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Genetic evidence for the occurrence of assimilatory nitrate reductase in arbuscular mycorrhizal and other fungi

Abstract Using primers synthesized from two conserved regions and employing PCR, a DNA segment coding for part of the apoprotein of assimilatory nitrate reductase could be amplified from the fungi *Aspergillus nidulans*, *Pythium intermedium*, *Phytophthora infestans*, *Phytophthora megasperma* and *Glomus* D13. Sequencing of the amplificates as well as DNA hybridization revealed strong homologies with the nitrate reductase gene in all cases. The digoxigenin-labeled amplificate from *Glomus* hybridized with DNA isolated from *Glomus* spores. The data from these gene probing experiments are generally in accord with the published results from enzyme measurements. Thus assimilatory nitrate reductase occurs in saprophytic, parasitic as well as arbuscular mycorrhizal fungi. No amplificates with these primers were obtained with DNA isolated from *Mucor mucedo* and *Saprolegnia ferax*. Such results agree with the failure to detect nitrate assimilation physiologically in these two organisms.

Key words Nitrate reductase · Gene amplication in fungi · PCR with fungi · Arbuscular mycorrhiza Saprophytic and parasitic fungi

Introduction

Colonization of plant roots by arbuscular mycorrhizal (AM) fungi is known to improve the mobilization of nutrients from soils, and this often results in an enhancement of plant productivity and health (Harley and Smith 1983). Roots colonized by AM fungi have been amply demonstrated to mobilize phosphate more efficiently than control plants (Hayman 1983; Smith and Gianinazzi-Pearson 1988), and data for nitrate utilization have also been reported (Azcon et al. 1992; Johansen et al. 1992). Nitrate could be reduced inside the fungal cells by the assimilatory reduction pathway, implying that AM fungi have the gene set for assimilatory nitrate reduction. However, this is not a trivial statement, since many microorganisms, even *Escherichia coli* (Stewart 1988), cannot perform assimilatory nitrate reduction. Nitrate mobilized from soils by an AM fungus could be transferred directly as the anion to the root cells where reduction could proceed. However, two short communications of activity measurements indicate that spores of AM fungi might possess nitrate reductase (Ho and Trappe 1975; Sundaresan et al. 1988).

In the present study, the occurrence of the gene for assimilatory nitrate reductase was tested in AM and non-AM fungi. The PCR technique allows amplification of gene segments provided suitable oligonucleotides of conserved DNA regions can serve as primers. In the case of nitrate reductase, amplificates of about 1 kb length could be obtained by this technique, and cloning and sequencing as well as DNA-DNA hybridizations showed that a gene homologous to the apoprotein of nitrate reductase occurs in five out of seven fungi tested.

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Materials and methods

Organisms and their growth

Aspergillus nidulans (=*Emeriella nidulans*) DSM 820, *Mucor mucedo* DSM 809, *Pythium intermedium* DSM 62950 and *Saprolegnia ferax* DSM 964 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany (DSM). *Phytophthora infestans* and *Phytophthora megasperma* were kindly supplied by Dr. D. Scheel, Max-Planck-Institut für Züchtungsforschung, Cologne, Germany. *A. nidulans*, *M. mucedo* and *S. ferax* were grown in 2×YT medium (Sambrock et al. 1989). Stock cultures of *Pythium intermedium*, *Phytophthora infestans* and *Phytophthora megasperma* were grown on V-8 agar (20% V-8 juice, Campbell, 0.3% $CaCO_3$; 1.5% Bacto-agar, medium taken from the DSM catalogue). For DNA preparations,

