Mycorrhiza (1999) 9:61–64

SHORT NOTE

Marisa K. Chelius · Eric W. Triplett

Rapid detection of arbuscular mycorrhizae in roots and soil of an intensively managed turfgrass system by PCR amplification of small subunit rDNA

Accepted: 8 January 1999

Abstract Arbuscular mycorrhizal fungi (AMF) were detected in uninoculated soil and roots of turfgrass (*Agrostis palustris*) by direct extraction and PCR amplification of the small subunit rRNA gene. Sequence analysis of the cloned PCR product confirmed the identity of the amplified DNA as an AMF sequence having 95% identity to *Glomus intraradices*. The sensitivity of the method was gauged by comparison with the most probable number analysis of infective propagules in an intensively managed turfgrass system having 56 propagules per 100 g soil. In contrast to the heavily managed system, infective propagule numbers were high in systems under moderate and limited management. The method described may be useful for rapid investigations of genetic diversity and community structure of AMF.

Key words Arbuscular mycorrhizae · MPN · Turfgrass · Fungicide · DNA · PCR · SSU rDNA

Introduction

A rapid method for determining the presence or absence of AMF propagules in soil samples would be very useful for comparison of the effects of many land-use systems on mycorrhizal populations. In this present work, a rapid protocol for the isolation of DNA from soil developed in this laboratory (Borneman et al. 1996; Borneman and Triplett 1997) was adapted to the isolation of AMF rDNA from soil and root samples.

Comparative studies of the small subunit of rRNA (SSU rDNA) allow for the identification of arbuscular mycorrhizal fungi (AMF) (Woese 1987; Bruns et al. 1991; Simon et al. 1992). These comparisons are made with the nucleotide sequence of rDNA after PCR amplification (Saiki 1988) and sequencing. This approach is well suited to the study of organisms in their natural environment, such as AMF, that cannot be cultured apart from the plant host (Simon et al. 1992; Helgason et al. 1998).

Previous isolations of AMF SSU rDNA have been done from colonized roots (Simon et al. 1992, 1993; Clapp et al. 1995; Di Bonito et al. 1995; Helgason et al. 1998), individual spores (Sanders et al. 1995) or directly from soil (Claassen et al. 1996). The method presented here does not require AMF inoculation, subtraction hybridization to remove plant DNA, and/or a laborious DNA extraction procedure. In this work, SSU rDNA of AMF is isolated from soil directly and rapidly extracted from soil and root tissue of a managed turfgrass system. None of the samples used in this work were inoculated with AMF. To gauge the sensitivity of the method, comparisons were made between three adjacent turfgrass systems under different management regimes that may affect AMF inoculum potential.

Materials and methods

Soil collection and analyses, including Most Probable Number (MPN) determination of endomycorrhizae

In order to compare DNA extraction from soil and roots to the conventionally utilized MPN technique for assessing the presence of AMF, we collected soil and root samples from relatively uniform turfs of *Agrostis palustris* under three different management regimes at a Minnesota golf course. A skeet shooting range (Skeet) under limited management received fertilizer and herbicide approximately once per year, whereas the practice green (Practice) received these applications in addition to fungicide treatments once per month, and green number 1 (Green) re-

M.K. Chelius
Department of Agronomy and Brock Institute for Environmental Microbiology,
University of Wisconsin-Madison,
Madison, WI 53706, USA

E.W. Triplett (✉)
Department of Agronomy, University of Wisconsin-Madison,
1575 Linden Drive,
Madison, WI 53706-1597, USA
e-mail: triplett@facstaff.wisc.edu, Tel.: +1-608-262-9824,
Fax: +1-608-262-5217

ceived these treatments every 2 weeks. Serial applications of fungicide included treatments with vinclozolin, mancozeb, azoxystrobin, chlorothalonil, propiconazole, and metalaxyl. The soil samples were analyzed at the Soil and Plant Analysis Laboratory, Soil Science Department, University of Wisconsin (Shulte et al. 1987). We anticipated that these turfs would have different levels of occupancy by AMF, and confirmed this with an MPN assay (Halvorson and Ziegler 1933; Jarstfer and Sylvia 1997).

