Soil Sci. Plant Nutr., **51** (1), 125–128, 2005

Application of a Molecular Method for the Identification of a *Gigaspora margarita* Isolate Released in a Field

Kazuhira Yokoyama[1], Takahiro Tateishi*[,2], Masanori Saito**[,3] and Takuya Marumoto

*Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi, 753–8515 Japan; *Bio-Oriented Technology Research Advancement Institution, Saitama, 331–8537 Japan; and **Laboratory of Soil Ecology, Department of Grassland Ecology, National Institute of Livestock and Grassland Science, Nishinasuno-machi, Nasu-gun, Tochigi, 329–2793 Japan*

Received June 16, 2004; accepted in revised form November 8, 2004

A molecular technique for the identification of the *Gigaspora margarita* isolate CK based on the detection of a DNA sequence of 235 bp as its diagnostic marker was evaluated to investigate the survival and establishment of introduced arbuscular mycorrizal fungi (AMF) in a field ecosystem. In March 2001, roots and rhizosphere soil of *Eragrostis curvula* and *Miscanthus sinensis* were collected from the Mizunashi River at Mt. Fugendake (Nagasaki Prefecture, Japan), where plant seeds and AMF including *G. margarita* CK had been introduced for reforestation after the occurrence of repeated pyroclastic flows. We detected the marker sequence from DNA preparations of *E. curvula* roots and *Gigaspora* spores in the rhizosphere. This clearly showed that the isolate occurred at both hyphal and sporal stages. It was shown that the isolate survived and developed a life cycle in the revegetation area for 4 years. It was confirmed that the method was effective for tracing the isolate in samples collected from field ecosystems.

Key Words: **diagnostic DNA marker, field ecosystem, *Gigaspora margarita*, isolate-specific identification, reforestation.**

Arbuscular mycorrhizal fungi (AMF) form a symbiosis with a variety of plants. AMF transport phosphate and water from bulk soil to plant roots and improve the nutrient status of plants (Smith and Read 1997). As a result of this characteristic, AMF have been expected to enhance plant growth in destroyed and barren lands. Revegetation practices in destroyed and barren ecosystems, therefore, often include the introduction of AMF with plant seeds (Saito 2000). However, the efficiency of AMF inoculation on revegetation has thus far not been fully evaluated in situ due to the need for developing a strategy to identify and trace the introduced AMF in fields.

First, inoculated and naturally occurring AMF should be distinguished. Morphological discrimination of AMF spores would be difficult for general researchers. Moreover, the AMF in the symbiotic phase could not be identified morphologically at species and strain levels (Merryweather and Fitter 1998). Thus, molecular biological techniques are expected to enable to achieve this objective (Zézé et al. 1997; Bago et al. 1998; Lanfranco et al. 1999). Recently, AMF diversity in plant roots collected from natural ecosystems has been determined through PCR and sequencing analyses (Van Tuinen et al. 1998; Helgason et al. 1999; Husband et al. 2002; Kowalchuk et al. 2002; Vandenkoornhuyse et al. 2002). However, the genetic heterogeneity of AMF interferes with the identification of an isolate based on the sequences of variable DNA regions (Zézé et al. 1997; Lanfranco et al. 1999; Kjöller and Rosendahl 2000; Clapp et al. 2001).

An isolate of *Gigaspora margarita* Becker and Hall (*G. margarita* CK, hereafter) has been included in revegetation programs in Japan (Marumoto et al. 1996; Saito 2000). It consisted of a commercial isolate of *G. margarita* MAFF 520054. We found that a DNA sequence of 235 bp enabled to diagnose the isolate. The determination of the sequence as a marker was carried out through PCR analysis and probing. This was a simple method for the identification of the fungus at both sporal and infection stages in pot cultures (Yokoyama et al. 2002). This technique may enable to trace the isolate in field ecosystems. In the present study, we evaluated

[1] To whom correspondence should be addressed.
Present addresses: [2]Department of Agro-Bioscience, Faculty of Agriculture, Iwate University, Morioka, 020–8550 Japan; [3]Department of Environmental Chemistry, National Institute for Agro-Environmental Sciences, Tsukuba, 305–8604 Japan.

the applicability of the technique to investigations of the survival and establishment of *G. margarita* CK that was used in a revegetation program on a barren slope at Mt. Fugendake, Japan.

