Running title: Effect of modulated carbon availability on AM

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Regulation of arbuscular mycorrhization by carbon: The symbiotic interaction cannot be improved by increased carbon availability accomplished by root-specifically enhanced invertase activity

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Abstract
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-derived invertase, which was directed to different subcellular locations. Using *Nicotiana tabacum alc::cwINV* 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 palmitvaccenic acid (C16:1Δ11), 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 GUS-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, *N. tabacum rolC::ppa* plants with defective phloem loading and *N. tabacum pyk10::InvInh* plants with decreased acid invertase activity in roots exhibited a diminished mycorrhization.
Introduction

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 cytoplastm 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 arbuscular mycorrhiza-inducible phosphate transporters (Maeda et al., 2006; M. Harrison, personal communication) 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 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 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 glucose, 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). Glucose 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 glucose 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 glucose, 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 *Medicago 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 (Martin, 2005). The present 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 sucrose-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, sucrose is the major transport form of photosynthetically fixed carbon to sink organs. Utilization of sucrose requires cleavage which can either be performed by cytosolic sucrose synthases, producing UDP-glucose and fructose, or invertases, producing glucose and
fructose. 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 sucrose-cleaving enzymes have been identified so far, suggesting that plant invertases and sucrose 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, the current 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 *N. tabacum* or *M. truncatula* two general approaches were followed. To increase root hexose content, yeast-derived invertase(s) were expressed either under control of a chemically inducible promoter in *N. tabacum* or in hairy roots of *M. truncatula* following *Agrobacterium rhizogenes* transformation. Decreased root hexose content was achieved by either expression of a
phloem-specific *Escherichia coli* inorganic pyrophosphatase or root-specific expression of *A. thaliana* invertase inhibitor.

Here we report that an elevated root hexose content does not alter mycorrhization of *N. tabacum* 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 Nicotiana tabacum plants expressing a chimeric gene encoding a yeast-derived invertase which is translocated to the apoplast. In these NT 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 glucose (2-fold) and fructose (2.5-fold) were increased over a period of at least 6 weeks, whereas sucrose content decreased (0.6-fold) (Fig. 1B shows the increased hexose-to-sucrose 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 which 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 (not shown). Furthermore, no significant changes could be detected in the accumulation of the fungus-specific palmitvaccenic acid (C16:1Δ11) 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 metabolites, such as cyclohexenone derivatives (Maier et al., 2000) and mycorradianc 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 (Tab. 1). In non-mycorrhizal 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. 1; for metabolite accumulation, see also Fig. 1D and Table 1). Even testing different AM-fungi (*Glomus 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. 2 and 3). In addition, the analysis of normally grown (non-split root) plants also revealed no influence of increased root-specific apoplastic invertase activity on the mycorrhization (see also Supplemental Fig. 3).

Arbuscular mycorrhiza 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 non-split root plants in term of transcript accumulation of the salt stress-inducible genes *Osmotin* and *Tsi1* (*Tobacco Stress Induced Gene 1*; Park et al., 2001) were investigated. *G. intraradices*-colonized wild-type plants showed a slightly enhanced inorganic phosphate content in the leaves compared to non-mycorrhizal 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. 4).

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. 5).

Biomass analysis of non-mycorrhizal 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
Medicago truncatula
Because increased apoplastic and vacuole-located invertases was tested. In tobacco plants that
expressed the yeast gene coding for cytosolic invertase under control of the *alc* promoter (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. 2 and 3). To
compare all 3 types of different subcellular located invertases, *Agrobacterium rhizogenes-
mediated* root transformation of the model legume *Medicago 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-sucrose 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). Non-mycorrhizal 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, as
palmitvaccenic acid and palmitic acid (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 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 (PPi)-dependent uptake of sucrose 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 N. tabacum plants (NT pyk10::InvInh). Recombinant AtC/VIF2 protein was shown to affect apoplastic 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 apoplastic invertase activities in the root (Fig. 7A). Vacuolar invertase activity was inhibited in vitro only in one line at later developmental stages (Fig. 7B).
cytosolic invertase activity levels were not affected; the same was true for invertases in leaves (Supplemental Fig. 6). Non-mycorrhizal plants showed a similar affection of invertase activities (Supplemental Fig. 6). According to the reduced apoplastic invertase activity, the roots had lower contents of glucose and fructose (Fig. 7C shows the sum of both hexoses) and a reduced ratio of both hexoses to sucrose (Fig. 7D). However, in contrast to rolC::ppa tobacco, plants with root-specific overexpression of invertase inhibitor were not altered in their vegetative growth 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 (Figure 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 apoplastic invertases that deliver hexoses.
Discussion