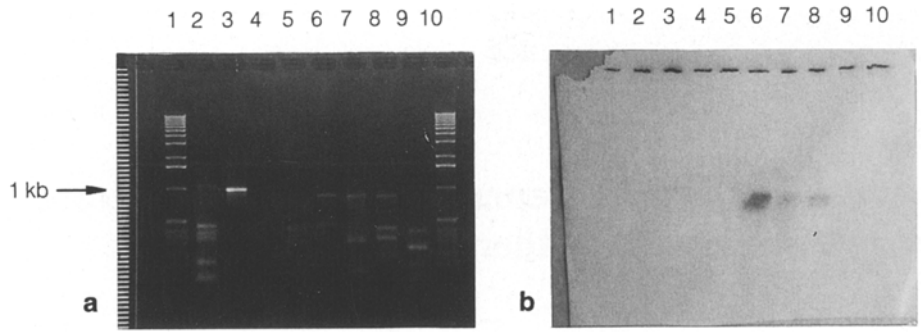

Fig. 1 **a** Amplification of DNA segments from nitrate reductase by PCR; **b** Southern hybridization of the gel shown in **a** using the amplificate from *Pythium intermedium* as probe. Lanes: *Aspergillus nidulans* (2), *Glomus* D13 (3), *Zea mays* (4), *Mucor mucedo* (5), *Phytophthora infestans* (6), *Phytophthora megasperma* (7), *Pythium intermedium* (8) and *Saprolegnia ferax* (9). Lanes 1 and 10 are molecular weight markers (1-kb ladder, Lift Technologies, Eggenstein, Germany). In lane 3, only a weak but clearly discernible signal is seen due to the weak homology between the *Pythium* probe and *Glomus* DNA. In lane 2, the signal is even weaker, but was visible on the fresh original filter

Pythium intermedium was grown in a medium containing in g/l: NH_4Cl 0.27, $MgSO_4 \cdot 7H_2O$ 0.2, NaCl 0.1, $CaCl_2 \cdot 2H_2O$ 0.02, $NaMoO_4 \cdot 2H_2O$ 0.02, $MnSO_4 \cdot H_2O$ 0.01, KH_2PO_4 0.61, K_2HPO_4 0.78, $FeSO_4 \cdot 7H_2O$ 0.0138, EDTA 0.0186 and sucrose 10. Phosphates and Fe-EDTA were autoclaved separately and added after cooling, whereas sucrose was added after sterile filtration. The pH of the medium was adjusted to 5.0.

The *Glomus* isolate D13 (kindly supplied by Dr. Dehne, Bayer AG, Leverkusen, Germany) was grown symbiotically with maize (*Zea mays* L. "Honeycomb" F-1 hybrid from Altofer Samen, Zürich, Switzerland). Details for the growth conditions in the greenhouse (Schmitz et al. 1991; Danneberg et al. 1992) and for the isolation of spores (Esch et al. 1994) were described previously.

DNA isolations

The DNA from *A. nidulans*, *Glomus*, *Pythium intermedium*, *Phytophthora infestans*, *Phytophthora megasperma* or *Z. mays* was isolated as described by Raeder and Broda (1985). The method of Doyle and Doyle (1990) was employed for isolating DNA from *M. mucedo* and *S. ferax*.

Computer analysis for conserved DNA sequences and synthesis of oligonucleotides

Conserved DNA sequences were identified after alignment of published nitrate reductase protein sequences using a computer program (written by Dr. T. Kentemich and Dr. W. Zimmer in this laboratory). Oligonucleotides were synthesized by a Pharmacia LKB Gene Assembler Plus (Pharmacia LKB, Uppsala, Sweden). After removal from the column, the oligonucleotides were purified by separation on a 1.5 ml NAP-10 column (Pharmacia LKB).

Polymerase chain reaction

The assays contained in a final volume of 50 µl: Taq polymerase (Boehringer Mannheim, Germany) 2 units, 10×Taq polymerase buffer (Boehringer Mannheim) 5 µl, deoxynucleotides 10 nmol of each, synthesized oligonucleotides 50 pmol of each, fungal DNA 2–10 ng. The reaction was performed in 32 cycles under the following conditions: 30 s denaturation at 92° C, 30 s annealing at 52° C (in the case of *A. nidulans*) or 46° C (all other fungi and maize) and 60 s polymerization at 72° C.

DNA sequencing

The amplified PCR products were cloned into pCRII (TA Cloning, Invitrogen, San Diego, Calif.). *Xba*I/*Hin*dIII fragments containing the entire PCR segments obtained from *A. nidulans* and *Pythium intermedium* were subsequently cloned into M13mp18 and M13mp19. The segment amplified from *Glomus* DNA possesses internal *Bam*HI and *Hin*dIII restriction sites. Therefore, subcloning of a *Sac*I/*Hin*dIII and a *Bam*HI/*Xba*I fragment into M13mp18/M13mp19 gave M13 clones with an overlap of 29 nucleotides. The sequences of the inserts with sizes of 0.28 kb and 0.79 kb, equivalent to about one-third of the gene (2.8–4.1 kb, depending on the introns), were determined by the dideoxy chain-termination technique using the TAQuence sequencing kit (United States Biochemical Corporation, Cleveland, Ohio).