DNA extraction and purification

DNA was extracted from 500 mg of soil using the protocol described in a soil DNA extraction and purification kit (BIO 101). Cell lysis was achieved by bead-beating soil for 30 s at speed setting 4 in a Savant (Farmingdale, N.Y.) mini-bead-beating system. Reagents used in the extraction included 978 µl of 200 mM phosphate buffer (pH 8.0) and 122 µl of MT buffer. Extracted DNA was partially purified by addition of an equal volume of DNA Binding Matrix (BIO 101) followed by one wash in a salt-ethanol wash solution. DNA was eluted in 100 µl water and purified using the Wizard Plus SV Miniprep Purification System (Promega, Madison, WI). DNA was added to neutralization solution in a 2:1 (v/v) ratio, spun in a SV Miniprep Spin Column (Promega) for 1 min at 14 000 *g*, and washed with 750 µl wash solution (Promega). After centrifugation at 14 000 *g* for 1 min, DNA was washed again in 250 µl of wash solution and eluted in 50 µl water. This purification step was necessary to remove substances inhibitory to PCR amplification. Using the same method, DNA was also extracted from roots of *A. palustris* that had been washed in distilled water and chopped fine with a razor blade.

PCR amplification

Oligonucleotide primers were synthesized by Operon Technologies, Calif.). Partial small subunit rRNA gene sequences were amplified from the purified total community DNA by PCR using NS1 and NS4, (White et al. 1990), NS21 and an AMF-specific primer, VANS1 (Simon et al. 1992).

DNA was amplified in a 50-µl reaction volume containing either 50 pmol of each primer (VANS1 and NS21), 10 pmol (NS1 and NS4), or 20 pmol (VANS1 and NS4), 5 µl of 10 × PCR reaction buffer (Promega), 5 µg BSA, 2.5 mM $MgCl_2$, 0.2 mM (each) dATP, dGTP, dCTP, dTTP, 2.5 U *Taq* polymerase (Promega) and approximately 100 ng of DNA. Capillary tubes were used with an Idaho Technology thermal cycle and having the following thermal cycling conditions for the VANS1 and NS21 primer pair: an initial 3-min denaturation at 95 °C, followed by 40 cycles of 35 s each of denaturation at 94 °C, annealing at 53 °C, extension at 72 °C, and a final 5 min extension at 72 °C. Conditions for PCR using NS1 and NS4 primer pairs included: an initial 3 min denaturation at 95 °C, followed by 40 cycles of 94 °C for 20 s, 55 °C for 35 s, and 72 °C for 1 min and a final 5 min extension at 72 °C. PCR amplification using VANS1 and NS4 primer pair followed the latter thermal profile for 25 cycles with 1 µl of PCR product from the NS1 and NS4 amplification used as the template. PCR product was visualized by electrophoresis on a 1.0% agarose gel followed by staining with ethidium bromide (0.5 µg/ml).

DNA cloning and sequencing

To confirm that the amplified DNA was of AMF origin, the PCR product was cloned and sequenced. Amplification products from PCR using the primers VANS1 and NS21 with Skeet soil as template were purified with a PCR purification kit (Qiagen) and then ligated into the pGEM-T Easy Vector (Promega) according to the manufacturer's instructions. The ligation products were transformed into competent *E. coli* JM109 (Promega) and the resulting plasmid clones were screened for the presence of insert sequence by restriction digestion analysis. The Biotechnology Center at the University of Wisconsin performed bi-directional sequencing on the plasmid using an ABI PRISM cycle sequencing kit with dye-labeled terminators (Perkin Elmer). rDNA sequences were assembled using GCG computer software and sequence similarities were determined using BLAST Sequence Similarity Search (Altschul et al. 1997).

The AMF sequence cloned from DNA extracted from soil was assigned a GenBank accession number AF082581.

Results

The MPN of mycorrhizae in the Green soil was 56 propagules per 100 g soil, and at least 590 propagules per 100 g soil for the Practice and Skeet soils (Table 1). The Green soil was under intensive management, receiving approximately 2-monthly applications of fungicide, and had a higher available P content than the other two soils (Table 1).

Plant DNA will co-extract with AMF DNA when using a direct DNA extraction procedure of plant tissue. This can result in low PCR amplification yields if plant DNA is in great excess (Clapp et al. 1995). Even though a direct DNA extraction method was used with *A. palustris* root tissue, PCR amplification was successful using all primer pairs tested (Fig. 1).

