Expression of Maize and Fungal Nitrate Reductase Genes in Arbuscular Mycorrhiza

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Accepted 9 February 1998.

The role of arbuscular mycorrhizal (AM) fungi in assisting their host plant in nitrate assimilation was studied. With polymerase chain reaction technology, part of the gene coding for the nitrate reductase (NR) apoprotein from either the AM fungus *Glomus intraradices* or from maize was specifically amplified and subsequently cloned and sequenced. Northern (RNA) blot analysis with these probes indicated that the mRNA level of the maize gene was lower in roots and shoots of mycorrhizal plants than in noncolonized controls, whereas the fungal gene was transcribed in roots of AM plants. The specific NR activity of leaves was significantly lower in AM-colonized maize than in the controls. Nitrite formation catalyzed by NR was mainly NADPH-dependent in roots of AM-colonized plants but not in those of the controls, which is consistent with the fact that NRs of fungi preferentially utilize NADPH as reductant. The fungal NR mRNA was detected in arbuscules but not in vesicles by in situ RNA hybridization experiments. This appears to be the first demonstration of differential formation of transcripts of a gene coding for the same function in both symbiotic partners.

The arbuscular mycorrhiza (AM) is probably the most common plant-microbe symbiosis. The vast majority of terrestrial plants are potential hosts for the highly specialized group of these obligate biotrophic fungi (Hayman 1982; Harley and Harley 1987), enabling the formation of a partnership of mutual benefit. Since colonization by AM fungi can increase growth rates, productivity, and stress tolerance, and allows plants to occupy broader ranges of habitats (Peat and Fitter 1993), the significance of the AM in agriculture attracts growing attention.

During colonization of the roots, many AM fungi produce vesicles that contain large amounts of lipids and that might serve as storage organs (Bonfante-Fasolo 1984). The arbuscules are even more characteristic structures of all AM fungi. These highly branched, treelike, hyphal formations play an important part in nutrient exchange between the fungus and the plant (Cox and Tinker 1976; Bonfante-Fasolo 1984). While the plant supplies the fungus with glucose and other

carbohydrates (Smith and Smith 1990; Shachar-Hill et al. 1995), the fungus exploits water and minerals from soils more efficiently, and effectively provides these to the plants, particularly under nutrient-limiting conditions (Hayman 1982; Kothari et al. 1990). Knowledge about the molecular basis and regulation of this mutual relationship is currently limited. Up to now, studies on mineral nutrition in AM have mostly concentrated on phosphate, which is taken up from the soil by the dense network of external fungal hyphae and transferred to the plant (Harley and Smith 1983; Jakobsen et al. 1992; Smith and Gianinazzi-Pearson 1988). A fungal phosphate transporter that is probably involved in the initial phosphate uptake from the soil into the external hyphae has recently been described (Harrison and van Buuren 1995).

The fungal assistance in nitrogen nutrition is as yet poorly defined in this symbiosis. Experiments with ¹⁵N-labeled ammonium indicate that the fungal hyphae take up NH₄⁺ and transport it to the plant (Ames et al. 1983; Barea et al. 1987; Johansen et al. 1992). In most soils, however, nitrate is the prevailing nitrogen form. In contrast to ammonium, nitrate is highly mobile in soils and may be rapidly replenished from the soil reservoir when taken up by the roots. Therefore, AM fungi were thought not to significantly contribute to nitrate assimilation of plants (Ames et al. 1983; Barea et al. 1987). This view has changed recently. Experiments with ¹⁵N-labeled nitrate indicated that this anion is taken up by *Glomus mosseae* hyphae (George et al. 1992) and is transferred from the fungus to the plant under water stress conditions (Tobar et al. 1994). Earlier investigators (Carling et al. 1978; Oliver et al. 1983) demonstrated increased total activity of nitrate reductase (NR) in both roots and shoots of AM plants and concluded that this enhancement is brought about by a relief of phosphate stress. Nitrate might be the preferential nitrogen source for AM fungi (Azcón et al. 1996).

In the present investigation, expression of NR genes from both *Glomus intraradices* and maize has been studied. Recently, by a polymerase chain reaction (PCR)-based approach, part of the gene coding for the NR apoprotein has been cloned from several *Glomus* isolates including *Glomus intraradices* (Kaldorf et al. 1994; Kaldorf 1996). A segment of the corresponding maize NR gene has also now been obtained by PCR. Sequencing revealed that the amplified products from maize and *G. intraradices* were sufficiently different to serve as probes for investigating the expression of NR genes from both symbiotic partners and for localizing their respective transcripts.

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RESULTS

Specificity of the amplified DNA segments of the genes coding for the NR apoprotein from either maize or the AM fungus.