Labeling

Purified *Eco*RI fragments of the pCRII vector, containing the subcloned PCR segments from *Pythium intermedium* or *Glomus*, were labeled by PCR as described above but with additionally 1 nmol of Dig-dUTP (Boehringer Mannheim).

DNA restriction, blotting and hybridization

For each lane of the Southern blots, 5 µg of DNA was digested overnight with 10 units of *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Sal*I or *Xba*I or by a combination of two of these enzymes. The digested DNA was separated on 0.7% (w/v) agarose gels and blotted onto Hybond-C nitrocellulose filters (Amersham Buchler, Braunschweig). After 16 h of transfer, DNA was immobilized by incubating for 2 h at 60° C. Hybridizations (without formamide at 68° C for 16 h) and colorimetric detection were performed according to the instructions of the manufacturer (Boehringer Mannheim).

Results

DNA sequences of the gene coding for the apoprotein of nitrate reductase have been communicated for many plants, e.g. tomato, spinach, rice, maize (Calza et al. 1987; Choi et al. 1989; Gowri et al. 1989; Prosser and Lazarus 1990), for the ascomycetes *Aspergillus nidulans* (Johnstone et al. 1990), *A. niger* (Unkles et al. 1992) and *Neurospora crassa* (Okamoto et al. 1991), but not for zygomycetes. The published sequences werc exam-

Table 1 Gene amplification and cloning of DNA segments of the assimilatory nitrate reductase from several fungi. The sequence homology was shown by cloning and sequencing the PCR amplificate. The homology in percent was referred to the *nia*D gene of *Aspergillus nidulans* (Johnstone et al. 1990). Homology by DNA-DNA hybridization was shown with the cloned PCR segment of nitrate reductase from *Pythium intermedium* as probe

Organism	Class	Size of the PCR amplificate (kb)	Homology to the nitrate reductase gene
Aspergillus nidulans	Ascomycetes	1.05	100% (by sequencing)
Pythium intermedium	Oomycetes	0.86	52% (by sequencing)
Phytophthora infestans	Oomycetes	0.90	DNA hybridization signal
Phytphthora megasperma	Oomycetes	0.90	DNA hybridization signal
Saprolegnia ferax	Oomycetes	none	—
Mucor mucedo	Zygomycetes	none	—
Glomus D13	Zygomycetes	0.99	53% (by sequencing)
Zea mays	Gramineae	none	—

ined for conserved regions by computer analysis. Two regions located at a distance of about 1.05 kb on the gene of *A. nidulans* were chosen to develop a 26mer primer with the sequence 5′-GCA GGC AAC AGA CGG AAA GA(AGCT) CA(AG) AA-3′ and a 29mer with 5′-ACC CGG AAC CAA GGG TTG TTC ATC AT(AGCT) CC-3′. When DNA from *A. nidulans*, *Pythium intermedium*, *Phytophthora infestans*, *Phythophthora megasperma* or *Glomus* D13 was used as template, the primers allowed amplification of a gene segment varying between 0.86 and 1.05 kb depending on the length of the introns. Amplificates were not obtained with *S. ferax*, *M. mucedo* or *Zea mays* (Fig. 1a, Table 1), or when the standard conditions for the PCR (see Material and methods) were varied and the stringencies were lowered (data not shown).

One half of the amplified segment from *A. nidulans* was sequenced and the region analyzed was identical to the published sequence (Johnstone et al. 1990). The amplificates of *Pythium intermedium* and *Glomus* D13 were sequenced from both sides until the overlapping regions (Fig. 2). A comparison of the deduced amino acid sequences showed that the homology was highest between *Glomus* and *A. nidulans* among the fungi, whereas *Pythium* showed only 40–50% sequence homology either with the other fungi or with plants. The sequence analysis indicated that the *Glomus* amplificate was derived from fungal DNA and not from plant material. The presence of introns in the DNA sequences from *Glomus* and *A. nidulans* indicated that the amplificates did not originate from bacterial contaminants.

The 0.86-kb segment from *Pythium intermedium* was cloned into pCRII and used as a probe for hybridizations with genomic DNA from the organism digested by restriction enzymes. The map obtained from this restriction analysis (Fig. 3) can be taken as another indication that the segment amplified by PCR is indeed part of the nitrate reductase gene of *Pythium intermedium*.

