cells by formation of intra- and intercellular hyphae and very characteristic haustoria-like structures, the highly branched intracellular arbuscules. When the carbon supply is sufficient, lipid-rich vesicles are formed intercellularly within the cortex, as fungal storage organs. Intracellular fungal structures are separated from the plant cytoplasm by an extension of the plasma membrane, forming the periarbuscular

membrane surrounding the arbuscule. The greatly increased surfaces of host and arbuscule plasma membranes offers optimized conditions for effective nutrient exchange via the established symbiotic interface (for review, see Gianinazzi-Pearson et al., 1996; Harrison, 1999).

The exchange of phosphate via the periarbuscular interface and the induction of AM-specific phosphate transporters are already well characterized (Karandashov and Bucher, 2005; Requena, 2005). The same holds true for the regulation of AM by the phosphate availability for the plant. High levels of available phosphate (Mosse, 1973; Jasper et al., 1979) as well as the inhibition of phosphate exchange by the knockout or down-regulation of AM-inducible phosphate transporters (Maeda et al., 2006; Javot et al., 2007) can suppress the mycorrhization. In contrast, there are only limited data about regulation of AM formation by the carbon aspect of this interaction, although previous studies revealed a link between phosphate and carbon availability. Analysis of monoxenic AM cultures of carrot (*Daucus carota*) roots fed with isotopically labeled carbon indicated an increased carbon supply of the fungus in roots that were undersupplied with phosphate (Olsson et al., 2002). Moreover, higher carbon availability was shown to stimulate the phosphate allocation (Bücking and

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Shachar-Hill, 2005). Conversely, limiting light intensity reduced mycorrhization and lead to decreased growth and phosphate uptake (Hayman, 1974; Tester et al., 1985; Son and Smith, 1988).

The fungal carbon uptake and metabolism has intensively been studied using isotopic-labeled substrates (for review, see Bago et al., 2000). Hexoses, in particular Glc, were found to be the major form in which carbon is taken up and metabolized by AM fungi (Solaiman and Saito, 1997; Pfeffer et al., 1999; Douds et al., 2000). Glc can then be directly incorporated into trehalose and glycogen, the first substantial fungal carbon pool (Shachar-Hill et al., 1995). Later on, storage lipids are synthesized. The most abundant form of lipid in AM fungi are triacylglycerols, which also serve as a carbon transport form in the fungus (Bago et al., 2002). In the symbiotic stage, carbohydrates can only be taken up within intraradical structures, no uptake of hexoses could be detected by the extraradical mycelium (Pfeffer et al., 1999; Douds et al., 2000). The high labeling levels in fungal metabolites suggested a direct uptake of isotopic-labeled hexoses from the root apoplast without dilution with the hexoses of the host sugar pool (Shachar-Hill et al., 1995; Douds et al., 2000). The uptake of carbon via the arbuscules is anticipated inter alia by the optimized formation and localization of these highly branched structures for nutrient exchange (for review, see Blee and Anderson, 1998). Moreover, the incorporation of isotopically labeled Glc into trehalose and glycogen was found to depend on the presence of arbuscules (Pfeffer and Shachar-Hill, 1996) and the latter could be correlated with spore formation (Douds, 1994). However, isolated intraradical hyphae showed efficient uptake of Glc, suggesting the putative involvement of hyphae in carbon uptake as well (Solaiman and Saito, 1997).

In addition to labeling experiments, the exchange of carbon in the form of hexoses is further supported by transcript accumulation of a mycorrhiza-induced hexose transporter from *M. truncatula* in colonized root areas (Harrison, 1996). This suggests an active transport of hexoses into and/or out of colonized cells driven by H^+ gradient generated by plasma membrane-located H^+ -ATPases, which were found to be induced upon mycorrhization (Marx et al., 1982; Gianinazzi-Pearson et al., 1991; Ferrol et al., 2002; Krajinski et al., 2002). The active hexose transport would supplement the potential passive efflux of carbohydrates through the plant plasma membrane into the symbiotic interface. The subsequent uptake of carbon by the AM fungus in form of hexoses is supported by the isolation of a fungal monosaccharide transporter from a member of the Glomeromycota that undergoes AM-like endosymbiosis with cyanobacteria (Schüssler et al., 2006). The presence of orthologous proteins from other glomeromycotan fungi within the arbuscular plasma membrane is very likely. Another indication of fungal uptake of hexoses is given by increased transcript and activity levels of plant Suc-cleaving enzymes in mycorrhizal roots (Wright et al., 1998; Blee and Anderson,

2002; Hohnjec et al., 2003; Ravnskov et al., 2003; Schubert et al., 2003; Schaarschmidt et al., 2006).

In plants, Suc is the major transport form of photosynthetically fixed carbon to sink organs. Utilization of Suc requires cleavage that can either be performed by cytosolic Suc synthases, producing UDP-Glc and Fru, or invertases, producing Glc and Fru. Plant invertases can be classified by their subcellular location and their pH optima into three groups: (1) acidic cell wall-bound apoplastic invertases, (2) acidic soluble vacuolar invertases, and (3) alkaline soluble cytosolic invertases (Tymowska-Lalanne and Kreis, 1998; Roitsch and González, 2004). No AM-fungal Suc-cleaving enzymes have been identified so far, suggesting that plant invertases and Suc synthases play an important role in delivering hexoses to the fungal partner. Particularly, extracellular invertases have a key function in supporting increasing sink strength, a feature of mycorrhizal roots, by phloem unloading (Godt and Roitsch, 1997; Tymowska-Lalanne and Kreis, 1998; Roitsch et al., 2003) and they may directly deliver utilizable carbohydrates to the apoplastic fungal structures. Increased transcript and activity levels of apoplastic invertases in mycorrhizal roots, which are particularly detectable at stages with high carbohydrate demand, corroborate this assumption (Wright et al., 1998; Schaarschmidt et al., 2006).

To analyze the regulatory function of the carbon supply during AM, this study focuses on the modulation of the carbohydrate status of the plant by root-specific overexpression of invertases, particularly an apoplast-located invertase. Increased extracellular hexose levels, achieved by an a priori enhanced apoplastic invertase activity in the root, might cause several alterations in the formation of the AM interaction. On the one hand, establishing a higher sink strength in the root before colonization by the AM fungus and the increase of available carbon might stimulate the mycorrhization and enhance the benefit to the plant as described for the phosphate supply (Bücking and Shachar-Hill, 2005). On the other hand, the supply of the AM fungus with hexoses for free could result in an excessive colonization, changing a mutualistic association into a parasitic one. If the fungus is able to take up hexoses via intraradical hyphae, as shown for isolated hyphae (Solaiman and Saito, 1997), the number of arbuscules might decrease, indicating that the fungus does not fulfill its part in the symbiosis any longer. Another possibility might include activation of defense-related mechanisms in the plant root via hexose sensing (Herbers et al., 1996; Rolland et al., 2002; Roitsch et al., 2003), which can interfere with the mutualistic interaction, resulting in diminished mycorrhization.

To study the impact of altered hexose availability on mycorrhization of tobacco or *M. truncatula* two general approaches were followed. To increase root hexose content, yeast (*Saccharomyces cerevisiae*)-derived invertase(s) were expressed either under control of a chemically inducible promoter in tobacco or in hairy roots of *M. truncatula* following *Agrobacterium rhizogenes*