The two primers FU 3 and FU 4 allowed specific amplification by PCR of an approximately 1-kb-long segment (Fig. 1) of a gene coding for the molybdenum-binding part of NR from DNA of *G. intraradices* spores but not from that of maize (Kaldorf et al. 1994; Kaldorf 1996). Likewise, the pair of primers PL 1 and PL 2, also derived from the molybdenum-binding domain, but this time taken from the rice gene sequence (Choi et al. 1989), have now been employed to amplify a 552-bp PCR segment from maize by PCR under highly stringent conditions (Fig. 1). The deduced amino acid sequence of this segment showed 92% identity to the NADH-dependent NR from rice (Choi et al. 1989) and 81% to this protein from barley (Schnorr et al. 1991) but only 76% to the NAD(P)H-dependent isoenzyme from the latter (Miyazaki et al. 1991), suggesting that the PCR product originated from the gene of the maize NADH-dependent NR.

The DNA sequence homology between the two PCR products from *G. intraradices* and maize was only 45% within the overlapping region (Fig. 2). The amino acid sequence deduced for the *G. intraradices* NR showed a similar sequence identity of 43% to the NADH-specific NR from rice (Choi et al. 1989) and of 47% to the NAD(P)H-dependent NR from barley (Miyazaki et al. 1991). Southern hybridizations between genomic maize DNA and the PCR product from *G. intraradices* gave no hybridization signals at higher stringencies (68 or 66°C) due to the low similarity between maize and *G. intraradices* NR genes (data not shown). Thus, the fungal probe did not hybridize with any of the genes coding for the maize NR isoenzymes. The fungal probe, however, strongly hybridized with *G. intraradices* DNA from spores, whereas the maize NR probe did not hybridize with fungal DNA (not documented). Weak cross-hybridization between *G. intraradices* and maize NR genes was detectable only in hybridization experiments between the two cloned NR gene segments (Fig. 3). The signal obtained in homologous hybridizations with 2 ng of either the *G. intraradices* (Fig. 3A, lane 1) or the maize (Fig. 3B, lane 1) PCR product and with the digoxigenin (DIG)-labeled probe from each of the organisms served as standard for comparing the signal intensity in the assays with increasing amounts of the heterologous PCR products (Fig. 3). In hybridizations

with the *G. intraradices* probe (Fig. 3A), 400 ng of the maize segment gave approximately the same signal intensity as the standard with the homologous DNA. Similarly, 800 ng of the *G. intraradices* segment showed a slightly weaker signal than 2 ng of the maize PCR segment in hybridizations with the maize PCR probe (Fig. 3B). Thus, the extent of the cross-hybridization between the maize and *G. intraradices* segments was below 1%. Both segments contain putative introns at different locations (*G. intraradices* 53 bp, maize 81 bp), explaining the low signal intensity in heterologous Southern hybridization. In Northern (RNA) experiments, the cross-hybridization was slightly stronger than in Southern but still low enough to monitor the differential gene expressions as follows.

Detection of maize and *G. intraradices* NR mRNA in AM-colonized maize.

For Northern blot analysis of the NR transcripts, maize plants were grown under phosphate-limiting conditions to ensure a 70 to 95% colonization of the root segments counted as described by Schmitz et al. 1991. Total RNA extracted from roots and leaves of AM-colonized and control plants was hybridized with the DIG-labeled antisense RNA transcripts of the NR segment from either maize or *G. intraradices* (Fig. 4). Northern experiments were performed at least three times, with RNA of different plant batches, with identical results. An equal amount of RNA from colonized and control maize was loaded in each comparison (controls in Figure 4). Since the fungal partner within an AM-colonized root contributes 4 to 20% of the total root mass (Hepper 1977), the large differences detected in plant NR transcript levels cannot be due to a dilution of plant RNA by fungal RNA.

In total RNA from maize leaves, a band of about 3 kb became visible with the maize-specific probe (Fig. 4C, lanes 3 and 4). This size was expected for mature mRNA of NR (Vaucheret et al. 1989). The weaker upper band seen on the blots (Fig. 4C) possibly represents premature NR mRNA transcripts. This band was seen even at the highest stringency employed (68°C). When the hybridization temperature was increased further, the 3-kb band also disappeared. The level of plant NR mRNA transcripts was lower in leaves of AM-colonized maize than in the controls (Fig. 4C).