Materials and methods

Description of study site. The slopes of Mt. Fugendake, Japan, were covered by repeated pyroclastic flows between 1992 and 1994, which completely destroyed the pre-flow ecosystems. A revegetation program was initiated in 1997, in which bags and pellets containing spores of AMF, plant seeds, bark compost and a small amount of slow-release fertilizers were introduced to the areas. These were spread downward by a helicopter (0.25 bag or 10 pellets m^{-2}) to cover a part of the Mizunashi River, running along the east side of Mt. Fugendake through Shimabara City. The cumulative doses of fertilizers were: N, 1.07 g; P, 1.88 g; and K, 3.39 g in a square meter for 2 years (1997 and 1998). The plant species introduced were *Indigofera pseudotinctoria* Matsum., *Lespedeza cuneata* (DuMont de Courset) G. Don, *L. bicolor* var. *japonica* Nakai, *Amorpha fruticosa* L., *Albizia julibrissin* Durazz., *Alnus sieboldiana* Matsum., *A. hirsute* var. *sibirica* C. K. Schneid., *Eragrostis curvula* (Schrad.) Nees (weeping love grass), *Miscanthus sinensis* Anderss. (Japanese pampas grass, susuki), and *Cosmos bipinnatus* Cav. Although weeping love grass was not a naturally occurring species around Mt. Fugendake, it had been widely used as a pioneer plant for revegetation programs in Japan. The spores of *G. margarita* CK, that were contained in a commercially available AMF product, Cerakinkong (Central Glass Co. Ltd., Tokyo), were mixed with the materials. The mean number of spores contained in the materials was 400 bag^{-1} or 5 $pellet^{-1}$, respectively. In addition, undefined numbers of AMF spores with a smaller diameter were contained in the materials as contaminants. The downstream part had been left untreated. It had already been reported that plant growth was promoted in the revegetation area (Marumoto et al. 1999; Saito 2000).

Sampling. On March 8 and 9, 2001, we collected plants from both the treated plots for revegetation (N 32°45′9 to 10″; E 130°19′31 to 32″, 400 to 500 m high) and the untreated (N 32°45′6″; E 130°19′33″, below 400 m) areas. Based on the plant mass, we selected ten and three individuals of weeping love grass and Japanese pampas grass from the revegetation site, four and two individuals of each plant from the untreated site and two individuals of Japanese pampas grass from the intact site, respectively. We dug out soil over a radius from 10 to 30 cm, up to the 15 cm depth, depending on the root mass of each plant. Both tops and roots with soil were packed in plastic bags and brought back to the laboratory in avoiding desiccation. The dry weight of the tops of weeping love grass ranged from 5.3 to 27.6 g.

Spore extraction. After removal of the non-rhizosphere soil by gentle shaking in air, whole roots were soaked into tap water to remove the rhizosphere soil (Gerdemann and Nicolson 1963). The suspension of rhizosphere soil was passed through a 1 mm then a 106 μm mesh sieve repeatedly. Spores collected on the 106 μm mesh sieve were counted under a microscope. Spores with a bulbous suspensor and without a germination shield were considered to be those of *Gigaspora* sp. (Walker and Sanders 1986) and stored in a refrigerator. The diameter of most of the *Gigaspora* spores ranged from 350 to 450 μm. We did not identify spores that had not been categorized into *Gigaspora*.

Processing of plant roots. Roots of each weeping love grass plant were cut and separated into two fractions, one for microscopic observation and the other for molecular analyses. For microscopic observation, roots were rinsed with tap water and stored in 50% ethanol until use. The other fraction was stored at −80°C. The infection rate was calculated by the method of McGonigle et al. (1990) with a slight modification. We counted the fields with the presence of either hyphae, arbuscules, vesicles, or coiling hyphae and summed them to calculate the infection rate. Roots of Japanese pampas grass were treated as described above but subjected only to molecular analyses.