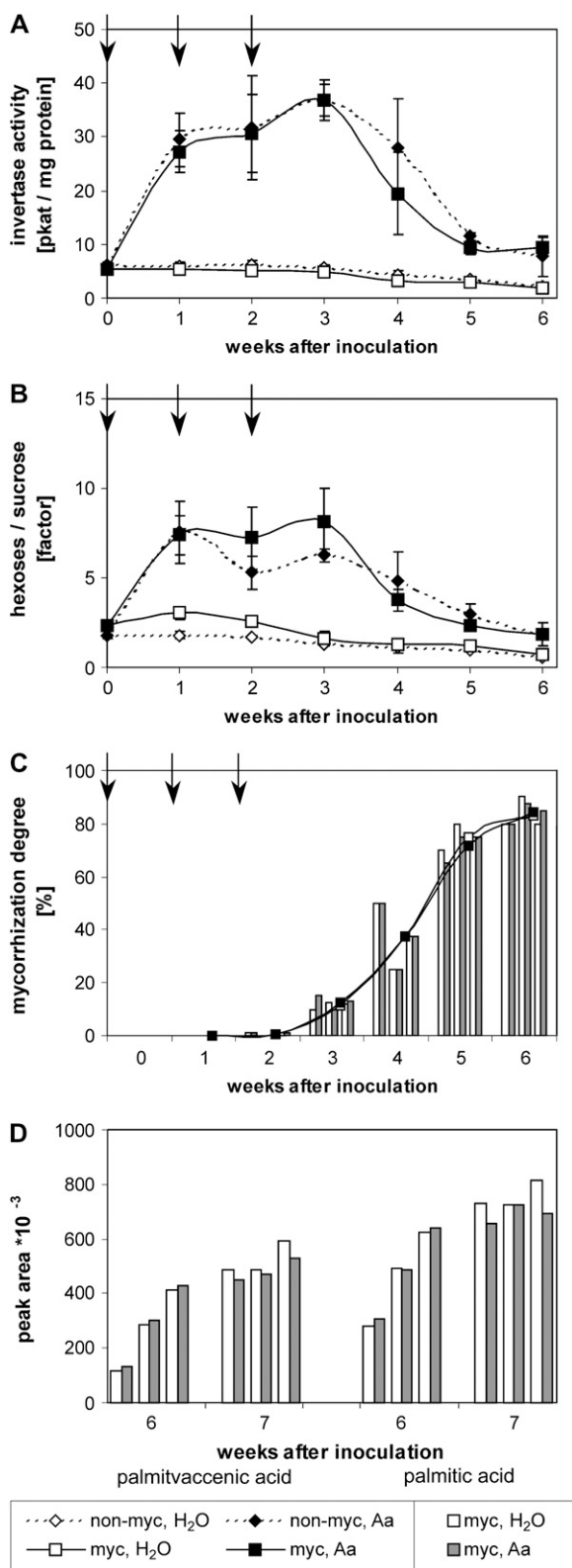


Figure 1. Induction of an apoplast-located invertase in root parts of transgenic tobacco plants and effects on mycorrhization. A to C, NT *alc::cwINV* plants were cultivated using the split-root system where both root parts were either inoculated with *G. intraradices* 6 weeks after sowing (myc) or left as controls without inoculation (non-myc). To

transformation. Decreased root hexose content was achieved by either expression of a phloem-specific *Escherichia coli* inorganic pyrophosphatase or root-specific expression of Arabidopsis (*Arabidopsis thaliana*) invertase inhibitor.

Here we report that an elevated root hexose content does not alter mycorrhization of tobacco or *M. truncatula* roots, indicating sufficient carbon supply in normal growth conditions. Reducing assimilate supply of roots as consequence of reduced phloem loading of photoassimilates, however, strongly decreases AM growth. By specifically inhibiting root invertase activity the requirement of sufficient hexose supply for AM growth could be documented.

RESULTS

Effect of Root-Specific Enhancement of Apoplastic Invertase Activity on AM Formation

To increase the carbon availability in the root and to analyze its role in the formation of AM, we used transgenic tobacco plants expressing a chimeric gene encoding a yeast-derived invertase that is translocated to the apoplast. In these NT (tobacco) *alc::cwINV* plants, the expression is under the control of the alcohol-inducible (*alc*) promoter system derived from *Aspergillus nidulans*. The *alc* promoter can easily be activated in roots and also in root parts by the specific application of low concentrations of acetaldehyde (Schaarschmidt et al., 2004). Drenching with 0.05% (v/v) acetaldehyde at weekly intervals results in long-lasting induction of the invertase and does not apparently affect plant vigor (Schaarschmidt et al., 2004).

To allow a direct comparison between control roots and roots with elevated invertase activity in one mycorrhizal plant, split-root plants were used, diminishing the high biological variance occurring in the analysis of an interaction between two different organisms. Apoplastic invertase was induced by weekly drenching of one part of the split root with 0.05% (v/v) acetaldehyde starting at the time of inoculation (Fig. 1A) and hexose levels increased. Levels of Glc (2-fold) and Fru (2.5-fold) were increased over a period of at least 6 weeks, whereas Suc content decreased (0.6-fold;

induce expression from the chimeric invertase gene, a defined root from the split-root plants was drenched with 100 mL of 0.05% (v/v) aqueous acetaldehyde solution (Aa). The control root was treated with water (H₂O). Soil drenching was performed in weekly intervals 0, 7, and 14 d after inoculation as indicated by arrows. A, Invertase activity of the root parts. B, Ratio of the Glc and Fru contents to the Suc content of the root parts. C, Degree of mycorrhization of the root parts of single plants. D, Content of C16:1 Δ^{11} and C16:0 in single root parts of mycorrhizal plants 6 and 7 weeks after inoculation. Plants harvested 6 weeks after inoculation are described above (A–C), plants harvested at week 7 were cultivated and inoculated in the same way but drenched with acetaldehyde 21, 28, and 35 d after inoculation. Data are expressed in A and B as means \pm SD. ($n = 3$). In C, data of single root parts and the mean values of the three parallel plants are given.

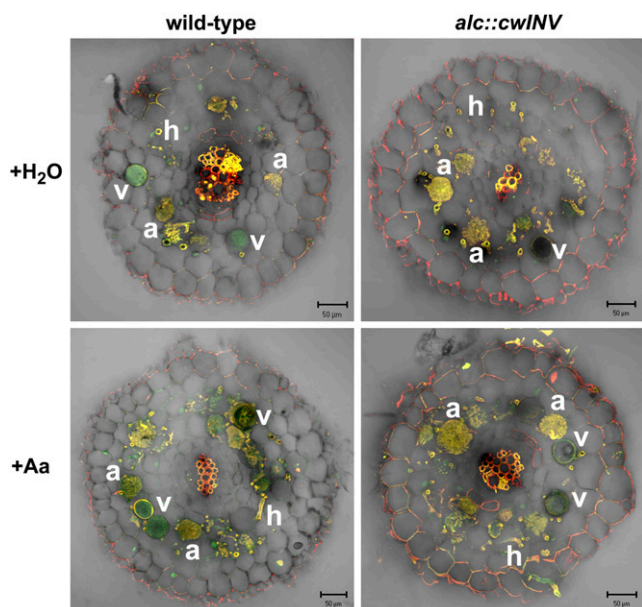


Figure 2. Formation of fungal structures in water- and acetaldehyde-treated wild-type and *alc::cwINV* roots. Cross sections of 140 μm thickness of *G. intraradices*-colonized roots were stained with two fluorescent-WGAs. The fluorescence of WGA-TRITC showing high affinity to arbuscules and hyphae is shown in red, and the fluorescence of WGA-Alexa Fluor 488, which additionally labeled fungal vesicles (v), is in green. In the overlay, structures labeled by both fluorescent WGAs as arbuscules (a) and hyphae (h) appear in yellow. Plants were harvested 6 weeks after inoculation. Drenching of the whole root system with 100 mL 0.05% (v/v) acetaldehyde for root-specific induction of apoplastic invertase was performed five times at weekly intervals starting with the time point of inoculation (+Aa). Control plants were water treated (+H₂O). Bars = 50 μm .

Fig. 1B shows the increased hexose-to-Suc ratio). Sugar levels of water-treated roots or leaves were not affected (data not shown). The acetaldehyde application did not detectably interfere with either the vigor of the plant or of the AM fungus (data not shown).

Surprisingly, comparing colonization of high-hexose and control roots with *Glomus intraradices* revealed no obvious difference (Figs. 1C and 2). The degree of mycorrhization and the formation of fungal structures did not differ from water-treated NT *alc::cwINV* roots or from wild-type plants that were drenched either with acetaldehyde or with water (Figs. 1C and 2). The number of arbuscules and fungal vesicles did not change and the colonization was restricted to the root cortex as usual; no fungal structures were found in the root central core (Fig. 2) or in the root tip (data not shown). Furthermore, no significant changes could be detected in the accumulation of the fungus-specific palmitvaccenic acid (C16:1 Δ^{11}) or of its precursor palmitic acid (C16:0; Fig. 1D), indicating that the fungal supply of carbon was unchanged. Obviously, the variance between different mycorrhizal plants was in most cases greater than between the differently treated root parts of one plant (Fig. 1D, see also Fig. 1C). Similarly, levels of AM-induced plant secondary me-

tabolites, such as cyclohexenone derivatives (Maier et al., 2000) and mycorradicin derivatives (also called yellow pigment; Klingner et al., 1995), which accumulate in mycorrhizal roots and are suggested to play a functional role in AM (Fester et al., 2002; Strack et al., 2003), were not affected by the increased invertase activity (Table I). In nonmycorrhizal plants these metabolites could not be detected (data not shown; see also Fester et al., 2002, 2005; Schliemann et al., 2006). Induction of invertase activity starting at the beginning of fungal colonization (3 weeks after inoculation) also had no effect on the mycorrhization (Supplemental Fig. S1; for metabolite accumulation, see also Fig. 1D and Table I). Even testing different AM fungi (*G. intraradices* versus *Glomus mosseae*) as well as different light conditions (high-light conditions with increased UV-[A + B] intensity or reduced light intensity to limit the carbohydrate supply of mycorrhizal wild-type roots) did not show any effect of increased hexose levels in root parts of NT *alc::cwINV* plants on AM formation (Supplemental Figs. S2 and S3). In addition, the analysis of normally grown (nonsplit root) plants also revealed no influence of increased root-specific apoplastic invertase activity on the mycorrhization (see also Supplemental Fig. S3).