The Practice and Green soils were used for comparisons of DNA extraction and amplification efficiency because they shared the same soil texture but differed in infective propagule number. Using VANS1 and NS21 PCR primers, amplification was detected only in the Practice soil. This soil has an at least 10-fold higher MPN than the Green soil, suggesting that in a sandy soil, infective propagule numbers may have to exceed 590 per 100 g soil to be detected using PCR with these primers. When the product of PCR amplification with NS1 and NS4 primers was used in a second round of amplification with VANS1 and NS4, a product was detected in all soils tested, even up to a 1:100 dilution of the original purified template. Sequence analysis of the 550-bp cloned PCR band (Fig. 1, lane 5) substantiated

Table 1 Physical and chemical soil analysis and number of mycorrhizal propagules per 100 g soil as determined by the Most Probable Number technique

	pH	Organic matter %	Available P ppm	Available K ppm	Soil texture	MPN
Skeet	7.6	7.1	2	110	Sandy loam	>590
Practice Green	8.0	2.5	3	35	Sand	>590
Green Number 1	7.3	4.2	150	105	Sand	56

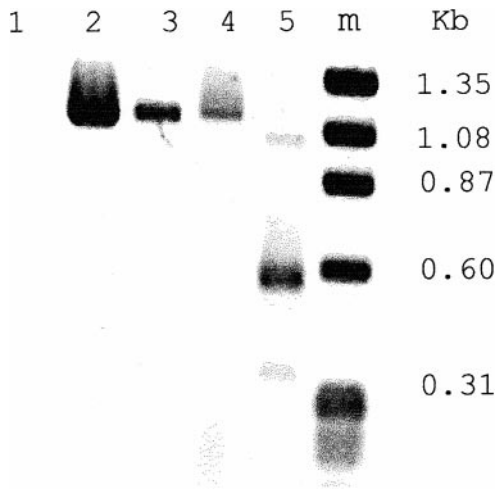

Fig. 1 PCR amplification of endomycorrhizal SSU rDNA directly extracted from soil and roots. Lanes 1–4: PCR with primers NS1 and NS4 followed by amplification using VANS1 and NS4. *Lane 1:* negative control; *Lane 2:* turfgrass roots; *Lane 3:* Green number 1 soil; *Lane 4:* Practice green soil; *Lane 5:* PCR of Skeet soil using primers VANS1 and NS21; *Lane m:* DNA molecular weight marker

the AMF specificity of the primer set VANS1 and NS21. *Glomus intraradices* was most similar to the AMF sequence, having a 95% identity.

Discussion

The intent of this study was to develop PCR-based methodology to determine the presence of AMF in soil and roots. The turfgrass soils were used because they contained a natural inoculum source that could be measured and used to determine the sensitivity of the PCR-based methods. rRNA sequence analysis has proven useful for similar objectives (Clapp et al. 1995), in part because multi-copy rRNA genes and multi-nucleated spores (Viera and Glenn 1990; Giovannetti and Gianinazzi-Pearson 1994) provide abundant target DNA. The DNA extraction and purification methods were rapid and effectively removed substances inhibitory to PCR amplification. The sensitivity of detection was improved when an initial PCR amplification using the primers NS1 and NS4 was followed by amplification of the *Glomales*-specific sequences using VANS1 and NS4.

In this study, a portion of the 18 S rRNA gene was used to identify the presence of AMF in soil. Analysis of the SSU rDNA can provide taxonomic information that may be lacking in examinations of spore morphology. However, this gene evolves relatively slowly and may not provide sufficient information to adequately characterize the AMF guild within a community. A more appropriate approach could rely on the sequence variability of the internal transcribed spacer regions (ITS) between the small and large subunits of the rRNA genes (Sanders et al. 1995; Redecker et al. 1997). As yet, few ITS sequences of a diversity of isolates have been deposited in the Genbank database to allow these comparisons.