Even in roots, hybridization signals were stronger with RNA from control plants (Fig. 4B, lane 3) than with RNA from AM-colonized material (Fig. 4B, lane 4). When the anti-

Fig. 1. Partial genomic organization of eukaryotic nitrate reductase (NR) apoprotein and position of polymerase chain reaction primers used for the amplification of segments from the maize and *Glomus intraradices* NR genes. Data for NR from *Aspergillus nidulans* are taken from Johnstone et al. (1990) and for NR from *Lycopersicon esculentum* from Daniel-Vedele et al. (1989).

sense RNA transcript of the DNA segment from *G. intraradices* was used as probe in Northern hybridization experiments with total RNA from roots, a band of about 3 kb appeared (Fig. 4A, lane 4). This size was to be expected since NR transcripts in plants and other fungi (Okamoto et al. 1991) have the same length. The weak signal at a similar position in the control blot with RNA from nonmycorrhizal roots (Fig. 4A, lane 3) can be ascribed to cross-hybridization of the *G. in-*

traradices probe with the maize NR mRNA. Such a weak signal at a similar position was also seen in hybridizations with RNA from control maize leaves and the *G. intraradices* probe, probably also due to cross-hybridization (not shown).

NR activities in mycorrhizal and nonmycorrhizal maize.

Enzyme activity measurements (Table 1) corroborated the findings of Northern analysis. Crude extracts from leaves of

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Glomus  GCAGGCAACAGACGGAAAGAGCAGAACATGGTCCGAAAATCACAAGGGTTCTCTTGGGG
Maize                                **T*****G*AGC*GA*GGTG*****AAC*****

TCCTGCTGGTCTCTCAACATCCCTCTGGACAGGACCTATGCTTTGCGACATTCTCGCA---TTAGCA
CG*C**G**CG*G**C**G***G*G***CGC**CG*CCGC**CC*****G*GC**CGCCGC*GC*GC

AAGCCAGACCGGCGGGCGGGGCTCGATATGTGTGTTTCGAGGGTGGAGATACTCTTCCCACAGGAG
*TCG*GCC***CAA*G***CC**C*TCA*C*****C*****C**CC**GGAC**C***GGG**T*

CT-----TATGGGACAAGCTTGAAGCTGAGCTGGTGCATGGATCCTATTCGGGGGATCATGCT
GCGGATCCAAG**C**C**C**G*C*C**GCGAG**GC*C*****GTGC*****AC*****

CGCCTACAAGCAGAACGGCGAAATGTTGACACCAGATCATGGAAGACCAATTAGAGTTGTGGTGCCG
*****T*****GCC*C**CTG**G**C**C**CTTC**CG*GC*C**CA*CA*C**C

GGAATGATTGGGGGGCGAAGTGTGAAGTGGTTGAAGAAAATTATCATCTCGGACCAGCCGAGCACGA
**CTGC**C**T**C**C**TG*CC*****C**C**CGC**CG**G**A*CCC*GCCGA*TC*GAC*

ATTATTACCACTACTACGACAATAAAGTCATGCCGACACATGTCACACCGCAGATTGCGAGTAGTGA
***C*****T*T*A*G*****CCGC***C*****T*C**C**GACG*C---GAGCTCGCC*AC*C

GGAGGGAAAGAAGT-----
A**A*-----gtaattagcagttcatgtttaatttattactactgaagcaaaaaacattgatc
-----TCTGGATGGATGAGAAGTGGTGTATCTTTGACTTGAAT
tgacttttgctattgcaaattgattgagcagCG**TG*T**A*ACCAGA**AC***A*CA*

GTCACCGCGCCATTGCGTATCCGGCACATGATGAGGTCTTGGAATCGCCCCAAAGCAGAGGAGAT

GGAGGTGGAAGAATATGTACACATTGCGGGCTACGCATATGGGGGTGGCGGAAGGAGGATTGAGAGA

GTGGAATCTCCCTCGACAAAGGAAAgtagcatagcctccacaattatttcatgaatatcatctaata
cttgtttgctacAGCATGGCGCCCTGCCTCCATCACCAACTACGAAGACAAATACCGCTCAAGAACA

TCCCCGAAAACACCTACCTCTTCGGGGGCAAACTCGACGTCGGCTGGCGTGATACCTGTTTCTGCTG

GTGTTTCTGGGACTTATCCGTCCCTCACTCCGACCTTGCCCTCATCAAACGTTGAAGACATTGTCGTT

CGTGCAATGGATGAAGCCCTCAATACCCAGCCAAGGGACATGTACTGGTCTTTACTCGGGATGATGA

ACAACCCTTGGTTCCGGGT

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Fig. 2. Nucleotide sequences of the polymerase chain reaction (PCR) products from the nitrate reductase genes of *Glomus intraradices* and maize. Sequences of PCR primers are in bold. Identical nucleotides are indicated by stars. Dashed lines mark putative introns (bases written in small letters).

AM-colonized maize plants reduced nitrate to nitrite (with NADH as the electron donor) with a rate of $1.4 \mu\text{mol per h} \times \text{g}$ of fresh weight, whereas this activity was $2.3 \mu\text{mol}$ in leaves of control plants. With NADPH as the electron donor, both

activities reached only 20 to 25% of the corresponding values with NADH. In roots of nonmycorrhizal plants, specific activity levels were essentially the same with NADH and NADPH. In contrast, NR activity was more than twofold higher with NADPH than with NADH in AM-colonized roots (Table 1).