DNA preparation. Total DNA was extracted from five or ten spores with a similar size to those of *G. margarita* (350 to 450 μm in diameter) independently, as described in our previous study (Yokoyama et al. 2002). After thawing, plant roots were soaked in sterilized water to avoid desiccation. Single roots were cut out with sterilized forceps and scissors. We chose relatively small and young taproots and attached secondary roots because old woody roots were likely to be somewhat broken and it would have been difficult to extract DNA from them. Each root was soaked in a 0.1% Tween 20 solution and kept at 50°C for 10 min, then rinsed twice in sterilized water. We treated five roots per plant, and DNA was prepared from each root by using a Plant DNA mini-kit (Qiagen, Maryland, USA). The total length and total fresh weight of the roots subjected to DNA extraction ranged from 328 to 752 mm and 37 to 242×10^{-3} g per individual weeping love grass plant, or 349 to 791 mm and 34 to 88×10^{-3} g per individual Japanese pampas grass plant, respectively.

PCR conditions. We tested the purity of the DNA preparations by PCR with the ITS1 and ITS4 primer set (White et al. 1990) at first. The preparations of sporal DNA giving the ITS product were used as templates for the amplification of the marker sequence with M13 mini-satellite (Zézé et al. 1997) and 639R primers

(Yokoyama et al. 2002). The DNA preparations of plant roots were first tested for the ITS sequence as described above. The presence of AMF-DNA in each preparation was assumed when we could amplify a product by a semi-nested PCR with VANS1 and AM-1 primers (Helgason et al. 1999), and then VANS1 and NS21 primers (Simon et al. 1992). Subsequently, the DNA preparations containing AMF-DNA were subjected to the double PCR with M13 mini-satellite and 639R primers to obtain the marker sequence. Conditions for PCR and probing were identical with those described in a previous study (Yokoyama et al. 2002).

Results and discussion

Weeping love grass was the most abundant plant species in both the revegetation and the untreated areas because the latter had received plant seeds from the revegetation area by erosion, wind and other mechanical agents. Hence, studies on the AMF that colonized weeping love grass were carried out at first. Infection rate of weeping love grass varied widely among the individual plants collected in both areas (Table 1). The mean value for each area did not differ significantly (25.7% for the revegetation area and 26.8% for the untreated area, respectively). There were no significant differences between the mean number of spores extracted from the individual rhizosphere in both areas (70.7 and 90.5 per rhizosphere, respectively). The frequency of the AMF-DNA detected and the intensity of either infection or spore production were not closely correlated. This might be attributed to the fact that the DNA analyses conducted on such a small scale could not be sufficiently quantitative and that we collected AMF spores from an undefined weight of rhizosphere soil. In addition, we might have underestimated the frequency of AMF-DNA in roots because a recent study revealed that the primers used were inadequate to cover the DNA sequences of various AMF species (Schüßler et al. 2001). However, three different parameters, i.e., spore number in the rhizosphere, microscopic observation of AMF in plant roots, and AMF-DNA in root DNA preparations indicated that AMF had infected all the weeping love grass plants collected (Table 1). Thus, plant-AMF symbiosis was widely developed during the 4-year period. Spores resembling those of *G. margarita* were collected from the rhizosphere of two individuals of weeping love grass in the revegetation area, and the number of spores varied considerably. There might be unknown factors stimulating spore production. In the case of the second individual of weeping love grass, the number of spores with the marker sequence accounted for half of those tested, unlike in the first individual (Table 1). In addition, we detected many *G. margarita*-like spores in the rhizosphere of *Polygonum cuspidatum* Sieb. et Zucc. in the revegetation area. These spores did not harbor the marker sequence (data not shown). Although Polygonaceae had been considered to be non-mycorrhizal (Tester et al. 1987), Wu et al. (2004) have recently reported AMF-infection of *P. cuspidatum*. These facts suggested that there was, at least, another isolate of *G. margarita* or a morphologically very similar AMF in the revegetation area. It is possible that these spores were contained in the materials for revegetation as well as *G. margarita* CK. This is suggested by the fact that we had identified *Glomus* and *Acaulospora* sp. but not *Gigaspora* sp. in a preliminary study conducted at another experimental site that had not been treated, and that these spores were detected with *G. margarita* CK in the case of weeping love grass in the revegetation area.