The method described here is rapid and simple, producing high quality DNA in about 1 h. The PCR amplification of AMF sequences in noninoculated soil emphasizes the potential utility of this method as a tool to identify indigenous AMF. Additionally, AMF in roots from an intensively managed system with a low abundance of propagules in the soil was easily amplified, even in the presence of plant root DNA. To assess the efficacy of methods for DNA extraction and purification of AMF in soil and roots, some studies have relied on a commercial inoculum and/or material grown under the artificial conditions of a greenhouse (Simon et al. 1992; Di Bonito et al. 1995; Claassen et al. 1996). This confounds interpretations of the sensitivity of the method, because the high concentration of inoculum used with optimized cultural conditions may not reflect the abundance of indigenous populations of AMF. The method described here also eliminates the necessity of isolating spores from soil prior to DNA extraction in order to obtain DNA of sufficient quality for PCR amplification.

Acknowledgements This work was supported by funds from the University of Wisconsin-Madison College of Agricultural and Life Sciences Hatch project 5201 and Toro Agronomics, Minn. We thank Syed Dara of Toro for his interest in and assistance with this project.

References

Altschul S F, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl Acids Res 25:3389–3402

Borneman J, Triplett EW (1997) Molecular microbial diversity in soils from eastern Amazonia: evidence for unusual microorganisms and population shifts associated with deforestation. Appl Environ Microbiol 63:2647–2653

Borneman J, Skroch PW, Jansen JA, O'Sullivan KL, Palus JA, Rumjanek NG, Nienhuis J, Triplett EW (1996) Microbial diversity of an agricultural soil in Wisconsin. Appl Environ Microbiol 62:1935–1943

Bruns TD, White TJ, Taylor JW (1991) Fungal molecular systematics. Annu Rev Ecol Syst 22:525–564

Claassen VP, Zasoski RJ, Tyler BM (1996) A method for direct soil extraction and PCR amplification of endomycorrhizal fungal DNA. Mycorrhiza 6:447–450

Clapp JP, Young JPW, Merryweather JW, Fitter AH (1995) Diversity of fungal symbionts in arbuscular mycorrhizas from a natural community. New Phytol 130:259–265

Di Bonito R, Elliott ML, Des Jardin EA (1995) Detection of an arbuscular mycorrhizal fungus in roots of different plant species with the PCR. Appl Environ Microbiol 61:2809–2810

Giovannetti M, Gianinazzi-Pearson V (1994) Biodiversity in arbuscular mycorrhizal fungi. Mycol Res 98:705–715

Halvorson HO, Ziegler NR (1933) Application of statistics to problems in bacteriology. J Bacteriol 25:101–121

Helgason T, Daniell TJ, Husband R, Fitter AH, JPW Young (1998) Ploughing up the wood-wide web? Nature 394:431

Jarstfer AG, Sylvia DM (1997) Isolation, culture and detection of arbuscular mycorrhizal fungi. In: Hurst CJ, Knudsen GR, McInerney MJ, Stetzenbach LD, Walter MV (eds) Manual of environmental microbiology. American Society for Microbiology, Washington, DC, pp 406–412

Redecker D, Thierfelder H, Walker C, Werner D (1997) Restriction analysis of PCR-amplified internal transcribed spacers of ribosomal DNA as a tool for species identification in different genera of the order Glomales. Appl Environ Microbiol 63:1756–1761

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erich HA (1988) Primer-directed enzyme amplification of DNA with a thermostable DNA polymerase. Science 239:487–491

Sanders IR, Alt M, Groppe K, Boller T, Wiemken A (1995) Identification of ribosomal DNA polymorphisms among and within spores of the Glomales: application to studies on the genetic diversity of arbuscular mycorrhizal fungal communities. New Phytol 130:419–427

Shulte EE, Peters JB, Hodgson PR (1987) Wisconsin procedures for soil testing, plant analysis and feed and forage analysis. Department of Soil Science, University of Wisconsin-Madison, Wisc

Simon L, LaLonde M, Bruns TD (1992) Specific amplification of 18 S fungal ribosomal genes from vesicular-arbuscular endomycorrhizal fungi colonizing roots. Appl Environ Microbiol 58:291–295

Simon L, Levesque RC, LaLonde M (1993) Identification of endomycorrhizal fungi colonizing roots by fluorescent single-strand conformation polymorphism-polymerase chain reaction. Appl Environ Microbiol 59:4211–4215

Viera A, Glenn MG (1990) DNA content of vesicular-arbuscular mycorrhizal fungal spores. Mycologia 82:263–267

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and application. Academic, San Diego, Calif, 99:315–322

Woese CR (1987) Bacterial evolution. Microbiol Rev 51:221–271