Histological localization of the NR mRNAs from maize and *G. intraradices*.

In situ RNA hybridizations of root sections were performed to disclose any cell-type specificity in the reduction of maize NR. In the cortex, the differences in the silver grain deposition obtained with the maize antisense and sense probes were clear-cut in nonmycorrhizal roots, and only small in mycorrhizal samples (Table 2). Comparing the results with the antisense probe in mycorrhizal and nonmycorrhizal roots, signals were about threefold stronger in nonmycorrhizal samples (Table 2), which correlates well with the results of the Northern hybridization experiments. The pattern of the silver grain distribution was not cell-type specific (Fig. 5), which is in general agreement with immunohistochemical data from others (Fedorova et al. 1994). Due to the long exposure, the radiation of the specifically bound antisense RNA probe resulted in an increased background signal on the glass surface in the vicinity of the root sections. The background was the same with sense and antisense probes at a distance of more than $100 \mu\text{m}$ from the sections (not documented). The differences in the labeling in AM-colonized and control plants cannot be attributed to different plant growth stages, since the flowering date was the same and since the fresh weight of colonized plants was only about 20% higher than that of the controls.

In leaf sections of 70-day-old maize, the silver grain density visually seemed to be higher in control plants than in the leaf tissue of mycorrhizal maize. This would corroborate the find-

Fig. 3. Cross-hybridization between the polymerase chain reaction (PCR) segments from *Glomus intraradices* and maize nitrate reductase genes. **A**, 2 ng of the *G. intraradices* PCR product (lane 1); 10 ng (lane 2), 100 ng (lane 3), 200 ng (lane 4), 400 ng (lane 5), and 800 ng (6) of the maize PCR product hybridized with approximately 500 ng of the digoxigenin-labeled PCR product from *G. intraradices* at 68°C . **B**, 2 ng of the maize PCR product (lane 1) and 10 ng (lane 2); 100 ng (lane 3), 200 ng (lane 4), 400 ng (lane 5), and 800 ng (lane 6) of the *G. intraradices* PCR product hybridized with approximately 500 ng of the PCR product from maize at 68°C .

Fig. 4. Northern (RNA) blot analysis of the nitrate reductase (NR) mRNA from either maize or *Glomus intraradices* in arbuscular mycorrhiza (AM)-colonized and in noncolonized plants. RNA was extracted from 70-day-old maize plants and $20 \mu\text{g}$ was loaded onto each lane. Lanes 1 and 2: amount of RNA used in the experiments; lanes 3 and 4: bands from the RNA-RNA hybridizations. Lanes 1 and 3: noncolonized maize; lanes 2 and 4: AM-colonized samples (70 to 95% colonization in the roots). Arrows indicate hybridization signals at 3.1 kb. **A**, RNA from maize roots hybridized with the 1.0-kb RNA probe of NR from *G. intraradices*. **B**, RNA from maize roots hybridized with the 0.55-kb RNA probe of NR from maize. **C**, RNA from maize leaves hybridized with the 0.55-kb RNA probe of NR from maize.

ings from the Northern analysis and the activity measurements. However, the pictures did not permit a quantification of the data, which are, therefore, not shown.

For localizing the *G. intraradices* NR mRNA transcripts in fungal structures growing within the cortex of maize roots, special care had to be taken to optimally preserve the tissue morphology. Root pieces were embedded in an acetone-soluble plastic resin and semithin, longitudinal sections were prepared. Despite the treatment with the rather harsh solutions of the in situ hybridization procedure, the fine hyphal network of fungal arbuscules was still discernible inside the cortical cells of the roots in such sections (Fig. 6A, B). In situ hybridizations with antisense or sense transcripts of the *G. intraradices* NR DNA segment indicated an accumulation of silver grains over the surface of the arbuscules that was visually stronger with the antisense than with the sense probe (Fig. 6C, D). A high density of silver grains was also seen over fungal vesicles, but with apparently no difference between the antisense and sense probes in this case (Fig. 6E, F). To verify these visual differences statistically, silver grains were counted that were covering the surface of vesicles and of cortical cells of about the same size depicting either arbuscules or being empty (Table 3). On the average, the number of silver grains was 2.7 ± 0.5 fold higher in cells with arbuscules than in empty ones in the in situ hybridizations with the antisense probe. The intensity of the labeling was significantly lower with the sense control. The ratio in the labeling between cells with and without arbuscules amounted to 1.5 ± 0.4 (Table 3) and was, thus, virtually the same. The fungal vesicles bound the antisense as well as the sense transcripts for unknown reasons, but to a similar extent and therefore unspecifically. The silver grains per vesicle were 125 ± 43 with the antisense probe and 100 ± 48 with the sense probe. In conclusion, the data revealed the fungal arbuscules but not the vesicles to be preferential sites for the presence of NR transcripts.