The influence of the carbohydrate status of the plant on arbuscular mycorrhiza (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 sucrose amounts an increased carbon transfer from the root to the fungus which 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 palmitvaccenic acid (C16:1Δ11) and its precursor palmitic acid (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 roots, and it can not 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 Par1, 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 sucrose synthase or hexokinases.
Nevertheless, increased sink strength by expression of yeast-derived apoplastic invertase
leading to higher yields has been shown, e.g. for potato 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 the present study induction of the apoplast-located yeast invertase resulted in root-
specifically enhanced hexose levels which 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 using sucrose with the same efficiency. In this case, 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 sucrose-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 glucose compared to sucrose and fructose (Solaiman and Saito, 1997). To vitiate this assumption, we have shown for the first time the importance of acid invertases in the AM association by repressing their enzymatic activity. Post-translational 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 et al., 2004). Here, transgenic tobacco plants with root-specific expression of \textit{AtC/VIF2} were analyzed. The inhibitory effect of \textit{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 \textit{pyk10::InvInh} 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-sucrose ratio, indicating the in vivo function of \textit{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 demonstrate 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 roots (Wright et al., 1998), which argues for a slower uptake of hexoses by the fungus compared to delivering sucrose and cleavage into hexoses by the plant. In our study, we were not able to detect significantly increased root hexose or sucrose levels in mycorrhizal wild-type or control transgenic plants as water-treated NT \textit{alc::cwINV} and GUS-transformed \textit{Medicago truncatula} (data not shown). However, constant (instead of decreasing) hexose or sucrose 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
sucrose into the phloem cells and translocation of sucrose 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.
Material and Methods

Plant material, growth conditions

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

Medicago truncatula cv. Jemalong seeds (obtained from AustraHort, Cleveland, Australia) were scarified by incubation in concentrated H₂SO₄ for 10 min, washed with water and surface-sterilized in 1:6-diluted sodium hypochlorite solution (12% Cl) (Roth, Karlsruhe, Germany) for 5 min. After washing, the seeds were placed on 0.8% (w/v) plant agar (Duchefa) and germinated 2 days at RT in the light and 4 days 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 µmol m⁻² s⁻¹) / 8 h dark, watered with distilled water and fertilized twice per week with 5 ml 10x Long Ashton (20% phosphate).

After inoculation with Glomus intraradices, plants were cultivated under the same conditions as before, but fertilized with 25 ml 10x 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) to generate pMB1-18. The cDNA encoding AtC/VIF2 (at5g64620) was amplified by RT-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 1467 bp *pyk10* promoter fragment was subcloned as Acc65I fragment from
pMB1-18 into the binary vector pMCG4, linearized by Acc65I, to generated pMCG6. The
*pyk10::InvInh* construct was transformed in tobacco (*Nicotiana tabacum* 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).

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::cytINV*, *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 NT *alc::cwINV*
plants root-specifically by soil-drenching with 100 ml of 0.05% (v/v) aq. acetaldehyde
solution (Schaarschmidt et al., 2004). Unless otherwise mentioned, acetaldehyde was applied
3 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 2 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 Glomus intraradices and staining of fungal structures**
The AM-fungus *Glomus intraradices* Schenk & Smith isolate 49 (Maier et al., 1995) was enriched by previous co-cultivation 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 estimation 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, Madison, USA) 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 (Zeiss, Jena, Germany) equipped with a video camera (Fujix Digital Camera HC-300Z, Fuji Photo Film, Tokyo, Japan) and were processed through Photoshop 7.0 (Adobe Systems, San Jose, USA).