As the infection rate and spore production of *G. margarita* CK were higher in Japanese pampas grass than in weeping love grass in the laboratory experiments (unpublished data), we tested the individuals of the former growing in the revegetation, untreated and adjacent undestroyed areas by PCR and probing methods. We did not find any molecular biological evidence for the infection of these plants by *G. margarita* CK, regardless of the sampling areas (data not shown). The reason for the uneven distribution of the isolate in the area remains to be elucidated and several possibilities related to the ability to survive and to form a symbiosis

Table 1. Infection rate and marker detection of arbuscular mycorrhizal fungi (AMF) for weeping love grass collected from Mizunashi River.

Plant number	Infection (%)	Spores in rhizosphere[a]		Root DNA[b]	
		Gigaspora	Non-*Gigaspora*	AMF-detected	Marker-positive
Revegetation area					
1	27.0	+ (3/3)[c]	+++	4	1
2	39.0	+++ (6/10)[c]	++	4	1
3	8.6	0	+	1	0
4	23.0	0	++	2	0
5	28.0	0	+	2	0
6	66.0	0	+	4	0
7	21.0	0	+	3	0
8	22.0	0	+	3	0
9	7.7	0	0	3	0
10	15.0	0	++	4	0
Untreated area					
1	15.0	0	+	1	0
2	60.0	0	++	1	0
3	23.0	0	+++	1	0
4	9.1	0	+++	2	0

[a]The number of "+" shows the abundance of spores extracted from rhizosphere soil in each plant: +, 1–50; ++, 51–100; +++, >101. [b]The number of DNA preparations giving the product in five replications. [c]Values in parenthesis show the number of spores tested as follows: (marker-positive / ITS-positive).

during competition with other AMF and plant succession are currently being examined.

Our technique enabled to identify *G. margarita* CK in a field ecosystem where various AMF, especially morphologically undistinguishable AMF, were present. The fact that the marker sequence was detected in the DNA preparations of both spore and plant roots indicated that the isolates developed their life cycle in the revegetation area, though to a limited extent. The sensitivity of detection by the DNA analysis might be improved if experiments could be conducted on a larger scale. This technique should enable to trace the isolate in revegetation programs conducted in Japan. This is the first study on the isolate-level identification of AMF artificially introduced into a natural ecosystem.

Acknowledgments. The present study was funded in part by a grant from the Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN), Bio-Oriented Technology Research Advancement Institution, Japan. The authors thank the staff members of the Shimabara District Office, Nagasaki Prefecture and Mr. Yasuji Nakaso in Central Glass Co., Ltd.

REFERENCES

Bago B, Bentivenga SP, Brenac V, Dodd JC, Piché Y, and Simon L 1998: Molecular analysis of *Gigaspora* (Glomales, Gigasporaceae). *New Phytol.*, **139**, 581–588

Clapp JP, Rodriguez A, and Dodd JC 2001: Inter- and intra-isolate rRNA large subunit variation in *Glomus coronatum* spores. *New Phytol.*, **149**, 539–554

Gerdemann JW and Nicolson TH 1963: Spores of mycorrhizal Endogone species extracted from soil by wet sieving and decanting. *Trans. Br. Mycol. Soc.*, **46**, 235–244

Helgason T, Fitter AH, and Young JPW 1999: Molecular diversity of arbuscular mycorrhizal fungi colonizing *Hyacinthoides non-scripta* (bluebell) in a seminatural woodland. *Mol. Ecol.*, **8**, 659–666

Husband R, Herre EA, Turner SL, Gallery R, and Young JPW 2002: Molecular diversity of arbuscular mycorrhizal fungi and patterns of host association over time and space in a tropical forest. *Mol. Ecol.*, **11**, 2669–2678