The PCR amplificates of the different organisms separated on the agarose gels (Fig. 1a) were blotted onto nitrocellulose filters. For DNA hybridizations with these amplificates, the 0.86-kb segment from *Pythium intermedium* labeled with digoxigenin served as a probe (Fig. 1b). Positive hybridization signals were obtained in the case of *A. nidulans*, *Glomus* D13, *Phytophthora infestans*, *Phytophthora megasperma* and *Pythium intermedium*, corroborating the evidence that these organisms possess the nitrate reductase gene. No hybridization bands could be detected with *M. mucedo*, *S. ferax* and *Z. mays* with this probe under the conditions of Fig. 1b.

In the case of *Glomus* D13, DNA was originally isolated from about 10000 spores of uniform size (probably representing *Glomus intraradices*) to obtain a PCR amplificate of 0.99 kb. The same amplificate was subsequently obtained also from only 10 spores. It apparently contained at least one intron of 0.13 kb, indicating that the DNA segment did not stem from contaminant bacteria living in or on the spores. To ascertain that the segment indeed originated from *Glomus*, it was labeled with digoxigenin and taken as a probe for hybridization with DNA extracted from about 5×10^6 *Glomus* spores of uniform size. The amount of DNA isolated from these spores (about 5 μg) was just sufficient to give a faint but clearly discernible signal in a Southern blot (data not shown).

Discussion

Both the sequence analysis and the DNA-DNA hybridization patterns strongly indicate that the amplificates of the nitrate reductase gene stem from the different fungi assayed. With the PCR method, small amounts of contaminant DNA are sometimes amplified, but this can be ruled out for the present study from the sequence data. The amplification products often contain a few false DNA bases which do not count in the sequence comparisons. The data show that even parasitic fungi possess the nitrate reductase gene. The sequence analysis revealed a coincidence between the properties of this gene and the taxonomic affinities of the fungi investigated (Table 2). The nitrate reductase gene is absent in *M. mucedo* and *S. ferax*, unless the conserved

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Aspergillus nidulans  GCAGGCAACAGACGGAAAGAGCAAAACACAGTACGGAAATCGAAAGGCTTCTCATGGGGCTCGGCCGCTCT
Pythium intermedium   GCAGGCAACAGACGGAAAGAGCAGAACATGATCAAGAAGTCGATCGGCTTCAGTTGGGGAGCCGGTGCTGC
Glomus D13            AGAGACAACAGACGGAAAGAGCAGAACATGGTCCGAAAATCACAAGGGTTCTCTTGGGGTCCTGCTGGTCT

GTCTACTGCGCTCTTCACCGGGCCCATGATGGCCGATATCATAAAGAGCGCCAAGCCCCTGCGAAGA------GCAAAGTATGTCTGCATGGA
GTCCACCGCGGTCTGGACTGGGATCCCGCTGCACGTTCTGCTTGCTCACTGCGGCGTCGACCGTCGAAAA---GCGCGCTTTGTGTGGTTTGA
CTCAACATCCTCTGACAGA---CCTATGCTTTGCGACATTCTCGCATTAGCAAAGCCAGACCGGCGGCGCGGGGCTCGATATGTGTGTTTCGA

GGGTGCCGATAACTTGgtatgtccgcgtgaatgaagccctgtcttagaggatcagggttagttgatccgtctaacgtttatagCCGAACGGAA
AGGCGCAGACGCCGTC                                                                  CCAAAGAACA
GGGTGGAGATACTCTT                                                                  CCCACAGGAG

ATTACGGGACATCCATCAAACTCAACTGGGCCATGGCCCGAATAGAGGCATTATGCTCGCGCACAAGATGAACGGCGAAGACCTCAGACCAAG
ACTACGGCACGTGCATCCCAGCTCACGTGGCGCTGGATCCGTCCTGCGATGTGCTCGTGGCGTGGAAGATGAACGGCGAGCACTTGAGTCCGG
CTTATGGGACAAGCTTGAAGCTGAGCTGGTGCATGGATCCTATTCGGGGGATCATGCTCGCCTACAAGCAGAACGGCGAAATGTTGACACCAG