AM not only improves the nutrient availability of the plant, but it can also enhance the plant's tolerance against drought and salt stress (Pfeiffer and Bloss, 1988; Ruiz-Lozano and Azcón, 2000; Cho et al., 2006). Thus, as criteria of the plant's benefit from the symbiotic interaction, the plant phosphate content and the salt stress tolerance of nonsplit root plants in term of

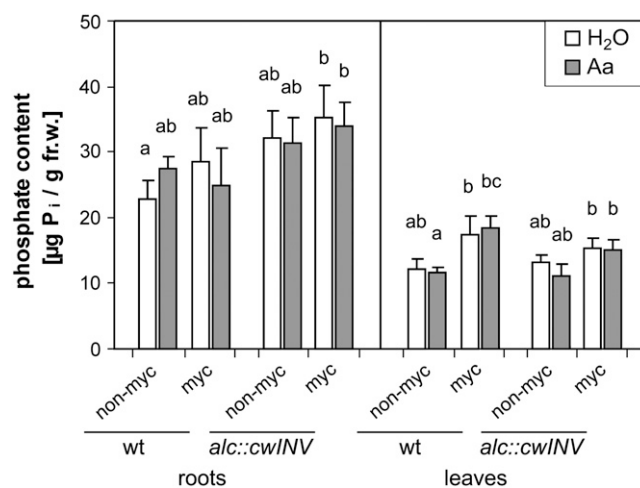


Figure 3. Inorganic phosphate content of roots and leaves of water- and acetaldehyde-treated wild-type and *alc::cwINV* plants. Six-week-old plants were either inoculated with *G. intraradices* (myc) or left without inoculation (non-myc). Invertase induction in the whole root system was performed as described in Figure 1 for root part-specific induction. Data are presented as mean values + SD (nonmycorrhizal plants: $n = 4$; mycorrhizal plants: $n = 6$) and are tested with multiple t tests, including Bonferroni correction. $P < 0.05$. Means sharing the same letters are not significantly different.

Table 1. AM-specific plant metabolites in tobacco *alc::cwINV* roots

Inoculation with *G. intraradices* and induction of invertase in root parts by acetaldehyde was performed as described in Figure 1. Compounds were detected in mycorrhizal roots via HPLC analysis of methanol extracts. Cyclohexenone and mycorradicin derivatives showed typical UV absorption maxima at 245 and 380 nm, respectively. Aa, Acetaldehyde-treated root parts; H₂O, water-treated root parts. Data are presented as mean values \pm SD of root parts of three plants harvested 6 and 7 weeks after inoculation and as mean \pm SD of the ratios of the acetaldehyde-treated root part to the water-treated root part.

Metabolite (Peak Area $\times 10^{-3}$)	6 Weeks			7 Weeks		
	+H ₂ O	+Aa	+Aa/+H ₂ O	+H ₂ O	+Aa	+Aa/+H ₂ O
Cyclohexenone derivatives						
RT 19.5	208.8 \pm 50.0	226.3 \pm 53.4	1.09 \pm 0.10	313.4 \pm 38.7	283.3 \pm 47.6	0.93 \pm 0.22
RT 22.0	73.3 \pm 21.9	68.2 \pm 19.5	0.94 \pm 0.14	93.7 \pm 14.2	79.4 \pm 16.1	0.88 \pm 0.26
RT 27.0	61.9 \pm 17.1	53.7 \pm 14.2	0.87 \pm 0.04	66.9 \pm 2.3	48.1 \pm 17.5	0.89 \pm 0.13
RT 27.7	152.5 \pm 5.4	143.5 \pm 1.3	0.94 \pm 0.03	142.1 \pm 21.4	153.8 \pm 11.4	1.12 \pm 0.27
RT 28.1	67.3 \pm 22.1	63.5 \pm 16.2	0.98 \pm 0.21	92.4 \pm 16.6	79.5 \pm 11.3	0.90 \pm 0.25
RT 30.4	43.9 \pm 14.1	41.3 \pm 16.3	0.94 \pm 0.23	67.4 \pm 11.8	55.8 \pm 13.5	0.86 \pm 0.26
Mycorradicin derivatives						
	372.7 \pm 104.6	359.7 \pm 125.1	0.96 \pm 0.26	540.8 \pm 120.4	455.0 \pm 105.2	0.91 \pm 0.32

transcript accumulation of the salt stress-inducible genes *Osmotin* and *tobacco stress-induced gene 1* (*Tsi1*; Park et al., 2001) were investigated. *G. intraradices*-colonized wild-type plants showed a slightly enhanced inorganic phosphate content in the leaves compared to nonmycorrhizal plants (Fig. 3). Phosphate content of the NT *alc::cwINV* plants was not altered by the expression of the invertase gene. Additionally, the induction of the yeast invertase had no effect on the gene expression of *Osmotin* and *Tsi1* (Supplemental Fig. S4).

To exclude an induction of defense reactions by elevated extracellular invertase activity in the root, transcript levels of defense-related genes, which are induced by constitutive expression of the apoplast-located yeast invertase (Herbers et al., 1996), were analyzed. In contrast to tobacco leaves with strongly increased invertase activity, the induction of the yeast-derived invertase in the root did not lead to enhanced transcript levels of *PAR1*, *PR-Q*, or *PR-1b* in roots or leaves (Supplemental Fig. S5).

Biomass analysis of nonmycorrhizal or mycorrhizal wild-type and *alc::cwINV* plants, treated either with water or acetaldehyde, did not indicate an increased sink function in roots with enhanced apoplastic invertase activity (Fig. 4). The root-to-shoot ratio of the fresh weight and of the dry weight (data not shown) did not change.

Summarizing, these data suggest that the sink function of the root and the supply of the AM fungus with carbon cannot be improved by root-specifically elevated apoplastic invertase activity leading to increased hexose levels.

Overexpression of Invertases Directed to Three Different Compartments in Root Cells of *M. truncatula*

Because increased apoplastic invertase activity of the root had no effect on AM formation, the influence of cytosol- and vacuole-located invertases was tested. In tobacco plants that expressed the yeast gene coding for cytosolic invertase under control of the *alc* pro-

moter (NT *alc::cytINV*; Caddick et al., 1998), cytosolic invertase induction in the root did not alter the extent of colonization with *G. intraradices* or *G. mosseae* (Supplemental Figs. S2 and S3). To compare all three types of different subcellular located invertases, *A. rhizogenes*-mediated root transformation of the model legume *M. truncatula* was performed using either the *35S::cwINV*, the *35S::cytINV*, or the *35S::vacINV* construct. Control plants were transformed with the *35S::uidA* construct expressing the gene coding for β -glucuronidase (GUS). Yeast invertase expressing roots showed on average an approximately 3- to 6-fold increased hexose level and an enhanced hexose-to-Suc ratio, both compared to GUS-transformed roots (Fig. 5A).

However, none of the overexpressing plants showed altered mycorrhization. The content of *G. intraradices*-specific rRNA did not differ significantly between GUS- and INV-transformed roots. The same was found for transcript levels of the AM-induced phosphate transporter *MtPT4* (Fig. 5B). Both parameters can be used for quantification of mycorrhization (Isayenkov et al., 2004). Nonmycorrhizal plants contained no fungal rRNA and negligible *MtPT4* transcript levels (data not shown). The inorganic phosphate levels in roots and leaves (Fig. 5, C and D) as well as AM-induced and fungus-specific metabolites, such as C16:1 Δ^{11} and C16:0 (Fig. 5E) or cyclohexenone and mycorradicin derivatives (data not shown), did not change in any of the INV-transformed roots compared to roots of GUS-transformed plants. Thus, it can be concluded that enhanced carbon availability in the root at specified subcellular location did not modify the physiological properties of AM. This finding suggests that the supply of the AM fungus is already optimal in the mutualistic association.