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 PBS for 30 min at RT and afterwards cut into 140 µm-thick cross-sections using a vibrating blade microtome (VT 1000 S, Leica Microsystems, Wetzlar, Germany). Cross sections were digested in 1% (w/v) cellulase, 0.1% (w/v) bovine serum albumin (BSA) and 0.01% (w/v) pectinase in PBS for 1 h at RT. After washing with PBS, staining was performed using 50 µg/ml WGA-TRITC and 50 µg/ml WGA-Alexa Fluor 488 (both from Molecular Probes, Leiden, NL) in PBS for 30 min at RT. The formation of mycorrhizal structures was analyzed with a confocal laser scanning microscope (LSM 510 Meta, Zeiss, Jena, Germany) 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 RT-PCR analysis**

Total RNA of Medicago root material was isolated using the Qiagen RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) 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, Madison, USA)
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 2x TaqMan Master Mix (Applied Biosystems, Foster City, USA) and 20x
TaqMan probe and primers (Assays-by-Design, Applied Biosystems) in a final volume of 10
µl in 3 independent replicates. TaqMan probes and primers for the mycorrhiza-induced
phosphate transporter MtPT4 of Medicago truncatula and for Glomus 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-alpha of M. truncatula were
measured (Isayenkov et al., 2004). Real-time PCR was performed using the Mx 3005P QPCR
system (Stratagene, La Jolla, USA) 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 to
500 mg fr.w.) was homogenized in liquid nitrogen and incubated under shaking with 800 µl
3% (v/v) perchloric acid for 20 min at RT. After centrifugation (14,000 g, 5 min, RT), 120 µl
of the supernatant were incubated with 80 µl reaction solution containing 10% (v/v)
ammonium molybdate stock solution [0.4 M (NH₄)₆Mo₇O₂₄ in 10 N H₂SO₄] and 0.18 M
FeSO₄ 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, Crailsheim,
Germany). The standard was KH₂PO₄. All measurements were carried out in 3 independent
replicates for each sample.

Metabolite analysis

Homogenized lyophilized root material (30 mg) was first extracted 3 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, Steinheim, Germany) 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 H₂O) (Sigma-Aldrich) were added.
Gas chromatography/mass spectroscopy GC/MS measurements were performed with an Trace 2000 GC equipped with an Autosampler 3000 and a single quadrupole Trace DSQ™ (ThermoElectron, Dreieich, Germany). Hexane extracts (125 µl aliquots) were derivatized after reduction to dryness in glass injection vials with 100 µl N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA; CS-Chromatographic Service, Langerwehe, Germany) for 30 min at 70°C and diluted with 400 µl hexane prior to injection. The following conditions were used: EI-voltage 70 eV; source temp. 240°C; column J&W DB-5 MS (30 m x 0.25 mm, i.d., 0.25 µm film thickness) (Agilent, Folsom, USA), carrier gas helium at constant flow of 1 ml/min; temp. program: 50°C (2 min), 50 to 260°C (6°C/min), 260°C (3 min), 260 to 300°C (10°C/min), 300°C (6 min); injection temp.: 240°C, splitless injection 1 µl, mass range of m/z 40 to m/z 800. Data acquisition and evaluation run with Xcalibur 1.4.1.

Methanol extracts were analyzed by high performance liquid chromatography (HPLC) according to Schliemann et al. (2006). A 10 µl aliquot of each extract was injected onto a 5 µm Nucleosil C18 column (250 x 4 mm i.d.; Macherey–Nagel, Düren, Germany). 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) aq. 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 (Waters, Eschborn, Germany). Data were collected and analyzed using the Millennium software 2010 (Millipore, Eschborn, Germany).
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Literature Cited


Lerchl J, Geigenberger P, Stitt M, Sonnewald U (1995) Impaired photoassimilate partitioning caused by phloem-specific removal of pyrophosphate can be complemented by
a phloem-specific cytosolic yeast-derived invertase in transgenic plants. Plant Cell 7: 259-270


Figure Legends

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 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 days after inoculation as indicated by arrows. A, Invertase activity of the root parts. B, Ratio of the glucose and fructose contents to the sucrose content of the root parts. C, Degree of mycorrhization of the root parts of single plants. D, Content of palmitovaccenic acid and palmitic acid in single root parts of mycorrhizal plants 6 weeks and 7 weeks after inoculation. Plants harvested 6 weeks after inoculation are described above (A to C), plants harvest at week 7 were cultivated and inoculated in the same way but drenched with acetaldehyde 21, 28 and 35 days after inoculation. Data are expressed in A and B as means ± 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 labeled wheat germ agglutinins (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 5-times at weekly intervals starting with the time point of inoculation (+Aa). Control plants were water-treated (+H2O). Bars = 50 µm.

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 Fig. 1 for root part-specific induction. Data are presented as mean values + SD (non-mycorrhizal plants: n = 4; mycorrhizal plants: n = 6) and
are tested with multiple t test including Bonferroni correction. P<0.05. Means sharing the same letters are not significantly different.

