SYMBIOSIS (2005) 40, 157–162 ISSN 0334-5114

Glycosidase and glycosyltransferase activity increase in arbuscular mycorrhiza infected legume roots

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(Received July 17, 2005; Accepted December 11, 2005)

Abstract
With four different cultivars of *Glycine max* (soybeans) a 3–10 fold increase in specific activity of alpha-mannosidase activity was observed in roots, inoculated with *Glomus mosseae* compared to the control plants without mycorrhiza development. The increase in α-mannosidase activity after infection with *Glomus intraradices* was slightly less but still significant. No such effect was found for the beta-D-glucosidase activity. With the model legume *Medicago truncatula* cultivar Jemalong A17 a more than 3 fold increase in the mRNA levels of two glycosyltransferases compared to the control plants was detected with a macroarray technique. RNA levels of 16 other enzymes involved in membrane and lipid metabolism, such as choline kinase, glycerol-3-phosphate dehydrogenase, phospholipase D and galactosyltransferase were not significantly increased.

Keywords: Arbuscular mycorrhiza, *Glycine max*, *Medicago truncatula*, glycosidases, glycosyltransferases

1. Introduction

The fungal arbuscule and the plant-derived periarbuscular membrane are the central structures for the function of the AM symbiosis. Compared to the bacteroid and the peribacteroid or symbiosome membrane in the legume-rhizobia symbiosis, the differentiation of the arbuscule and the periarbuscular membrane are much less understood (Werner, 1992; Udvardi and Day, 1997; Whitehead and Day, 1997; Bonfante, 2003; Barea et al., 2005, Gresshoff, 2005).

After the completion of more than 100 genome projects for bacteria and fungi it was found that of all prokaryotes studied the symbiotic bacteria *Braydyrhizobium japonicum*, *Mesorhizobium loti* and *Sinorhizobium meliloti* have the largest genomes, and parasites such as *Borrelia burgdorferi* and *Ricketsia prowazekii* have the smallest genomes (Varma et al., 2004; Werner et al., 2002). This means that the symbiotic interaction apparently needs, or allows due to energetic advantages, a much larger genome than those of nonsymbiotic or parasitic partners.

Key genes involved in the establishment and the regulation of the AM symbiosis and other symbioses have been identified such as the Ljsym4-1 gene (Wegel et al., 1998; Bonfante et al., 2000) and kinase-like receptors (Endre et al., 2002; Stracke et al., 2002; Searle et al., 2003). The dialogue between symbionts and pathogens with their host plants reveals some similarities and many differences (Parniske, 2000; Bonfante, 2001; Werner et al., 2002). Flavonoids apparently are non-essential plant signals in arbuscular mycorrhiza symbiosis (Becard et al., 1995). A hyphae branching factor in the root exudates of *Lotus* was identified as 5-deoxy-strigol (Akiyama et al., 2005). In this symbiosis, a very interesting individual genetic variation has been observed (Pawlowska and Taylor, 2004). Besides the arbuscules and vesicles, also within the hyphae cellular differentiation to a tubular vacuole system has been described (Uetake et al., 2002). In soybeans, specific mutations have been found with nomycorrhizal autoregulation (Meixner et al., 2005).

Most of the genes involved in these structural and functional differentiations remain unknown, mainly because of the absence of a genetic system based on axenic mycorrhizal fungal growth. Thus descriptive, molecular

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techniques based on measuring the abundance of either RNA or proteins were employed. With macroarray techniques, a more than 2.5 fold increase of a trypsin inhibitor RNA in mycorrhizal roots compared to control roots was found (Grunwald et al., 2004). With the same technique, 12 plant genes (in *Medicago truncatula*) and 6 fungal genes (in *Glomus mosseae*) were found to be upregulated in AM roots, with the exact functions still to be determined (Brechenmacher et al, 2004). Due to the comprehensive knowledge of the biochemistry of plant-pathogen interaction, in *Medicago truncatula*, infected with *Glomus intraradices* the induction of a chalcone synthase (Bonanomi et al., 2001a) and of class III chitinase was detected (Bonanomi et al., 2001b).