ACCACGGTCGTCCGCTGAGGGCCGTCGTACCGGGCCAGATTGGAGGGCGCAGTGTCAAGTGGCTGAAGAAGCTTATCATCACCGACGCGCCCA
ACCACGGGTTCCCGCTGCGTCTCATTGTTCCTGGCCACATTGCGGGGCGGATGGTCAAGTGGCTCACGCGGATCCACGTCAGCGAGCGCGAGT
ATCATGGAAGACCAATTAGAGTTGTGGTGCCGGGAATGATTGGGGGGCGAAGTGTGAAGTGGTTGAAGAAAATTATCATCTCGGACCAGCCGA

GCGATAACTGGTACCATATTTATGACAACAGGGTGCTACCgtacgtctcatctcgttgactgttatttctcactcatactaatggtctgcagT
CTGACAACCACCACCACCTGCACGACAACCGCGTGCTTCC                                                       A
GCACGAATTATTACCACTACTACGACAATAAAGTCATGCC                                                       G

ACAATGGTCACGCCA---GATATGTCGTCCCAAAACCCGTCA---TGGTGGCGCGATGAACGCTACGCCATCTACGATCTGAACGTCAATTCC
TCCATGTGGACGCTG---AGAGAGCTACGGCGAGAATTG------TGGCCAAGCCTGAGTCGC------ATCCAGGAGCTCAACGTCAACTCG
ACACATGTCACACCGCAGATTGCGAGTAGTGAGGAGGGAAAGAAGTTCTGGATGGATGAGAAGTGGTGTATCTTTGACTTGAATGTCAACAGC

GCCGCCGTCTACCCCCAGCACAAGGAAACCCTGGATCTG------GCCGCCGCGAGGCCGTTC------------------TACACA---GCA
GCTGCTGTCTCCAGCTCACACGGAGGAACTGAACCTGAACAATTTCTCAGTGCGTCGGGGTCACAAATGATGTTCCCGTCATACAAA---GTG
GCCATTGCGTATCCGGCACATGATGAGGTCTTGGAAATCGCCCCCAAAGCAGAGGAGATGGAG---------GTGGAAGAATATGTACACATT

AAAGGGTATGCCTATGCAGGAGGCGGACGAAGAATAACCCGTGTCGAGATCTCGCTGGATAAGGGCAAATgtacgcttcctaactaccaagcc
AGCGGCTATGCGTACTCTGGCGGTGGACGGCGCATCATCCGCGTCGAAGTCACGCTGGACGACGGCGCGA
GCGGGCTACGCATATGGGGGTGGCGGAAGGAGGATTCAGAGAGTGGAAATCTCCCTCGACAAAGGAAAGTaccatagcctccacaattatttc

cataaatctgcatcccgttgaatccactgctaacatcttacagCATGGCGCCTTGCGCGGATC---GAATACGCCGAAGACAAATACCGCGAC
                                            CCTGGGACAACTGCCGCATC---AACTACAAGGAG---AAGCCCAACGCC
atgaatatcatctaatcttgtttgctacag              CATGGCGCCCTGCCTCCATCACCAACTAC---GAAGACAAATACCGCTCA

TTCGAAGGTACG------------CTCTATGGCGGCCGCGTCGACATGGCCTGGCGAGAAGCCTGCTTCTGCTGGTCCTTCTGGTCCCTCGAC
--------------------------TACGGG-----------AAGATGTGG---------------TGCTGGGTGCAGTACGAGCTGGCG
ATGAACATCCCCGAAAACACTCACCTCTTCGGGGGCAAACTCGACGTCGGCTGGCGTGATACCTGTTTCTGCTGGTGTTTCTGGGACTTATCC

ATTCCCGTCTCGGAACTCGCAAGCAGTGAT---GCCCTACTC---GTCCGCGCAATGGACGAAGCACTTAGTCTCCAACCAAAGGATATGTAC
ATCCCACTCCAGCGCCTGACTCAAGCAAGC---GAGATCTGC---GTGCGCGCCTGGGACTCGTCGATGAACACCCAGCCAGAGTTCCCGACC
GTCCCTCACTCCGACCTTGCCTCATCAAACGTTGAAGACATTGTCGTTCGTGCAATGGATGAAGCCCTCAATACCCAGCCAAGGGACATGTAC