Effect of an Undersupply of the Root on AM

Because increased hexose levels in the root had no effect on mycorrhization, as proof of concept, transgenic tobacco plants with an undersupply of carbon in the root were analyzed. The effect of low root carbohydrate

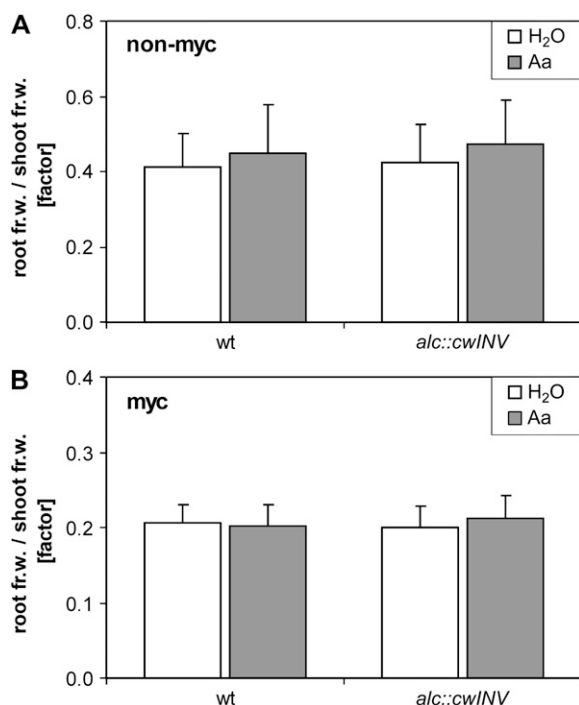


Figure 4. Biomass analysis of nonmycorrhizal and mycorrhizal wild-type and transgenic tobacco plants exhibiting increased apoplasmic invertase activity in the root. **A**, Root-to-shoot ratio of the fresh weight of 8-week-old nonmycorrhizal wild-type and *alc::cwINV* plants. These plants were soil drenched twice with 100 mL 0.05% (v/v) acetaldehyde (Aa) or water (H₂O) 1 and 2 weeks before harvest. Mean values of two independent experiments + SD are given (wild type: $n = 22$; *alc::cwINV*: mean values of three independent lines with each $n = 30$). **B**, Root-to-shoot ratio of the fresh weight of 13-week-old wild-type and *alc::cwINV* plants inoculated with *G. intraradices* 7 weeks before harvest. Acetaldehyde (Aa) or water (H₂O) was applied as described for A, but was carried out weekly four times starting 7 d after inoculation. Results are the mean + SD (wild type: $n = 24$; *alc::cwINV*: mean values of three independent lines with each $n = 16$).

content on AM formation was studied in transgenic tobacco plants expressing the *ppa* gene from *E. coli*, encoding inorganic pyrophosphatase, under control of the phloem-specific *rolC* promoter (NT *rolC::ppa*). Phloem-specific expression of *ppa* inhibits the inorganic pyrophosphate-dependent uptake of Suc into the phloem cells resulting in sugar accumulation in source leaves and an undersupply of the sink organs (Lerchl et al., 1995). Here, heterozygous NT *rolC::ppa* plants were investigated. These plants were classified according to the degree of growth reduction (large, intermediate, and small; Fig. 6A). All plants showed stunted growth, and in addition, the root-to-shoot ratio of the fresh weight (Fig. 6B) and of the dry weight (data not shown) was decreased compared to wild-type plants. This clearly indicates an insufficient carbohydrate supply in the root. Furthermore, all *rolC::ppa* plants exhibited decreased carbohydrate levels in the root and/or increased levels in the shoot (Fig. 6C shows the decreased root-to-shoot ratio of hexoses).

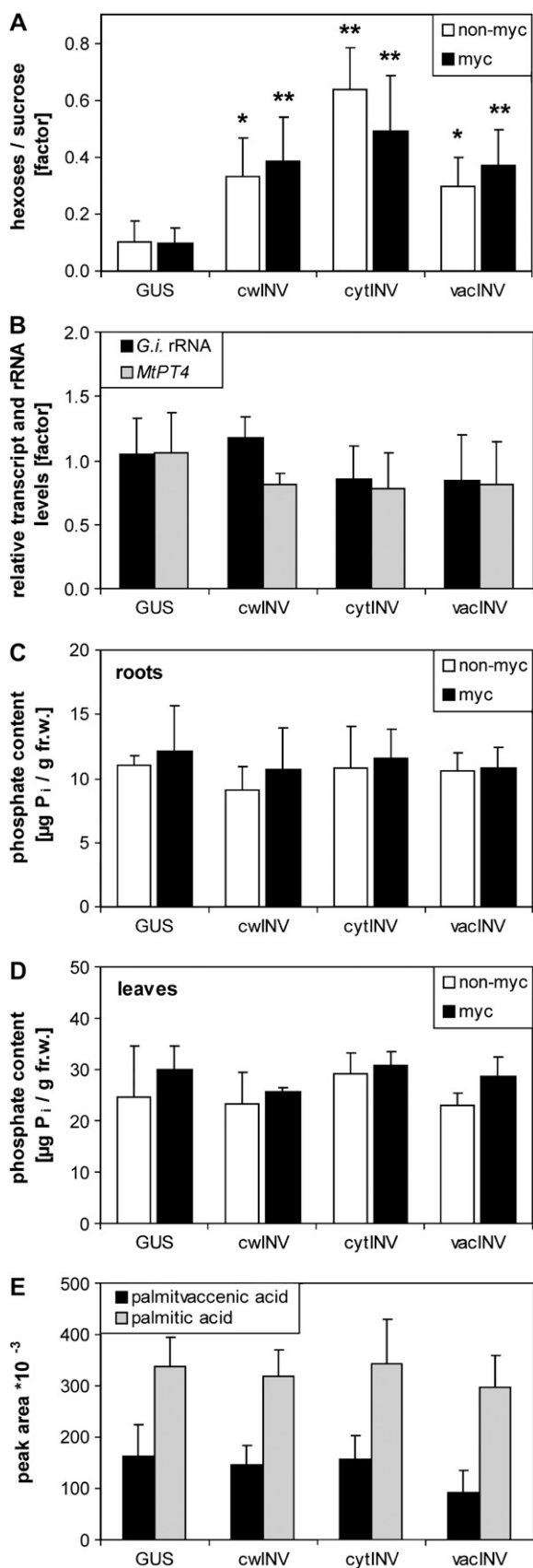
With increasing growth reduction and therefore lower carbohydrate supply of roots of the heterozygous NT *rolC::ppa* plants, decreasing mycorrhization rates were observed upon *G. intraradices* inoculation (Fig. 6D). Moreover, fewer fungal vesicles and spores were found compared to wild-type plants (Fig. 6, E and F). This indicates an undersupply of the AM fungus leading to decreased formation of fungal storage organs, which rely on the carbon allocation by the plant.

Effect of Root-Specifically Decreased Invertase Activity on AM

Because a general undersupply of the root with carbon by defective phloem loading resulted in decreased mycorrhization, the analysis of plants with reduced invertase activity and decreased phloem unloading complement this study. This aspect was implemented by expressing the Arabidopsis gene *AtC/VIF2* coding for an inhibitor of acid invertases (Link et al., 2004) under control of the root- and seedling-specific *pyk10* promoter from Arabidopsis (Nitz et al., 2001) in transgenic tobacco plants (NT *pyk10::InvInh*). Recombinant AtC/VIF2 protein was shown to affect apoplasmic and vacuolar invertase activities in vitro (Link et al., 2004). Plants of the two independent NT *pyk10::InvInh* lines, 98-1-10 and 98-4-1, showed reduced apoplasmic invertase activities in the root (Fig. 7A). Vacuolar invertase activity was inhibited in vitro only in one line at later developmental stages (Fig. 7B). Neutral cytosolic invertase activity levels were not affected; the same was true for invertases in leaves (Supplemental Fig. S6). Nonmycorrhizal plants showed a similar affection of invertase activities (Supplemental Fig. S6). According to the reduced apoplasmic invertase activity, the roots had lower contents of Glc and Fru (Fig. 7C shows the sum of both hexoses) and a reduced ratio of both hexoses to Suc (Fig. 7D). However, in contrast to *rolC::ppa* tobacco, plants with root-specific overexpression of invertase inhibitor were not altered in their vegetative growth. There were no alterations in their total biomass (data not shown) and their root or shoot biomass compared to wild-type plants (Fig. 8 shows the root-to-shoot ratio of the fresh weight). Nevertheless, corresponding to the lower hexose levels in roots of *pyk10::InvInh* plants we found a lower mycorrhization level with *G. intraradices* (Fig. 7E). Moreover, colonized roots of NT *pyk10::InvInh* plants showed a lower density of fungal structures compared to the wild type (Fig. 7F), reflected by a significant decrease in fungus-specific rRNA 5 weeks after inoculation (data not shown). This indicates that the carbon supply in the AM interaction depends on the activity of apoplasmic invertases that deliver hexoses.