Glycosidases and glycosyltransferases are essential for membrane biogenesis, best studied in yeast and in mammalian cells. N-glycosylation is involved in such basic cellular processes as secretion, cytoskeleton development and cell division. But very little is known of the involvement of these enzymes in arbuscular mycorrhiza development. In *Allium schoenoprasum* infected with *Glomus intraradices* a two-fold increase in alpha-mannosidase activity compared to control roots has been found (Abrecht et al., 1996), with no increase in beta-glucosidase and arabinosidase activity.

The major aim of the present study was to evaluate, if this effect is also found with other host plant species with different cultivars and also for the combination with two different *Glomus* species, so that it can be more generalized. In addition, we wanted to identify by macroarray techniques, whether glycosyltransferases were also specifically increased in the expression during AM-development.

2. Materials and Methods

Plant cultivars and growth

Seeds of *Glycine max* cultivar Sathiya were received from the Central Department of Botany, Tribhuvan University Kathmandu (Nepal) by Prof. B.N. Prasad, seeds of the cultivars NRC-7, NRC-12 and JS-335 from the Department of Botany, University of Ajmer (India) by Prof. S.K. Mahna, seeds of the cultivar Maple Arrow of *Glycine max* from Agriculture Canada, Ottawa. Seeds of *Medicago truncatula* Jemalong A 17 were received from the Mol-Myc Group in Bielefeld (Germany). Plants were grown as described by Werner et al. (1975) and Vinuesa et al. (2005). The nutrient solution used was modified as following (per l): $MgSO_4.7H_2O$: 246.5 mg; $NH_4H_2PO_4$: 23 mg; $Ca(NO_3)_2.4H_2O$: 944.6 mg; KNO_3: 606.6 mg.

Arbuscular mycorrhiza inoculum

Glomus mosseae spores were received from Biorhize (Dijon, France), spores of *Glomus intraradices* from Premier Tech Biotechnology (Riviere du Loup, Canada). Inoculation of *Glycine max* was done as described previously for *Allium schoenoprasum* (Abrecht et al., 1996).

For *Medicago truncatula* the following steps were modified: The seeds were surface-sterilized by treatment with diluted sodium hypochlorite (10%). After thorough washing with sterile water, the seeds were left to germinate on agar (1.2%) water plates at 26°C in the dark for 2–3 days. The germinated seeds were transferred to pots of 500 ml filled with sterile vermiculite and perlite in the ratio 1:1 with three germinated seeds per pot. After one week only one plant was left in each pot.

The *G. mosseae* fungus was inoculated in the pots with a full tablespoon (approx. 8 ml) of the general inoculum per pot. For the experiments with *G. intraradices*, plants were inoculated with a spore suspension (approx. 900 spores in 1 ml) per pot. In both cases the inoculation was at planting. Plants were cultivated in a greenhouse (20–25°C temperature range). When required, the pots were watered with tap water, and twice a week with the Hoagland nutrient solution.

Staining the roots and estimation of colonization and arbuscular density

At harvest, the roots were washed with tap water and boiled in 10% (wt/vol) KOH for 20 min, washed with tap water again and placed in HCl (0.2 M) for 2 min. Then the HCl was poured off, and the roots were incubated at 90°C for at least 20 min in 0.02% (wt/vol) trypan blue in lactic acid. For the estimation of the mycorrhizal colonization, aliquots of the stained root material were washed with water and the degree of the colonization was determined using the gridline intersection method (Varma et al., 2004). Data are given as percentages of root length containing blue-stained mycorrhizal structures within the root. The number of arbuscules was counted, and referred only to the total of the colonized roots.

RNA isolation

Total RNA was isolated using the "RNeasy Plant Mini Kit". The root tissues used for the RNA isolation were derived from *M. truncatula* plants (20, 30 and 40 days old). Approximately 150 mg of frozen root material were ground to a fine powder with a mortar and pestle and the RNA isolated according to the manufacturer's instruction. The quality and quantity of the RNA extraction was measured with the RNA 6000 Nano Assay from the LabChip Kit.

PCR-labeled probes (^{33}P)

To generate the first strand of cDNA from the RNA samples, the SMART Synthesis Kit (Clontech) was used according to the manufacturer's instructions. The enzyme

SuperScript Reverse Transcriptase (Invitrogen) was used. The labelling with (^{33}P)-dATP (3000 Ci/mmol from Hartmann Analytik) of the first strand was carried out using the Advantage PCR Kit (Clontech) and the primers from the SMART Synthesis Kit (Clontech).