DISCUSSION

The use of maize with a high degree of mycorrhizal colonization was a prerequisite to study gene expression of NR. In non-nutrient-limited soils, particularly in the field (Peat and Fitter 1993), plants are at best poorly colonized. Therefore, plants had to be grown under stress, which was achieved by phosphate-limiting conditions in the present study. Remarkably, although the nutrient solution used contained abundant nitrate, the mRNA levels of maize NR are significantly reduced both in roots and leaves of mycorrhizal plants, compared with the situation in noncolonized controls. This is correlated with a reduced specific activity of the enzyme in

leaves of the mycorrhizal maize. In roots of maize, both NADH- and NAD(P)H-utilizing, bispecific isoenzymes occur (Redinbaugh and Campbell 1981). The enzyme in ascomycetes (*Neurospora* sp.) preferentially utilizes NADPH (Crawford and Arst 1993). In line with this, NADPH-dependent nitrite formation was higher in AM-colonized roots than in the controls, and the hybridization experiments with the *G. intraradices* NR probe show that the fungal-specific NR is transcribed in colonized maize. The different findings presented here relate well to one another, indicating that the fungus might take over nitrate reduction at least to a major part in mycorrhizal roots.

Investigations on the same subject are scarce in the literature. The specific NR activity in shoots of *Trifolium subterraneum* was reported also to be higher in controls than in AM-colonized plants (Oliver et al. 1983) although total NR activity per plant was higher in AM plants in that study. At first glance, conflicting results have been published by Cliquet and Stewart (1993). In their experiments with maize where nutrient supply was optimal, NR activity was twofold higher, referred to per g of fresh weight, and about the same per mg of protein in AM-colonized maize as in control material. Such plants were, however, apparently poorly colonized, as indicated by the determination of the chitin content of the AM-colonized roots in that study (Cliquet and Stewart 1993). Further experiments have to show whether stress conditions other than phosphate limitation also cause an decrease of plant NR transcripts in maize in parallel with a high degree of mycorrhizal colonization.

In regard to the localization of NR transcripts within *G. intraradices*, the silver grain deposition reveals the arbuscules, but not the vesicles, to be major sites of NR transcripts. Arbuscules are formed by extensive membrane folding by the fungal partner, thus the fungal cytoplasm per square unit is concentrated there sufficiently for detecting fungal RNA by in situ hybridization. The sections used for the in situ hybridizations showed only short segments of intraradical hyphae that did not allow quantifying of any silver grain depositions here. External hyphae had not been preserved during the preparation of the material used. The intensive labeling at the arbuscules is a demonstration of the occurrence of NR transcripts there, but does not exclude their presence in fungal hyphae. Although a high level of NR transcripts must not always coincide with a high activity, the extensive labeling of the arbuscules by in situ hybridizations is consistent with the general view that arbuscules in particular are metabolically active in

Table 1. Influence of arbuscular mycorrhiza colonization on the specific activity level of maize nitrate reductase

Source of plant material	Specific activity of NR in $\mu\text{mol per g of fresh weight} \times \text{h}$	
	NADH-dependent ^a	NADPH-dependent ^a
Leaves, control plants	2.34 ± 0.37	0.48 ± 0.06
Leaves, mycorrhizal plants	1.40 ± 0.26	0.37 ± 0.06
Roots, control plants	0.13 ± 0.03	0.12 ± 0.02
Roots, mycorrhizal plants	0.09 ± 0.03	0.21 ± 0.05

^a Data represent average values of six independent measurements.

Table 2. Quantification of the signals of in situ hybridization in root cortical cells with the maize probe

Exp. no.	Antisense RNA probe		Sense RNA probe	
	Root			
	Mycorrhizal	Nonmycorrhizal	Mycorrhizal	Nonmycorrhizal
1	14.8 ± 4.4	39.5 ± 12.7	11.7 ± 4.4	12.0 ± 5.2
2	13.2 ± 4.1	47.9 ± 20.5	9.7 ± 1.5	11.3 ± 3.3
3	26.4 ± 8.2	49.5 ± 11.6	17.9 ± 4.0	16.8 ± 2.9

^a Number of silver grains was counted for maize cortical cells of equal size. Each value represents mean of 10 cells from one slide. Differences between signals in mycorrhizal roots obtained with antisense probe and sense control are statistically significant (Student's *t* test, *P* < 0.05).

the nutrient transfer to and from the plant (Cox and Tinker 1976; Bonfante-Fasolo 1984) and in respiration (Smith and Gianinazzi-Pearson 1988; Smith and Smith 1990).