TGGTCTGTCCTTGGTATGATGAACAACCCTTGGTTCCG
TGGAACGTCATGGGCATGATGAACAACCCTTGGTTCCG
TGGTCTTTACTCGGGATGATGAACAACCCTTGGTTCCG
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Fig. 2 Alignment of 1072 bp ofthe *nia*D gene from *Aspergillus nidulans* (Johnstone et al. 1990) and the DNA sequences of PCR segments obtained from *Pythium intermedium* and *Glomus* D13 with oligonucleotide primers for assimilatory nitrate reductase. Bases 1–285 and 821–1049 of the amplificate from *A. nidulans* have been sequenced and found to be identical to the published sequence (Johnstone et al. 1990)

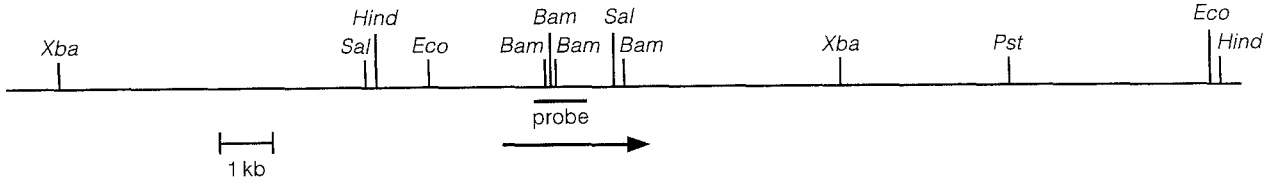

Fig. 3 Map of the restriction sites in the *Pythium intermedium* DNA region adjacent to the locus identified by the 861-bp PCR segment of the gene for assimilatory nitrate reductase from *Pythium intermedium*. Bam *Bam*HI, Eco *Eco*RI, Hind *Hin*dIII, Pst *Pst*I, Sal *Sal*I, Xba *Xba*I. The expected size and orientation of the nitrate reductase gene are shown by the *arrow* below the map

regions used for the primer synthesis happen to be strongly modified in the genes of these two organisms.

The occurrence of the nitrate reductase gene in parasitic fungi is somewhat surprising. The observation is, however, generally in accord with the results from enzyme measurements published years ago. *Pythium intermedium* but not *M. mucedo* were reported to reduce nitrate (Lilly and Barrett 1951). These data should, however, be taken with some caution, since in this publication several fungi were claimed to perform N_2 fixation, probably due to insufficiencies of the methods employed at that time. According to Webster (1983), *A. nidulans* but not *S. ferax* and *M. mucedo* perform nitrate reduction, whereas no data are available for *Phytophthora infestans* or *Phytophthora megasperma*. For arbuscular fungi, circumstantial evidence was forwarded that spores and hyphae of *G. mosseae* and *G. macrocarpus* reduce nitrate to nitrite (Ho and Trappe 1975), although the reaction could have been performed by contaminant bacteria in these assays.

In comparison to the biochemical assay for nitrate reductase, the primers synthesized and PCR as well as hybridization with the developed gene probe allowed faster screening for nitrate reductase in fungi using smaller amounts of cell material. At present, the molecular approach is of course more expensive. The present investigation showed that both approaches come to the same conclusions in the case of fungi, and confirmed an earlier report that DNA can be isolated from spores of arbuscular fungi for molecular applications (Cummings and Wood 1989).

The observation that *Glomus* possesses the gene coding for assimilatory nitrate reductase does not rule out the possibility that the plant root cells mainly reduce nitrate in the arbuscular mycorrhizal symbiosis. The development of a gene probe that specifically recognizes the fungal nitrate reductase sequence will presumably allow specific labeling of the expression of the nitrate reductase gene by in situ hybridization. Future work will also show which fungal structures (e.g. arbuscules) are the most active sites of nitrate reduction.

Table 2 Comparison of the amino acid sequences deduced from the DNA sequences of nitrate reductase genes. The total length of the segment amplified between the two primers is 287–296 amino acids, depending on the organism

Source of the nitrate reductase	No. of common amino acids	Homology (%)
Tomato + spinach	244	85
Tomato + rice	224	78
Tomato + *Aspergillus*	134	48
Aspergillus + *Neurospora*	194	68
Pythium + tomato	150	52
Pythium + *Aspergillus*	133	46
Glomus + *Aspergillus*	192	65
Glomus + *Pythium*	122	42
Glomus + tomato	137	47
Pythium, tomato, *Glomus* and *Aspergillus*	94	32

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