Hybridization of 6k-RIT macroarrays

The hybridization and washing steps of the membranes were carried out in hybridization glass tubes under slow rotation in an hybridization oven. 2 x SSC was added in the prehybridization step and incubated at 65°C for at least 2 hours. The ^{33}P labelled probe was heated at 100°C for 10 min in a water bath and immediately placed on ice for 2 min. The denatured probe was added to the hybridization tube and incubated overnight (at least 18 hours) at 42°C. After the incubation, the membrane was washed twice using 2 x SSC/0.1% (w/v) SDS at 65°C for 5 min the first time and 0.2% x SSC/0.1% (w/v) SDS at 65°C for 15 min the second time. The membrane was sealed with plastic film on a solid support and placed in an exposure cassette with the DNA facing up. This cassette was exposed to a phosphoimager screen (GP, Typhoon imager-Amersham) overnight (at least 8 hours). For recording and storage of images, software by ImageQuant was used. The spot identification and signal quantification was carried out with appropriate software (ImageMaster Array, Amersham).

Glycosidase assays

Alpha-mannosidase and beta-glucosidase activities were tested in photometric assays in root extracts from mycorrhized compared to non-mycorrhized control plants as described by Abrecht et al. (1996).

3. Results

Glycosidases

With a mycorrhization index between 70–90%, the mycorrhized roots of 4 different cultivars of *Glycine max* were assayed for alpha-mannosidase activities after inoculation with *Glomus mosseae* (LMSS) and *Glomus intraradices* (LINR) compared to the uninoculated control plants. The results are summarized in Figs. 1a–d.

After inoculation with LMSS, the specific activity in the cell-free extracts increases over the control plants by a factor of 2.5 to more than 10, depending on the cultivars. The increase after inoculation with LINR is also significant, but in general smaller than with LMSS. The highest specific activity of alpha mannosidase was found in LMSS infected roots of the *Glycine max* cultivar NRC-12 with 700 mU per mg of protein with an average figure for the control plants of around 100 mU per mg protein. This is a much higher specific activity than found previously in *Allium schoenoprasum* roots, colonized with LMSS or LINR, with a specific activity of around 40 mU and an increase over the control plants by a factor of 2. In Table 1 these data are compared with tissues from *Bradyrhizobium* infected roots and nodules of *Glycine max* and tissue cultures of *Glycine max*.

We can see that the here reported activities for arbuscular mycorrhiza colonized roots are by far the highest specific alpha mannosidase activities reported for any soybean tissue. The comparison with the bacterial symbiosis in nodules will be discussed later.

The specific activities of beta-glucosidase were not increased in mycorrhized roots compared to the control plants.

Table 1. Alpha-mannosidase activities from symbiotic and nonsymbiotic tissues of *Glycine max*. Data from tissue cultures and *Bradyrhizobium*-symbiotic organs (nodules) are from Kinnback et al. (1987).

	Specific activity (mU.mg^{-1} protein)
Tissue culture	183
B. japonicum uninfected roots	87
B. japonicum infected roots	161
Nodules (strain 61-A-101 infected)	22
Nodules (strain RH 31 infected	27
Nodules (strain 61-A-24 infected)	11
LMSS infected roots *Glycine max* cv NRC-12	700
LMSS infected roots *Glycine max* cv JS 335	450

Table 2. Induction of genes, encoding enzymes involved in lipid and membrane biosynthesis and modification in roots from *Medicago truncatula* infected with *Glomus mosseae*.

Enzyme	Code EC	Induction
Phosphatidylinositol-phosphatidyl-choline-transfer-protein-IV	2.7.1.67	0.97
Galactosyltransferase	2.4.1.-	0.96
Glycosyltransferase	2.4.1.-	0.72
CTP-phosphoethanolamine-cytidylyltransferase	2.7.7.14	0.53
UDP-glucose-glucosyltransferase	2.4.1.-	0.46
UDP-glucose-glucosyltransferase	2.4.1.-	0.34
Glycosyltransferase	2.4.1.-	3.24
UDP-glucose-glucosyltransferase	2.4.1.-	3.54
Glycerol-3-phosphate-dehydrogenase	1.1.99.5	1.12
Glycerol-3-phosphate-dehydrogenase	1.1.99.5	1.54
Cholinephosphate-cytidylyltransferase	2.7.7.15	1.30
13-beta-glucansynthase-component-I-cell wall	2.4.1.34	1.26
UDP-glucose-glucosyltransferase	2.4.1.-	1.44
Glucosyltransferase	2.4.1.-	0.82
Choline kinase	2.7.1.32	0.80
Long-chain-fatty-acid-COA-ligase		0.95
Omega-6-fatty-acid-desaturase-endoplasmic reticulum		0.80
Phospholipase		0.93