DISCUSSION

The influence of the carbohydrate status of the plant on AM formation is still poorly understood. In this



study, the effect of elevated root hexose levels on the mutualistic interaction in AM was investigated. This was achieved by increased invertase activities in different subcellular locations in the root due to the expression of chimeric genes encoding yeast-derived invertase (Sonnewald et al., 1991). Surprisingly, we were not able to detect any change in the mycorrhization of tobacco or *Medicago* plants with increased apoplastic, cytosolic, or vacuolar invertase activities in the root, either on the fungal or plant side.

We intensively analyzed NT *alc::cwINV* plants with root (part)-specifically increased apoplastic invertase activity, because extracellular invertases have been suggested to play a crucial role in the carbohydrate supply of the obligate biotrophic AM fungus (Schaarschmidt et al., 2006). However, no evidence was found either for forcing the mutualistic association, or for changing it into a parasitic one, or for suppressing AM by activation of defense reactions. All of the measured parameters for AM formation and for the plant's benefit of AM remained unaffected, both in normally grown and in split-root plants. On the first glance this might contradict to previous feeding experiments indicating a stimulation of the phosphate allocation in mycorrhizal roots by increased carbohydrate availability (Bücking and Shachar-Hill, 2005). However, in these experiments with axenic root cultures the authors observed due to feeding of higher Suc amounts an increased carbon transfer from the root to the fungus that was suggested to stimulate P uptake and transfer by the fungus. Here, no indices for a higher carbon transfer from the plant to the AM fungus, deduced from the content of fungus-specific C16:1 Δ^{11} and its precursor C16:0 (Trépanier et al., 2005; van Aarle and Olsson, 2005), were found, although levels of available carbon increased. Moreover, the finding of Bücking and Shachar-Hill (2005) was obtained from experiments with axenic cultures of transformed carrot

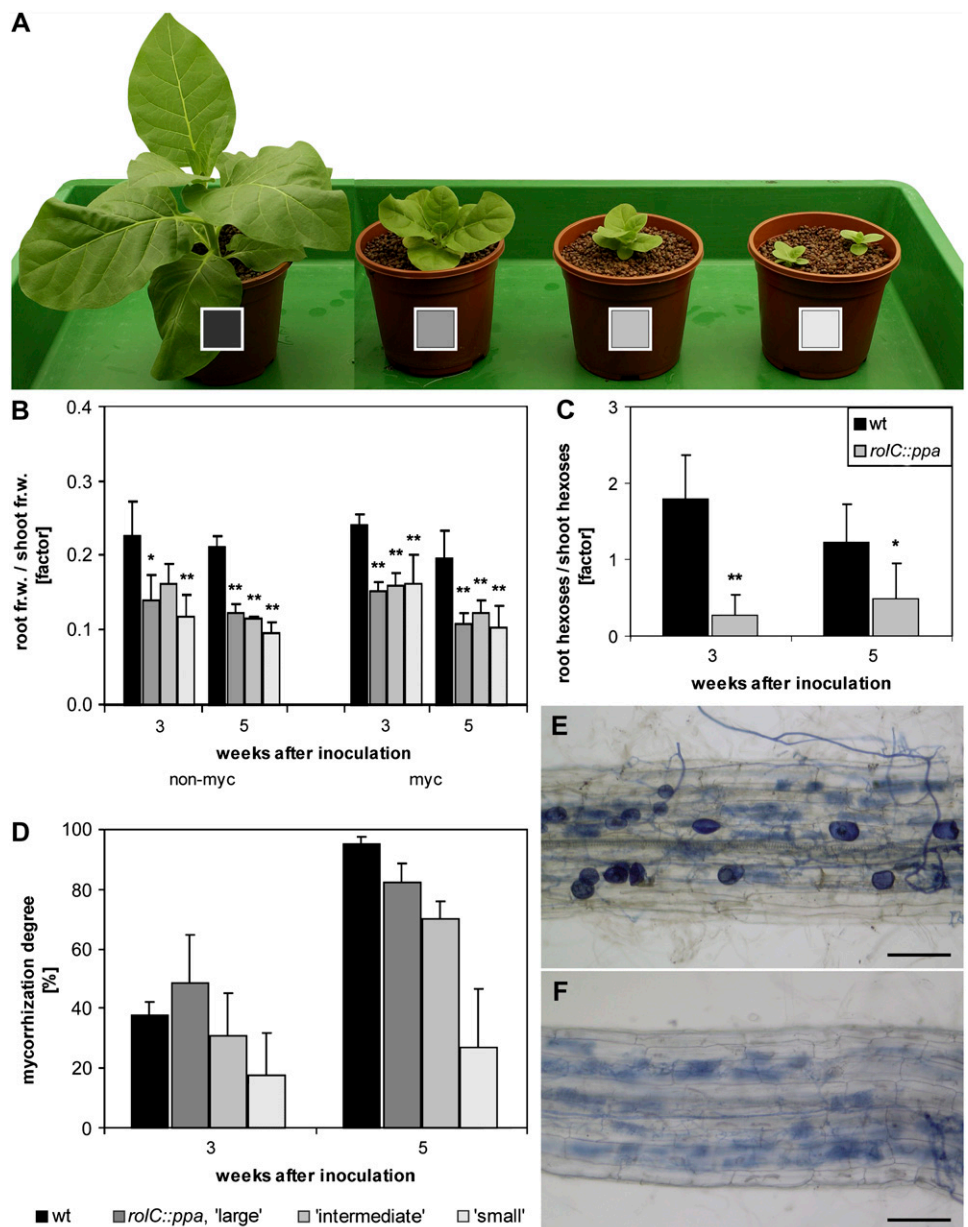
Figure 5. Analysis of root-transformed *M. truncatula* plants expressing genes encoding yeast-derived invertase directed to different subcellular locations. A, Ratio of the sum of Glc and Fru to the Suc content of the transformed roots. B, Relative levels of *G. intraradices* rRNA and of transcripts of the mycorrhiza-induced phosphate transporter *MtPT4* in mycorrhizal roots. Transcript and rRNA levels of roots transformed with the GUS construct were set to 1. C and D, Inorganic phosphate content of roots (C) and leaves (D). E, Content of C16:1 Δ^{11} and C16:0 in mycorrhizal roots. A. *rhizogenes*-mediated root transformation was performed using constructs containing the gene coding for yeast invertase, which is either translocated to the apoplast (*cwINV*), the cytosol (*cytINV*), or the vacuole (*vacINV*), or the gene coding for GUS, all expressed under control of the *35S* promoter. Five weeks after transformation, plants were inoculated with *G. intraradices* (*myc*) or left without inoculation (*non-myc*) and were harvested 5 weeks later. All results are presented as means \pm sd (*nonmycorrhizal* plants: $n = 4$; *mycorrhizal* plants: $n = 6$). Data of *nonmycorrhizal* and *mycorrhizal* plants root transformed with plasmids coding for the different located invertases were compared to *nonmycorrhizal* and *mycorrhizal* plants root transformed with the GUS construct, respectively, using the Student's *t* test. * $P < 0.05$, ** $P < 0.01$.