**Figure 4.** Biomass analysis of non-mycorrhizal and mycorrhizal wild-type and transgenic tobacco plants exhibiting increased apoplastic invertase activity in the root. A, Root-to-shoot ratio of the fresh weight of 8-week-old non-mycorrhizal wild-type and *alc::cwINV* plants. These plants were soil-drenched twice with 100 ml 0.05% (v/v) acetaldehyde (Aa) or water (H₂O) one and two 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 4 times starting 7 days after inoculation. Results are the mean + SD. (wild-type: n = 24; *alc::cwINV*: mean values of three independent lines with each n = 16).

**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 glucose and fructose to the sucrose content of the transformed roots. B, Relative levels of *Glomus 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 palmitvaccenic acid (C₁₆:₁Δ₁₁) and palmitic acid (C₁₆:₀) in mycorrhizal roots. *Agrobacterium 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 β-glucuronidase (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 result are presented as means + SD. (non-mycorrhizal plants: n = 4; mycorrhizal plants: n = 6). Data of non-mycorrhizal and mycorrhizal plants root-transformed with plasmids coding for the different located invertases were compared to non-mycorrhizal and mycorrhizal plants root-transformed with the GUS-construct, respectively using the Student t test. *P<0.05, **P<0.01.
**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 3 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 glucose and fructose 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 ≥ 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 *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.

**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, Glucose and fructose content of the roots. D, Ratio of the glucose and fructose contents to the sucrose 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 *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::InvInh plants. Root-to-shoot ratio of the fresh weight of 6-week-old non-mycorrhizal wild-type SR1 plants and plants of two independent NT pyk10::InvInh lines (98-1-10 and 98-4-1). Mean values of + SD are given. (n ≥ 33).
Supplemental Figure 1. Mycorrhizal formation in \textit{alc::cwINV} tobacco plants after invertase induction in root parts. NT \textit{alc::cwINV} plants were cultivated in parallel to the plants shown in Fig. 1 using the split root system in which both root parts were inoculated with \textit{G. intraradices} 6 weeks after sowing. The induction of the invertase gene was performed by drenching one root in the split root plants with 100 ml of 0.05\% (v/v) aqueous acetaldehyde solution (Aa) whereas the other root was treated with water (H$_2$O). Soil-drenching was performed at weekly intervals starting with the onset of mycorrhizal colonization 21, 28 and 35 after inoculation as indicated by arrows. Mycorrhization degree of the root parts of single plants and the mean value ± SD is given (n = 3).

Supplemental Figure 2. 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. A, Influence of enhanced UV intensity on the mycorrhization of acetaldehyde-treated wild-type and \textit{alc::cwINV} plants. Plants were grown under 250 µmol m$^{-2}$ s$^{-1}$ as described in "Material and Methods" and were inoculated with \textit{G. intraradices} 6 weeks after sowing. Subsequently, at 6, 13, and 20 days, the whole root system of the plants was drenched with 100 ml of 0.05\% (v/v) aqueous acetaldehyde solution to induce apoplast-located yeast invertase. Two days after the beginning of invertase induction, half of the plants were carefully transferred into an analogous growth chamber with the same conditions but equipped accessorially with Osram Eversun lamps [L 100/79 Super, emission spectrum: 310 to 400 nm peaking at 355 nm, UV-A (75 W/m$^2$) and UV-B (1.5 W/m$^2$); Osram, München, Germany] to increase UV intensity mimicking the sun light (+ UV). The other plants were cultivated under the same light conditions without additional UV light as before (- UV). Plants were harvested 2 weeks after the beginning of UV exposure and 2 days after the last acetaldehyde treatment. Mean values + SD are given. (n = 5). Data tested with one-way ANOVA and post-hoc Tukey HSD test. P<0.01. Means sharing the same letters are not significantly different. Note the increased mycorrhization upon UV exposure. In contrast, increased invertase activity of roots or root parts did not affect the mycorrhization degree. B and C, Influence of reduced total light intensity on the mycorrhization of wild-type, \textit{alc::cwINV} and \textit{alc::cytINV} plants treated with acetaldehyde (Aa) or water (H$_2$O). In the \textit{alc::cytINV} plants, the yeast-derived invertase is cytosol directed (Caddick et al., 1998). Plants were grown up either as split root (B) or as non-split root plants (C) as described in "Material and Methods" under 250 µmol m$^{-2}$ s$^{-1}$. When inoculation with \textit{G. intraradices} occurred (6 weeks after sowing), the total photon irradiance intensity was reduced to 60 µmol
m$^{-2}$ s$^{-1}$. Invertase induction was induced by soil-drenching of root parts (B) or of the whole root system (C) with 100 ml of 0.05% (v/v) aqueous acetaldehyde solution 0, 7, 14 and 28 days after inoculation. Control plants were water-treated. Data are presented as mean $\pm$ SD of 3 to 4 parallel plants.