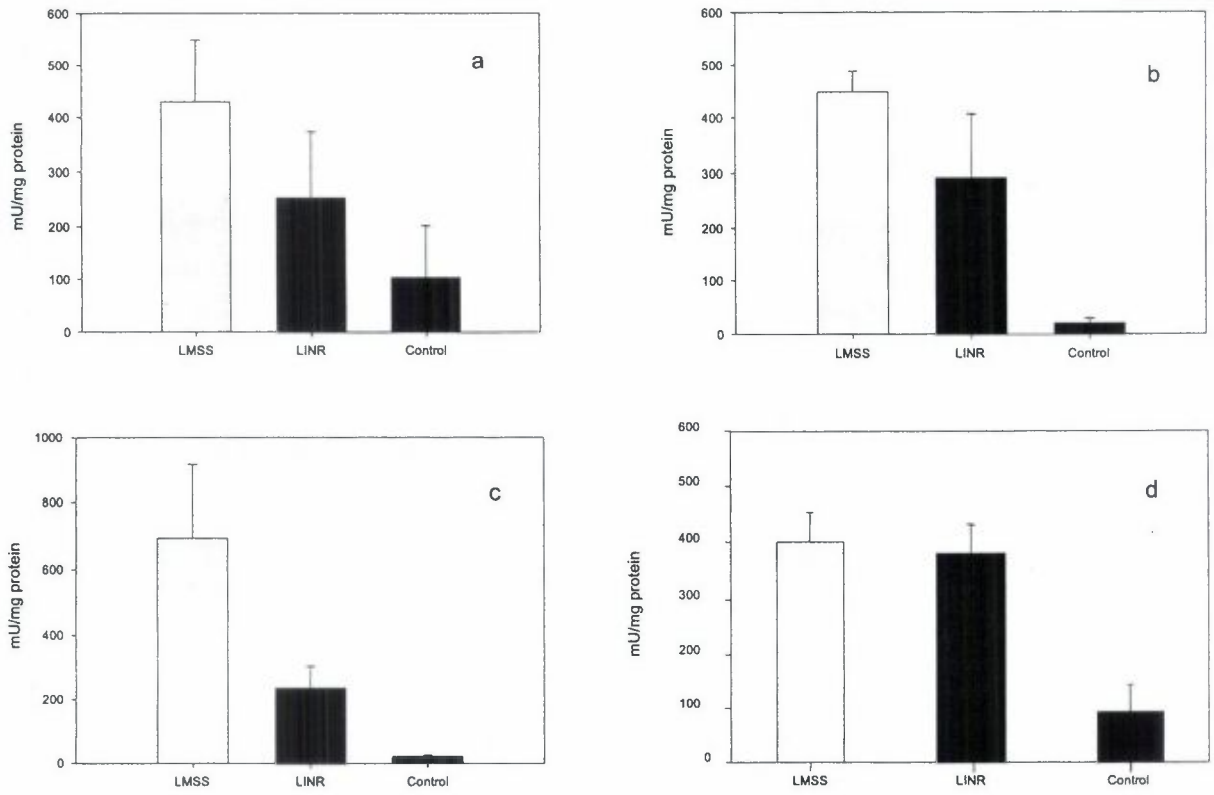

Figure 1. Specific α-mannosidase activity in *Glycine max* (a) cv Maple Arrow, (b) cultivar JS-335, (c) cultivar NRC-12 and (d) cultivar NRC-7.

Glycosyltransferases

With the macroarray technique available for the partly finished genome project of *Medicago truncatula*, we could compare *Glomus mosseae* (LMSS) infected roots with the control roots for a list of candidate genes encoding enzymes, involved in lipid- and membrane biosynthesis and modification. The quantification of these results shows a 3.2 and 3.5 fold induction of a general gylcosyltransferase and an UDP-glucose-glucosyltransferase (Table 2). A value over three is considered as significant in this method (Hohnjec et al., 2005). It is quite remarkable, that two other UDP-glucose-glucosyltransferases are with a rate of 0.34 and 0.46 down-regulated in mycorrhized roots.