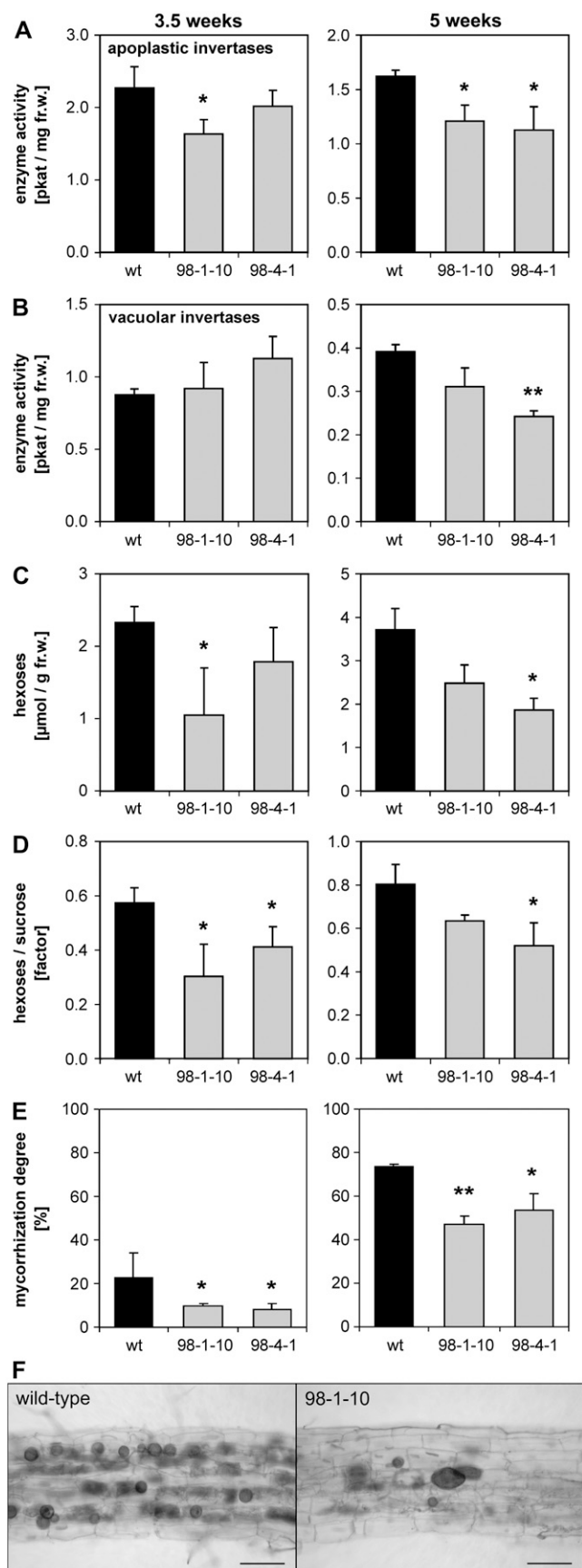
roots, and it cannot be directly translated to the situation in a normal mycorrhizal root.

A strong induction of extracellular invertases in plants and the severe modulation of source-sink activities could also lead to an activation of defense-related mechanisms via sugar-mediated gene expression (Rolland et al., 2002; Roitsch et al., 2003). As shown previously, constitutive expression of the apoplast-located yeast invertase resulted in a strong accumulation of PR protein transcripts in tobacco leaves, encoded by *PARI*, *PR-1b*, and *PR-Q* (Herbers et al., 1996). During root-specific expression of apoplast-located yeast invertase, we did not observe transcript accumulation of those PR genes, indicating that there was no activation of plant defense responses interfering with the mutualistic interaction. To check the

intensity of sink-source modulation in tobacco plants with elevated extracellular invertase activity in the root, some markers for sink strength were analyzed. However, there was no effect of enhanced apoplastic invertase activity detected on the starch content (data not shown) or on the root biomass. The root-to-shoot ratio of fresh weight and dry weight did not change; the same was found for the total plant biomass (data not shown). This might suggest that the increased apoplastic invertase activity did not change the sink strength. Especially in storage sinks, as rhizomes, tap roots, or tubers, the storage is often limited by the activity of enzymes involved in starch and oil accumulation, like Suc synthase or hexokinases. Nevertheless, increased sink strength by expression of yeast-derived apoplastic invertase leading to higher yields has been

Figure 6. Analysis of transgenic tobacco plants with phloem-specific expression of a pyrophosphatase. A, Phenotypes of 9-week-old non-mycorrhizal wild-type (left-most position) and *rolC::ppa* plants. Heterozygous NT *rolC::ppa* plants were classified by their growth reduction into three groups: large, intermediate, and small (from left to right; see different shadings). B, Biomass analysis. Ratio of root fresh weight to shoot fresh weight of non-mycorrhizal and mycorrhizal wild-type and *rolC::ppa* plants 3 and 5 weeks after inoculation. Wild-type and *rolC::ppa* plants were inoculated with *G. intraradices* 6 weeks after sowing (myc) or left without inoculation (non-myc). C, Root-to-shoot ratio of the sum of Glc and Fru in the mycorrhizal plants. Ratios of NT *rolC::ppa* plants comprehend data of large, intermediate, and small plants. D, Mycorrhization degree of the inoculated plants. Data in B, C, and D are given as means + SD ($n \geq 6$). For each developmental stage, the fresh weight and hexose ratios of NT *rolC::ppa* plants were compared to wild-type plants using the Student's *t* test. * $P < 0.05$, ** $P < 0.01$. E and F, Ink-stained fungal structures in a wild-type (E) and an intermediate *rolC::ppa* plant (F) 4 weeks after inoculation. Bars represent 100 μm .





shown, e.g. for potato (*Solanum tuberosum*) tubers (Sonnewald et al., 1997; Hajirezaei et al., 2000) and for Arabidopsis seeds (Heyer et al., 2004). In contrast, transgenic tobacco plants expressing the apoplast-located yeast invertase with a seed-specific promoter did not show increased accumulation of storage products in the oilseeds, even with additional overexpression of hexokinase, indicating no change in the sink strength of these storage sinks (Tomlinson et al., 2004). In Arabidopsis, the root-specific expression of yeast-derived apoplastic invertase had no detectable effect on the plant biomass as well (von Schweinichen and Büttner, 2005). In contrast, Arabidopsis plants expressing an apoplastic invertase of *Chenopodium rubrum* with the root-specific *pyk10* promoter showed only slightly enhanced invertase activity and no changes in the soluble sugar contents, but there was an indirect influence on the whole plant development and an increased total plant biomass (von Schweinichen and Büttner, 2005). The authors observed a similar phenotype when the extracellular-located yeast invertase was driven under a meristem-specific promoter, which was observed in several other studies (e.g. Lerchl et al., 1995; Fukushima et al., 2001; Canam et al., 2006), confirming the principal function of a yeast-derived invertase in plants.

In this study induction of the apoplast-located yeast invertase resulted in root-specifically enhanced hexose levels that were suggested to affect AM. To test whether the subcellular location of increased invertase activity in the root plays an essential role for improving the carbon supply of the AM fungus, *A. rhizogenes*-mediated root transformation was carried out. In addition, the model legume *M. truncatula* was used to exclude potential tobacco-specific features in the formation of AM. However, even in *Medicago*, none of the differently located invertases had an observable effect on AM formation. These results could suggest that hexoses might not be the favored form of AM-fungal carbon uptake and that the fungus is capable of using Suc with the same efficiency. In this case,

Figure 7. Analysis of transgenic tobacco plants with root-specific expression of an invertase inhibitor. A and B, Cell wall (A) and vacuolar (B) invertase activity in roots of wild-type SR1 plants and NT *pyk10::InvInh* plants of two independent lines (98-1-10 and 98-4-1) 3.5 and 5 weeks after inoculation with *G. intraradices*. C, Glc and Fru content of the roots. D, Ratio of the Glc and Fru contents to the Suc content of the roots. E, Degree of mycorrhization. To allow statistical analysis, the degree of mycorrhization in percent of the root length was determined for every root system in 50 to 100 root pieces of each 1 cm length. Plants were in two independent experiments inoculated with *G. intraradices* either 2.5 weeks after sowing and harvested 3.5 weeks later or inoculated 4 weeks after sowing and harvested 5 weeks later. Data are presented as mean values + SD (at 3.5 weeks: $n = 5$; at 5 weeks: $n = 3$). The data from the transgenic lines were pairwise compared to the wild type by the Student's *t* test. * $P < 0.05$, ** $P < 0.01$. F, Ink-stained fungal structures in a wild-type and a NT *pyk10::InvInh* plant of line 98-1-10, each 5 weeks after inoculation. Bars represent 100 μm .