**Supplemental Figure 3.** Colonization of transgenic tobacco plants, exhibiting root part-specifically increased apoplastic or cytosolic invertase in roots, with *G. mosseae*. A, Mycorrhization degree of NT *alc::cwINV* plants, expressing apoplast-located yeast-derived invertase. B, Mycorrhization degree of NT *alc::cytINV* plants, in which the yeast-derived invertase is located in the cytosol. Root part-specific induction of apoplast- and cytosol-located yeast invertase in NT *alc::cwINV* and *alc::cytINV* plants, respectively was performed using the split root system. Both root parts were inoculated with *G. mosseae* Nicholson & Gerdemann (Biorize R&D, Dijon, France) 6 weeks after sowing and one part was drenched with 100 ml of 0.05% (v/v) aqueous acetaldehyde solution (Aa) whereas the other part was water-treated (H$_2$O). Soil-drenching was performed 5-times at weekly intervals starting 7 days after inoculation as indicated by arrows. Mycorrhization degree of the root parts of single plants and the mean value is given. (in A: n = 3; in B: n = 4).

**Supplemental Figure 4.** Expression analysis of the salt stress-inducible genes *Osmotin* and *Tsi1* in wild-type and *alc::cwINV* tobacco plants. Plants were inoculated with *G. intraradices* 5 weeks after sowing (myc) or stayed uninoculated (non-myc). Root-specific induction of the apoplastic yeast invertase was performed by drenching the whole root system with 100 ml 0.05% (v/v) acetaldehyde, which was applied 0, 7 and 14 d after inoculation (+Aa). Control plants were water-treated (+H$_2$O). Two days after the last treatment, all plants were soil-drenched with 100 ml 300 mM NaCl. Roots and middle-aged leaves were harvested 0, 1, 3 and 24 h after NaCl-application. Total RNA was isolated from pooled samples of three parallel plants. After RT, semi-quantitative PCR was performed with different amounts of cDNA using the following primers and annealing temperatures: *Osmotin*: forward primer: 5’–act atc gag gtc cga aac – 3’, reverse primer: 5’ – ctt cca ggc att tcc aag – 3’, 56$^\circ$C; *Tsi1*: forward primer: 5’ – gag gac gaa cga cag atc ac – 3’, reverse primer: 5’ – cat cgt tac gga gaa ctc gac – 3’, 56$^\circ$C; *Ubiquitin*: forward primer: 5’ – tga ctg gga gca cca tca c – 3’, reverse primer: 5’ – tag aca cca cag aga c – 3’, 55$^\circ$C.

Note that in roots and leaves, *Osmotin* was clearly induced by drenching with NaCl. *Tsi1* showed only low transcript accumulation in both tissues after NaCl treatment. Both genes
showed slightly lower expression levels in mycorrhizal plants than in non-mycorrhizal plants. However, acetaldehyde-treated wild-type showed decreased expression levels of *Osmotin* in roots and leaves compared to water-treated wild-type plants, possibly due to slight effects of acetaldehyde on *Osmotin* gene expression. Moreover, *Osmotin* and *Tsi1* accumulation in leaves and roots of mycorrhizal *alc::cwINV* plants was in general lower than in wild-type plants – independent of acetaldehyde application. Thus, there was no obvious reduction in salt stress-inducible gene expression due to the invertase induction.