Kjöller R and Rosendahl S 2000: Detection of arbuscular mycorrhizal fungi (Glomales) in roots by nested PCR and SSCP (single stranded conformation polymorphism). *Plant Soil*, **226**, 189–196

Kowalchuk GA, De Souza FA, and Van Veen JA 2002: Community analysis of arbuscular mycorrhizal fungi associated with *Ammophila arenaria* in Dutch coastal sand dunes. *Mol. Ecol.*, **11**, 571–581

Lanfranco L, Delpero M, and Bonfante P 1999: Intrasporal variability of ribosomal sequences in the endomycorrhizal fungus *Gigaspora margarita*. *Mol. Ecol.*, **8**, 37–45

Marumoto T, Kohno N, Ezaki T, and Okabe H 1999: Reforestation of volcanic devastated land using the symbiosis with mycorrhizal fungi. *Soil Microorg.*, **53**, 81–90 (in Japanese with English summary)

Marumoto T, Okabe H, Ezaki T, Nishiyama M, and Yamamoto K 1996: Application of symbiotic microorganisms to soil conservation and reforestation. BioJapan '96 Symposium. Proceedings, p. 242–250

McGonigle TP, Miller MH, Evans DG, Fairchild GL, and Swan JA 1990: A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. *New Phytol.*, **115**, 495–501

Merryweather J and Fitter A 1998: The arbuscular mycorrhizal fungi of *Hyacinthoides non-scripta* I. Diversity of fungal taxa. *New Phytol.*, **138**, 117–129

Saito M 2000: Use of VA mycorrhizal fungi. *In* Biseibutsu no Shizaika: Kenkyu no Saizensen (Microorganisms Resources: Its Characterization and Utilization), Ed. T Suzui et al., p. 55–70, Soft Science Incorporation, Tokyo (in Japanese)

Schüßler A, Gehrig H, Schwarzott D, and Walker C 2001: Analysis of partial *Glomales* SSU rRNA gene sequences: Implications for primer design and phylogeny. *Mycol. Res.*, **105**, 5–15

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

Smith SE and Read DJ 1997: Mineral nutrition, heavy metal accumulation and water relations of VA mycorrhizal plants. *In* Mycorrhizal Symbiosis, 2nd Ed., p. 126–160, Academic Press, San Diego

Tester M, Smith SE, and Smith FA 1987: The phenomenon of "nonmycorrhizal" plants. *Can. J. Bot.*, **65**, 419–431

Vandenkoornhuyse P, Husband R, Daniell TJ, Watson IJ, Duck JM, Fitter AH, and Young JPW 2002: Arbuscular mycorrhizal community composition associated with two plant species in a grassland ecosystem. *Mol. Ecol.*, **11**, 1555–1564

Van Tuinen D, Jacquot E, Zhao B, Gollotte A, and Gianinazzi-Pearson V 1998: Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. *Mol. Ecol.*, **7**, 879–887

Walker C and Sanders FE 1986: Taxonomic concepts in the Endogonaceae: III. The separation of *Scutellospora* gen. nov. from *Gigaspora* Gerd. & Trappe. *Mycotaxon*, **27**, 169–182

White TJ, Bruns T, Lee S, and Taylor J 1990: Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* PCR Protocols. A Guide to Methods and Applications, Ed. MA Innis et al., p. 315–322, Academic Press, San Diego

Wu B, Isobe K, and Ishii R 2004: Arbuscular mycorrhizal colonization of the dominant plant species in primary successional volcanic deserts on the Southeast slope of Mount Fuji. *Mycorrhiza*, **14**, 391–395

Yokoyama K, Tateishi T, Marumoto T, and Saito M 2002: A molecular marker diagnostic of a specific isolate of an arbuscular mycorrhizal fungus, *Gigaspora margarita*. *FEMS Microbiol. Lett.*, **212**, 171–175

Zézé A, Sulstyowati E, Ophel-Keller K, Barker S, and Smith S 1997: Intersporal genetic variation of *Gigaspora margarita*, a vesicular arbuscular mycorrizal fungus, revealed by M13 minisatellite-primed PCR. *Appl. Environ. Microbiol.*, **63**, 676–678