Several other enzymes are unaffected, such as a galactosyltransferase, the glycerol-3-phosphate dehydrogenases, a cholinephosphate-cytidyltransferase, a choline kinase and a phospholipase D (Table 2).

4. Discussion

More than 50 per cent of all eukaryotic proteins are glycoproteins, according to the SWISS-PROT database, with an average of 1.9 N-linked glycans per polypeptide chain (Helenius and Aebi, 2004). The core structure of the glycans is a branched oligosaccharide unit, with 3 glucoses, 9 mannoses and 2 N-acetylglucoseamines. The biosynthesis of this unit is located in the ER membrane with several glycosyltransferases involved. Then it is transfered by oligosaccharyltransferases to the growing polypeptide at glycosylation sequons (Asn-X-Ser/Thr). Without proper glycosylation many proteins are not correctly folded and do not reach their native structure. In the degradation of glycoproteins, mannosidases play an important role, since 9 out of 14 sugar residues are mannose. In a mannosidase mutant of yeast the degradation of glycoproteins is drastically reduced (Liu et al., 1999). Different mannosidases are involved in the step by step degradation of the oligosaccharide units (Frenkel et al., 2003; Kitzmüller et al., 2003). Different oligosaccharides with 5 to 9 mannose units from rice gluteolin can be digested by alpha mannosidases (Kishimoto et al., 2001).

The three to more than ten fold encrease in α-mannosidase activity in AM infected roots of *Glycine max* cultivars (Fig. 1) is an indication that the turnover of glycoproteins may be a very important reaction during the establishment of this symbiosis. This was also seen very recently for the gene expression in ectomycorrhizal roots in the system *Paxillus involutus-Betula pendula*, where a 4-

fold increase for alpha mannosidase has been reported (Morel et al., 2005). This means, we have now three independent reports about a large increase of alpha-mannosidase as a general biochemical reaction for mycorrhiza development.

In comparison with different types of soybean tissues, the symbiosis of *Glycine max* with *Bradyrhizobium japonicum* reveals some interesting similarities and major differences (Table 1). Infected roots with infections thread penetrations have about a twofold increase over uninfected roots. At the fully functional stage of the symbiosis, however, nodules with up to 10^{10} to 10^{11} symbiosomes per g of nodule tissue have an extremely low alpha mannosidase activity, indicating at this stage a probably rather low glycoprotein degradation. The even much lower activity in nodules compared to the uninfected roots may be mainly due to the fact that the high protein content of bacteroids is going in the reference parameter (mU per mg protein). If there is more protein in nodules than in roots, the specific activity can drop from 80 to 20 mU (Table 1). The reaction in mycorrhized roots is the opposite. In mycorrhized roots, which may also have a higher protein content than unmycorrhized roots, the specific activity is much higher.

The functions of the increased activities of the glycosyltransferases (Table 2) are less obvious, since they may be involved not only in the biosynthesis of glycoproteins but also in cell wall modifications, during the differentiation of arbuscules and the interfaces to the matrix of the plant cell wall and the periarbuscular membrane. In a comprehensive study with *Medicago sativa* roots transcriptional profiling, Hohnjec et al. (2005) found a 2-fold increase of a β-1,3-glucanase, but only a small increase in a glucosyltransferase. Several key functions of the AM-symbiosis are related to this interface and the specific cell biology of both partners: nutrient transport across the interfaces (Ferrol et al., 2002) tubulin and actin functions (Jun et al., 2002), cytoskeleton and cell wall modifications (Bonfante, 2001), reorganisation of plastids (Fester et al., 2001) and the activation of plant defence genes (Bonanomi et al., 2001; Garcia-Garido and Ocampo, 2002).

Comprehensive reviews on arbuscular mycorrhizal development and cytoskeleton (Raudaskoski et al., 2004), functional genomics approaches (Podilla and Lanfranco, 2004) and functional diversity (Solaiman and Abbott, 2004) have recently been published in Plant Surface Microbiology (Varma et al., 2004).

Acknowledgements

The authors thank the Deutsche Forschungsgemeinschaft for support of project We 200/27-1 in the Schwerpunktprogramm "Mol-Myc" and the EU for support in the project INCO-DEV ICA 4-CT-2001-10057.

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