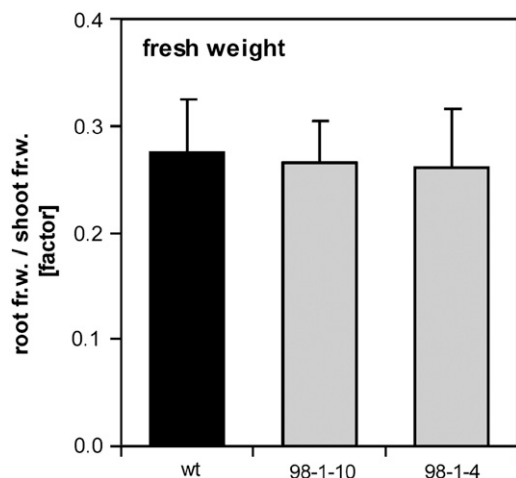


Figure 8. Biomass analysis of NT *pyk10::Invlnh* plants. Root-to-shoot ratio of the fresh weight of 6-week-old nonmycorrhizal wild-type SR1 plants and plants of two independent NT *pyk10::Invlnh* lines (98-1-10 and 98-4-1). Mean values of \pm SD are given ($n \geq 33$).

increased invertase activity in the root would not influence the availability of carbon for the fungus. Nevertheless, this assumption would be in strong contrast to several other studies that examine the induction of Suc-cleaving enzymes in mycorrhizal roots (e.g. Dehne, 1986; Snellgrove et al., 1987; Wright et al., 1998; Hohnjec et al., 2003; Schaarschmidt et al., 2006) and also to the fact that isolated intraradical hyphae showed a high preference for the uptake of Glc compared to Suc and Fru (Solaiman and Saito, 1997). To vitiate this assumption, we have shown the importance of acid invertases in the AM association by repressing their enzymatic activity. Posttranslational inhibitor-mediated inactivation of enzymes represents a powerful tool for analyzing the physiological role of enzyme classes. Proteinaceous inhibitors of extracellular and acid invertases are essential to control acid invertase activities and their regulatory functions in plants (Balibrea et al., 2004; for review, see Rausch and Greiner, 2004). Here, transgenic tobacco plants with root-specific expression of *AtC/VIF2* were analyzed. The inhibitory effect of *AtC/VIF2* has been demonstrated in vitro using recombinant protein (Link et al., 2004). From this, a higher in vitro affinity of vacuolar invertases compared to apoplastic invertases was shown; however, the inhibitors may behave differently in vivo. Using in vitro assays, we found in the roots of two independent NT *pyk10::Invlnh* lines a significantly reduced extracellular invertase activity but less inhibition of vacuolar invertases. Along with decreased apoplastic invertase activity, the roots were characterized by reduced hexose contents and a decreased hexose-to-Suc ratio, indicating the in vivo function of *AtC/VIF2* in tobacco roots. The plants, however, did not show an altered vegetative growth and root or shoot biomass. Reduced colonization rates with a lower density of fungal structures clearly dem-

onstrate the important role of apoplastic invertases in supplying sufficient amounts of hexoses in the mycorrhizal root.

In conclusion, we suggest that under normal growth conditions the supply of the fungal symbiont with carbon is already optimal in the AM symbiosis and that it cannot readily be improved. This hypothesis is supported by increased levels of soluble sugars in mycorrhizal clover (*Trifolium repens*) roots (Wright et al., 1998), which argues for a slower uptake of hexoses by the fungus compared to delivering Suc and cleavage into hexoses by the plant. In our study, we were not able to detect significantly increased root hexose or Suc levels in mycorrhizal wild-type or control transgenic plants as water-treated NT *alc::cwlNV* and GUS-transformed *M. truncatula* (data not shown). However, constant (instead of decreasing) hexose or Suc levels in mycorrhizal tobacco and Medicago roots indicate the sufficient availability of carbohydrates in wild-type plants under normal growth conditions. In contrast, according to previous experiments that show a reduced mycorrhization upon limiting light intensity (Hayman, 1974; Tester et al., 1985; Son and Smith, 1988), suppression of AM could be observed if the root was severely undersupplied with carbohydrates as shown for NT *rolC::ppa* plants. During the disturbed inorganic pyrophosphatase-dependent uptake of Suc into the phloem cells and translocation of Suc to the sink tissue (Lerchl et al., 1995), the formation of AM was characterized by less density of fungal storage organs, indicating a deficiency of carbon.

Summarizing, our findings clearly demonstrate the fungal dependency on the carbohydrate supply of the root controlled by plant invertases and the general regulation of AM formation by carbon availability. Nevertheless, in the functional symbiotic interaction in AM, the carbon supply seems not to be the limiting factor. It is tempting to speculate that other mechanisms such as the phosphate supply of the plant or plant defense responses to limit fungal growth to the root cortex may be of higher importance in regulating that interaction.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Wild-type tobacco (*Nicotiana tabacum* cv Samsun NN) plants were obtained from Vereinigte Saatzuchten eG and germinated on solid Murashige and Skoog medium (Duchefa). Transgenic tobacco *alc::cwlNV* (Schaarschmidt et al., 2004) and *rolC::ppa* plants (Lerchl et al., 1995) were selected by sowing on solid Murashige and Skoog medium containing 50 mg/L kanamycin A (Duchefa). All tobacco plants were cultivated in a growth chamber with 16 h light ($250 \mu\text{mol photons m}^{-2} \text{s}^{-1}$; Philips Powerstar HQI 250/D lamps) at 25°C and 50% relative humidity. After 3 weeks, plants were transferred into pots filled with expanded clay of 2 to 5 mm particle size (Original Lamstedt Ton, Fibo ExClay). They were watered with distilled water and fertilized twice per week with 10 mL $10 \times$ Long Ashton (20% phosphate corresponding to 2.8 mM; Hewitt, 1966).

Medicago truncatula cv Jemalong seeds (obtained from AustraHort) were scarified by incubation in concentrated H_2SO_4 for 10 min, washed with water, and surface sterilized in 1:6 diluted sodium hypochlorite solution (12% Cl;

Roth) for 5 min. After washing, the seeds were placed on 0.8% (w/v) plant agar (Duchefa) and germinated 2 d at room temperature (RT) in the light and 4 d at 4°C in the dark. The seedling were root transformed as described below and transferred to pots filled with expanded clay as described for tobacco. Plants were cultivated in a growth chamber at 20°C, 50% relative humidity, and 16 h light ($250 \mu\text{mol m}^{-2} \text{s}^{-1}$)/8 h dark, watered with distilled water, and fertilized twice per week with 5 mL $10\times$ Long Ashton (20% phosphate).

After inoculation with *Glomus intraradices*, plants were cultivated under the same conditions as before, but fertilized with 25 mL $10\times$ Long Ashton (20% phosphate) twice per week in case of tobacco and 10 mL twice per week in case of Medicago. Roots and middle-aged leaves of tobacco and roots and all leaves of Medicago plants were harvested at the end of the light period.

Plasmid Constructions, Stable Plant Transformation, and Determination of Plant Invertase Activities

The *pyk10* promoter was amplified by PCR using genomic DNA and subcloned into the vector pTF2-6 (T. Fatima and T. Roitsch, unpublished data) to generate pMB1-18. The cDNA encoding AtC/VIF2 (at5g64620) was amplified by reverse transcription-PCR using total RNA, initially cloned into the vector pBluescript KS+ to generate pMCG2, and subsequently subcloned thereof as Acc65I-*KpnI* fragment into the binary vector pTF2-6 to generate plasmid pMCG4. To generate a transcriptional fusion between the *pyk10* promoter and the cDNA encoding AtC/VIF2, a 1,467 bp *pyk10* promoter fragment was subcloned as Acc65I fragment from pMB1-18 into the binary vector pMCG4, linearized by Acc65I, to generate pMCG6. The *pyk10::InvInh* construct was transformed in tobacco (cv SR1) using *Agrobacterium tumefaciens* strain LBA4404 and standard transformation procedures (Horsch et al., 1985). Transgenic lines expressing the *pyk10::InvInh* fusion were characterized by PCR (M. Gonzalez and T. Roitsch, unpublished data).

Determination of apoplastic (cell wall bound) and vacuolar plant invertase activities was performed as described by Greiner et al. (1999).

Agrobacterium rhizogenes-Mediated Root Transformation

Construction of plasmids containing the *35S::cwINV*, *35S::cylINV*, and *35S::vacINV* constructs was described previously (von Schaewen et al., 1990; Sonnewald et al., 1991). A plasmid containing the *35S::uidA* construct was kindly provided by Stanislav Isayenkov (IPB Halle; Isayenkov et al., 2005). *Agrobacterium rhizogenes* Arqua1 (Quandt et al., 1993) was transformed by electroporation. The induction of transgenic hairy roots was performed according to standard procedures (Vieweg et al., 2004). After development of hairy roots, all roots that did not emerge from the infection site and roots that did not show a hairy root phenotype were removed.