**Supplemental Figure 5.** Expression analysis of *PR* genes in wild-type and *alc::cwINV* tobacco plants. Transcript levels of *PAR1*, *PR-Q* and *PR-1b* were analyzed in leaves and roots of water- (H₂O) and acetaldehyde-treated (Aa) wild-type and NT *alc::cwINV* plants by RT PCR. Cultivation, inoculation with *G. intraradices* and acetaldehyde-treatment of plants was performed as described for Supplemental Figure 4, but without NaCl treatment. non-myc: plants left without inoculation, myc: plants inoculated with *G. intraradices*. As positive control, leaves of plants with constitutive expression of the chimeric gene encoding apoplast-located yeast invertase with the 35S promoter were used (A41-10; von Schaewen et al., 1990; Herbers et al., 1996). In addition, the leaves and roots of non-mycorrhizal wild-type plants harvested 24 h after soil-drenching with 300 mM NaCl as described in Supplemental Figure 4 were analyzed (NaCl). Total RNA of roots and middle-aged leaves was isolated from pooled samples of three parallel plants. After RT, semi-quantitative PCR was performed with 25 cycles and an annealing temperature of 56°C using the following primers: *PAR1*: forward primer: 5’ – gaa gcg ttg cgt gtt aga g – 3’, reverse primer: 5’ – cac tgg tcg gtt tca atc c – 3’; *PR-Q*: forward primer: 5’ – ttg gca caa ggc att ggt tc – 3’, reverse primer: 5’ – ctt gtt gtc ctg tgt tgc cat c – 3’; *PR-1b*: forward primer: 5’ – gta ggc gtt gaa cca tta ac – 3’, reverse primer: 5’ – gca ctt aac cct agc aca tc – 3’; *Ubiquitin*: forward primer: 5’ – tga ctt gga aga cca tca c – 3’, reverse primer: 5’ – tag aaa cca cca cgg aga c – 3’.

Note the strong accumulation of *PR* transcripts in leaves of plants with constitutive expression of the apoplast-located yeast invertase (A41-10). No increased mRNA levels were found in plants with root-specifically induced yeast invertase. Plants treated with NaCl showed no enhanced accumulation of *PR* gene transcripts 24 h after treatment.

**Supplemental Figure 6.** Invertase activities in non-mycorrhizal and mycorrhizal NT *pyk10::InvInh* plants. A, Cell wall bound invertase activities in roots. B, Vacuolar invertase activities in roots. C, Cytosolic invertase activities in roots. D, Apoplastic invertase activities
in leaves. Wild-type SR1 plants and plants of the two NT pyk10::InvInh lines 98-1-10 and 98-4-1 were inoculated with *G. intraradices* 4 weeks after sowing and harvested 3 and 5 weeks later. Data are presented as mean values + SD (n = 3). The data from the non-mycorrhizal and mycorrhizal transgenic plants were compared to the non-mycorrhizal and mycorrhizal wild-type plants, respectively, using the Student *t* test. *P*<0.05, **P**<0.01.
Table 1. AM-specific plant metabolites in *N. tabacum 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 nm and 380 nm respectively. Aa, acetaldehyde-treated root parts. H2O, water-treated root parts. RT, retention time. Data are presented as mean values ± SD of root parts of 3 plants harvested 6 and 7 weeks after inoculation and as mean ± SD of the ratios of the acetaldehyde-treated root part to the water-treated root part.

<table>
<thead>
<tr>
<th>metabolite [peak area <em>10^-3</em>]</th>
<th>6 weeks</th>
<th>7 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+H2O</td>
<td>+Aa</td>
</tr>
<tr>
<td>cyclohexenone derivatives</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT 19.5</td>
<td>208.8 ± 50.0</td>
<td>226.3 ± 53.4</td>
</tr>
<tr>
<td>RT 22.0</td>
<td>73.3 ± 21.9</td>
<td>68.2 ± 19.5</td>
</tr>
<tr>
<td>RT 27.0</td>
<td>61.9 ± 17.1</td>
<td>53.7 ± 14.2</td>
</tr>
<tr>
<td>RT 27.7</td>
<td>152.5 ± 5.4</td>
<td>143.5 ± 1.3</td>
</tr>
<tr>
<td>RT 28.1</td>
<td>67.3 ± 22.1</td>
<td>63.5 ± 16.2</td>
</tr>
<tr>
<td>RT 30.4</td>
<td>43.9 ± 14.1</td>
<td>41.3 ± 16.3</td>
</tr>
<tr>
<td>mycorradicin derivatives</td>
<td>372.7 ± 104.6</td>
<td>359.7 ± 125.1</td>
</tr>
</tbody>
</table>
A

Phosphate content [μg P_i / g fr.w.]

H_2O  Aa

non-myc  myc  non-myc  myc  non-myc  myc  non-myc  myc

wt  alc::cwINV  wt  alc::cwINV

roots  leaves