The present investigation suggests that the fungus in AM-colonized roots performs nitrate uptake (probably by specific carriers) and nitrate reduction at least to a large part. Due to its toxicity, the nitrite formed is probably not exported from the fungal to the plant cells. Other enzymes of nitrate assimilation have been described to occur in AM

fungi (Smith et al. 1985). Thus, nitrite reductase, glutamine synthetase, glutamate synthase (GOGAT), and/or transaminase(s) may also be differentially expressed in AM-maize. A similar study with fungal- and plant-specific gene probes of these enzymes may indicate which *N* compound (ammonia, glutamine, glutamate, and/or others) is transferred from the arbuscules to the maize cells. On the other hand, a specific, carrier-dependent transfer of *N* compounds from arbuscules to the plant must not necessarily exist. Arbuscules are con-

Fig. 5. Distribution of the maize nitrate reductase (NR) transcripts in root tissue. Fixed and paraffin-embedded pieces of roots from arbuscular mycorrhiza (AM)-colonized (A, C) and control plants (B, D) were cross-sectioned and hybridized in situ with radioactive-labeled antisense (A, B) or sense (C, D) in vitro transcripts of the maize NR DNA segment. Bars represent 140 μm.

Fig. 6. Localization of the nitrate reductase (NR) mRNA of *Glomus intraradices* in arbuscular mycorrhiza (AM)-colonized maize roots by in situ hybridizations. Pieces of AM-colonized roots were fixed and embedded in acetone-soluble methacrylate resin. Semithin longitudinal sections were prepared and, after removal of the resin with acetone, hybridized in situ with ^{35}S -labeled antisense (**C**, **E**) or sense (**D**, **F**) in vitro transcripts of the *G. intraradices* NR DNA segment. Morphology of arbuscules (**A**, **B**) was visualized by epifluorescence microscopy (excitation filter, 450 to 490 nm; dichroic mirror, 510 nm; barrier filter, 520 nm). **A–D**, arbuscules. **E–F**, vesicles. Bars represent 30 μm .

tinuously synthesized and degraded, and the degradation products may be absorbed by the plants.

In the past, some genes, from either the fungal or the plant side, have been described that are differentially expressed during mycorrhizal colonization (Golotte et al. 1993; Franken and Gnädinger 1994; Harrison and van Buuren 1995; Lambais and Mehdy 1995; Gianinazzi-Pearson et al. 1996). In the case of NR, the enzyme occurs in maize (Redinbaugh and Campbell 1981) and in the AM fungus, as demonstrated in spores (Ho and Trappe 1975; Sundaresan et al. 1988). PCR probes that specifically recognized either maize or *G. intraradices* NR transcripts were developed for the present study. The amino acid sequence identity between the NR PCR products from *G. intraradices* and the other fungus, *Aspergillus nidulans*, was 65% and thus high (Kaldorf et al. 1994), but only 51% between those from *G. intraradices* and maize, which explains the specificity of the NR probes used. In addition, each of the two PCR products contained one intron of different size and composition (Fig. 2). This also shows that the segment from *G. intraradices* was amplified from the DNA of the fungus and not of the bacteria abundantly occurring in spores (Bianciotto et al. 1996). The demonstration of differential distribution of NR mRNA appears to be the first example where one and the same enzyme is shut down in the plant and is present in the microsymbiont during symbiosis. The factors that cause these differences in the transcript levels of a gene coding for one and the same enzyme function in the two partners are unknown.

MATERIALS AND METHODS

Mycorrhizal colonization of plants.

Seeds of *Zea mays* L. 'Honeycomb' F₁ hybrid (Altörfer Samen, Zürich, Switzerland) and the AM isolate *Glomus intraradices* Schenck & Smith INVAM Sy167 (kindly supplied by S. Reinhard, Universität Hohenheim, Hohenheim, Germany) were used for the experiments. Fungal inoculum was produced with *Tagetes patula* L. 'Bonita Del Sol' (Samen Walz, Stuttgart, Germany) as host plant (Dehne and Backhaus 1986). Maize was inoculated and grown in the greenhouse under phosphate-limiting conditions. To achieve this, plants were watered daily, and Hoagland's nutrient solution (with 10 mM K-nitrate, but without phosphate) was supplied once a

week. In addition, the nutrient solution was supplemented with 1 mM K₂HPO₄ once a month, which ensured growth under phosphate-limiting conditions (for further details see Schmitz et al. 1991; Esch et al. 1994). The degree of mycorrhizal colonization was microscopically determined after staining the roots with lactophenol blue for visualizing the fungal structures (Schmitz et al. 1991). The AM-colonized and control plant material used for activity measurements, RNA extraction, and in situ hybridization was harvested after 70 days, thus immediately before the onset of flowering.

Determination of NR activity.