Invertase Induction and Determination of Yeast Invertase Activity

Using the *alc* promoter system, the yeast-derived invertase was induced in *NTalc::cwINV* plants root specifically by soil drenching with 100 mL of 0.05% (v/v) aqueous acetaldehyde solution (Schaarschmidt et al., 2004). Unless otherwise mentioned, acetaldehyde was applied three times at weekly intervals starting with the time point of inoculation. Control plants were drenched in the same way with distilled water. Root part-specific invertase induction was carried out using the split-root system, in which the root system was divided onto two pots. Here, only one root part of mycorrhizal or non-mycorrhizal plants was treated with acetaldehyde, whereas the other was drenched with water. Determination of invertase activity was performed as described before (Schaarschmidt et al., 2004).

Inoculation with *G. intraradices* and Staining of Fungal Structures

The AM fungus *G. intraradices* Schenk & Smith isolate 49 (Maier et al., 1995) was enriched by previous cocultivation with leek (*Allium porrum* cv Elefant). For inoculation, plants were transferred after careful removal of the previous substrate to new pots filled with expanded clay containing 5% to 10% (v/v) *G. intraradices* inoculum freshly harvested from mycorrhizal leek plants. Non-mycorrhizal plants were transferred in the same way to pure expanded clay. All tobacco plants were inoculated 6 weeks after sowing; inoculation of Medicago was performed 5 weeks after root transformation. For the estima-

tion of *G. intraradices* colonization a representative cross section of each root system was taken. The mycorrhizal structures in the root pieces were stained according to Vierheilig et al. (1998) using 5% (v/v) ink (Sheaffer Skrip jet black, Sheaffer Manufacturing) in 2% (v/v) acetic acid and analyzed using a stereomicroscope. The degree of mycorrhization is given in percent of root length. Micrographs of ink-stained roots were taken using a Zeiss Axioplan microscope equipped with a video camera (Fujix Digital Camera HC-300Z, Fuji Photo Film) and were processed through Photoshop 7.0 (Adobe Systems).

For fluorescent staining of fungal structures with wheat germ agglutinin (WGA), coupled to tetramethyl rhodamine isothiocyanate (TRITC) or Alexa Fluor 488, root pieces of 3 mm were fixed with 4% (w/v) paraformaldehyde and 0.1% (v/v) Triton X-100 in phosphate-buffered saline (PBS) for 30 min at RT and afterward cut into 140- μm thick cross sections using a vibrating blade microtome (VT 1000 S, Leica Microsystems). Cross sections were digested in 1% (w/v) cellulase, 0.1% (w/v) bovine serum albumin, and 0.01% (w/v) pectinase in PBS for 1 h at RT. After washing with PBS, staining was performed using 50 $\mu\text{g}/\text{mL}$ WGA-TRITC and 50 $\mu\text{g}/\text{mL}$ WGA-Alexa Fluor 488 (both from Molecular Probes) in PBS for 30 min at RT. The formation of mycorrhizal structures was analyzed with a confocal laser-scanning microscope (LSM 510 Meta, Zeiss) using the 488 nm (Alexa Fluor 488) and 543 nm (TRITC) laser lines for excitation. Series of optical sections (*z* series) were acquired by scanning 19 sections with a distance of 0.2 μm on the *z* axis; *z*-series projections were done with the LSM Image Examiner software (Zeiss).

Real-Time Reverse Transcription-PCR Analysis

Total RNA of Medicago root material was isolated using the Qiagen RNeasy plant mini kit including a DNase digestion (RNase-free DNase Set, Qiagen). First-strand cDNA synthesis of 1 μg RNA in a final volume of 20 μL was performed with M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega) according to the supplier's protocol using an oligo dT (T19) primer for gene expression analysis of *MtPT4* and a random hexamer primer for determination of fungal rRNA levels.

For real-time PCR, 4.5 μL of 1:9 diluted cDNA (25 ng reverse-transcribed total RNA) were mixed with $2\times$ TaqMan master mix (Applied Biosystems) and $20\times$ TaqMan probe and primers (Assays-by-Design, Applied Biosystems) in a final volume of 10 μL in three independent replicates. TaqMan probes and primers for the mycorrhiza-induced phosphate transporter *MtPT4* of *M. truncatula* and for *G. intraradices*-specific rRNA were used as described previously (Isayenkov et al., 2004). As internal control for both cDNA syntheses transcript levels of *elongation factor 1- α* of *M. truncatula* were measured (Isayenkov et al., 2004). Real-time PCR was performed using the Mx 3005P QPCR system (Stratagene) according to the Assays-by-Design protocol (Applied Biosystems). Data were evaluated with the MxPro software (Stratagene) and calculated by the comparative Ct method.

Determination of Soluble Sugars and Inorganic Phosphate

The soluble sugar contents were measured photometrically by a coupled enzymatic assay as described previously (Schaarschmidt et al., 2004). The determination of inorganic phosphate was performed according to Taussky and Shorr (1953). Frozen root and leaf material (100–500 mg fresh weight) was homogenized in liquid nitrogen and incubated under shaking with 800 μL 3% (v/v) perchloric acid for 20 min at RT. After centrifugation (14,000g, 5 min, RT), 120 μL of the supernatant was incubated with 80 μL reaction solution containing 10% (v/v) ammonium molybdate stock solution [0.4 M $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ in 10 N H_2SO_4] and 0.18 M FeSO_4 in a microtitre plate. When the reaction reached a plateau the absorbance was measured at 750 nm using a 96-well microtitre plate reader (Sunrise, Tecan). The standard was KH_2PO_4 . All measurements were carried out in three independent replicates for each sample.

Metabolite Analysis

Homogenized lyophilized root material (30 mg) was first extracted three times with 500 μL hexane. The supernatant (in total 1.5 mL) was collected and 15 μL of a methyl nonadecanoate stock solution (2 mg/mL hexane) (Sigma-Aldrich) were added as internal standard. After washing the pellet with hexane and drying it, the polar components were extracted in the same way using 80% (v/v) aqueous methanol. As internal standard, 75 μL of a ribitol stock solution (2 mg/mL water; Sigma-Aldrich) was added.

Gas chromatography/mass spectroscopy measurements were performed with an Trace 2000 GC equipped with an Autosampler 3000 and a single quadrupole Trace DSQ (ThermoElectron). Hexane extracts (125 μ L aliquots) were derivatized after reduction to dryness in glass injection vials with 100 μ L *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (CS-Chromatographie Service) for 30 min at 70°C and diluted with 400 μ L hexane prior to injection. The following conditions were used: EI-voltage 70 eV; source temperature 240°C; column J&W DB-5 MS (30 m \times 0.25 mm, i.d., 0.25 μ m film thickness; Agilent); carrier gas helium at constant flow of 1 mL/min; temperature program: 50°C (2 min), 50°C to 260°C (6°C/min), 260°C (3 min), 260°C to 300°C (10°C/min), 300°C (6 min); injection temperature: 240°C, splitless injection 1 μ L, mass range of mass-to-charge ratio 40 to 800. Data acquisition and evaluation run with Xcalibur 1.4.1.

Methanol extracts were analyzed by HPLC according to Schliemann et al. (2006). A 10 μ L aliquot of each extract was injected onto a 5 μ m Nucleosil C₁₈ column (250 \times 4 mm i.d.; Macherey-Nagel). Separation was achieved using a 40-min linear gradient at 1 mL/min from 5% to 25% (v/v) acetonitrile in 1.5% (v/v) aqueous H₃PO₄ followed by a gradient from 25% to 80% (v/v) acetonitrile in 20 min. Cyclohexenone and mycorradicin derivatives were detected photometrically at 245 nm and 380 nm, respectively, by a Waters 2996 photodiode array detector. Data were collected and analyzed using the Millennium software 2010 (Millipore).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number NM_125858.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Mycorrhizal formation in *alc::cwlNV* tobacco plants after invertase induction in root parts.

Supplemental Figure S2. Influence of UV and total photon irradiance intensity on the mycorrhization of wild-type and transgenic tobacco plants with increased invertase activity in the root.

Supplemental Figure S3. Colonization of transgenic tobacco plants, exhibiting root part-specifically increased apoplastic or cytosolic invertase in roots, with *G. mosseae*.

Supplemental Figure S4. Expression analysis of the salt stress-inducible genes *Osmotin* and *Ts1l* in wild-type and *alc::cwlNV* tobacco plants.

Supplemental Figure S5. Expression analysis of *PR* genes in wild-type and *alc::cwlNV* tobacco plants.

Supplemental Figure S6. Invertase activities in nonmycorrhizal and mycorrhizal NT *pyk10::Ino1nh* plants.

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