For assaying NR in crude extracts, shoots or roots of maize were frozen and ground in liquid nitrogen. Two hundred to 400 mg of the powder was extracted in 1 ml of buffer containing 100 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-KOH pH 7.5, 10% glycerol, 1 mM EDTA, 5 mM Mg-acetate, 0.1% Triton X100 (vol/vol), 0.5% bovine serum albumin (wt/vol), 5 µM Na-molybdate, 1% polyvinyl pyrrolidone (vol/vol), 5 mM dithiothreitol, 25 µM leupeptin, 0.5 mM phenyl methyl sulfonyl fluoride (PMSF) and 20 µM flavine adenine dinucleotide (FAD; modified from Kaiser and Huber 1994; Lohaus et al., *in press*). After centrifugation (30 s, 18,300 × g, 4°C), NR activity was determined by adding 50 µl of crude extract to 300 µl of reaction mixture (100 mM HEPES-KOH pH 7.5, 6 mM KNO₃, 6 mM EDTA, 0.5 mM NADH or NADPH). The samples were incubated (30 min, 30°C), and nitrite in the supernatant was then determined colorimetrically at 540 nm with sulfanilic acid/α-naphthylamine (Snell and Snell 1949).

Protocol for the PCR.

Maize DNA was isolated from 3-day-old maize seedlings as described by Raeder and Broda (1985). For amplifying a segment of maize NADH-dependent NR, oligonucleotides (PL 1: TTG ATG ATG TAC TCT GGC(T) TTA(G) TAC CAC CA and PL 2: AAC(T) ATG GTG CAG CAG ACG GTG GGA(CGT) TTC AA) were synthesized with a Pharmacia LKB Gene Assembler Plus (Pharmacia LKB, Uppsala, Sweden) and purified with 1.5-ml NAP-10 columns (Pharmacia LKB). The PCR assays contained the following in a final volume of 50 µl: 2 U of *Taq* DNA polymerase (Promega, Heidelberg, Germany), 5 µl of 10× *Taq* DNA polymerase buffer (Promega); 4 µl of 25 mM MgCl₂; 10 nmol of each deoxynucleotide, 50 pmol of each oligonucleotide primer, 30 ng of maize DNA. The reaction was performed in 34 cycles employing 30 s of denaturation at 92°C, 30 s of annealing at 54°C, and 60 s of polymerization at 72°C.

PCR products were cloned into pCR II (Invitrogen, San Diego, CA). *Xba*I/*Hind*III fragments containing the complete PCR segment from *Zea mays* were subcloned into M13mp18 and M13mp19. The M13 clones were sequenced by the dideoxy chain termination technique with the TAQuence kit (US Biochem. Corp., Cleveland, OH).

The protocol for the amplification of segments of *G. intraradices* NR has already been described in preceding publications (Kaldorf et al. 1994; Kaldorf 1996). With the codons of the *Aspergillus nidulans* gene (Johnstone et al. 1990), the pair of primers FU 3: ACC CGG AAC CAA GGG TTG TTC ATC ATA(CGT) CC and FU 4: GCA GGC AAC AGA CGG AAA GAA(CGT) CAA(G) AA allowed amplification of an

Table 3. Quantification of the signals of in situ hybridization with the *Glomus intraradices* probe^a

Exp. no.	Antisense RNA probe			Sense RNA probe		
	Silver grains in cells			Silver grains in cells		
	With arbuscules	Without arbuscules	Ratio	With arbuscules	Without arbuscules	Ratio
1	37	20	1.9	82	93	0.9
2	111	36	3.1	54	33	1.6
3	141	48	2.9	42	25	1.7
4	120	43	2.8	68	42	1.6
5	148	49	3.0			
Avg.			2.7 ± 0.5			1.5 ± 0.4

^a Number of silver grains was counted for maize cortical cells with and without fungal arbuscules. Each value represents mean of 15 cells from one slide. Difference between signals obtained with antisense probe and sense control at the arbuscules is statistically highly significant (Student's *t* test, *P* < 0.01)

approximately 1-kb segment specifically from DNA of *G. intraradices*.

Filter blot hybridization.

For the Southern hybridizations, the PCR segments of NR were separated by agarose gel electrophoresis and blotted on Nylon membranes (Qiagen, Hilden, Germany). The NR-DNA segments were labeled with DIG-11-dUTP by PCR. The reaction (5 min of denaturation at 95°C, 36 cycles with 30 s at 92°C, 30 s at 52°C and 60 s at 72°C) contained 1 to 10 ng of DNA template, the oligonucleotide primers (concentrations as above), and a mixture of dATP, dCTP, dGTP (2.5 nmol each), dTTP (1.63 nmol), and DIG-11-dUTP (0.88 nmol). The filters were hybridized with 100 ng to 1 µg of labeled probe in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.5% (wt/vol) blocking reagent (Boehringer, Mannheim, Germany), 0.1% *N*-lauroylsarcosine, and 0.02% sodium dodecyl sulfate (SDS) at 68°C overnight, and washed with 2× SSC, 0.1% SDS (2 × 5 min, 68°C). The DIG-labeled, hybridized DNA was detected immunologically by alkaline phosphatase conjugated antibodies with nitroblue tetrazolium as substrate (manufacturer's protocol; Boehringer).

For Northern blot hybridization, total RNA was isolated from maize tissue by guanidinium thiocyanate/phenol/chloroform extraction (Chomczynski and Sacchi 1987). Plant and fungal material was homogenized in an extraction mixture containing 50% H₂O saturated phenol, 49.5% of 4.23 M guanidinium thiocyanate, 18.4 mM *N*-lauroylsarcosine, 0.7% β-mercaptoethanol, and 25 mM Na-acetate, pH 7.0. The solution was supplemented with a half volume of chloroform/isoamyl-alcohol (24/1, vol/vol), incubated on ice for 30 min and centrifuged (15 min, 18,300 × *g*). The supernatant was extracted with an equal volume of phenol/chloroform/isoamylalcohol (25/24/1, vol/vol/vol) and then with chloroform. RNA was precipitated from aqueous phase with isopropanol (20 min, -20°C), centrifuged (15 min, 18,300 × *g*), washed with 70% ethanol/3 M Na-acetate pH 5.2 and then with 70% ethanol solely. The pellet was dried under vacuum and dissolved with 20 µl of diethyl pyrocarbonate (DEPC)-treated water (Sambrook et al. 1989). The final yield per g of plant fresh weight was 100 to 400 ng of RNA with an OD 260/280 nm of about 1.8. RNA was separated on denaturing agarose gels and transferred onto Hybond-N membranes (Amersham, Braunschweig, Germany). Hybridization of the filters with DIG-labeled RNA probes and chemiluminescence detection followed the protocol of the manufacturer (Boehringer).

DIG- or ³⁵S-labeled (for in situ hybridization) sense and antisense RNA probes were generated by in vitro transcription. The vector pCR II containing the cloned PCR products of the maize or the *G. intraradices* NR genes was linearized and used as template for in vitro transcription with Sp6 or T7 RNA polymerase (Melton et al. 1984). [³⁵S]-UTP was purchased from Amersham, and DIG-UTP from Boehringer.

In situ hybridization.

Fixation of maize root and leaf tissue with 1% glutaraldehyde, embedding in paraffin, and sectioning of paraffin-embedded specimen (see Figure 5) were performed as already described (Schmelzer et al. 1989). AM structures were poorly preserved when embedding was performed in paraffin. Therefore, AM-colonized maize roots were fixed with 1% glutaral-

dehyde and embedded in acetone-soluble methacrylate (Baskin et al. 1992) for the in situ localization of the *G. intraradices* NR mRNA. The resin (kindly provided as ready-to-use mixture by S. Fink, Freiburg, Germany) polymerized within 24 h at 45°C. Semithin (2 µm) longitudinal sections (see Figure 6) were prepared and adhered to poly L-lysine-coated microscope slides. The resin was removed by 10 min of treatment with acetone, and the sections were air dried. Both the paraffin and resin sections were treated with pronase and fixed with 1% glutaraldehyde. For in situ hybridization, sections were covered with 10 µl of hybridization buffer (50% formamide, 10% dextran sulfate, 0.3 M NaCl, 1× Tris-EDTA, 1× Denhardt's reagent, 45 mM dithiothreitol [DTT]) and 10 ng of ³⁵S-labeled sense or antisense in vitro transcripts of the maize or *G. intraradices* NR DNA segment, respectively. Coverslips (18 × 18 mm) were placed over the sections, and edges were sealed with rubber cement (Fixogum, Marabu, Tamm, Germany). The slides were incubated in a moist chamber overnight at 50°C, then washed (1 × 30 min and 2 × 45 min at 45°C in 1× SSC, 50% formamide, 10 mM DTT), treated with 20 µg of RNase A per ml (Boehringer) in 0.5 mM NaCl, 10 mM Tris/HCl, pH 8.0, 1 mM EDTA for 30 min at 37°C, followed by washing (2 × 45 min at 45°C in 1× SSC, 50% formamide, 10 mM DTT), and finally 2 × 5 min at room temperature in 1× SSC, 10 mM DTT. Sections were dehydrated through a graded series of alcohol. Signals were detected by microautoradiography as described previously (Schmelzer et al. 1988).

ACKNOWLEDGMENTS

We are indebted to Ila Rouhara for skillful technical assistance and to Vijaya Khisty for critically reading the manuscript. This work was kindly supported by grants from the BMBF (through BEO of the KFA Jülich) and the Deutsche Forschungsgemeinschaft.

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