Intersporal Genetic Variation of *Gigaspora margarita*, a Vesicular Arbuscular Mycorrhizal Fungus, Revealed by M13 Minisatellite-Primed PCR

ADOLPHE ZÉZÉ,^{1,2*} EMY SULISTYOWATI,³ KATHY OPHEL-KELLER,^{1,2} SUSAN BARKER,³
AND SALLY SMITH²

Cooperative Research Centre for Soil and Land Management,¹ Department of Plant Science,³ and Department of Soil Science,² University of Adelaide, Glen Osmond 5064, Adelaide, South Australia, Australia

Received 6 May 1996/Accepted 6 December 1996

Spores of vesicular arbuscular mycorrhizal (VAM) fungi contain thousands of nuclei. In order to understand the karyotic structure of a VAM fungus spore, the genetic variation of the first generation of spores from a VAM fungus (*Gigaspora margarita*) was examined. Spores originating from both single- and multisporous inoculations of the species *G. margarita* were analyzed by M13 minisatellite-primed PCR. In both cases, different fingerprints were obtained from individual spores with few spores exhibiting similar fingerprints. These results can be explained only by a heterokaryotic status of the nuclear population within a spore.

Vesicular arbuscular mycorrhizal (VAM) fungi belonging to the order Glomales (8) and presumed to be members of the Zygomycetes form a symbiotic association with at least 90% of land plants. The taxonomy of these fungi is based mainly on the structure and morphology of their reproductive spores, often incorrectly called azygospores or chlamydospores (4).

During their life cycle, typical zygomycetes can reproduce either sexually or asexually. For most zygomycetes, the spore which is the main means of formation of new individuals is uninucleate in both sexual and asexual reproduction. In contrast to this general situation, the spores formed by VAM fungi are multinucleate, with estimates of the number of nuclei ranging from 2,000 to 20,000 depending on species and investigation (1, 2, 13). There is still doubt about the way in which the multinucleate spores of VAM fungi are formed. Is it a simple packaging of genetically identical nuclei after serial mitosis of one nucleus derived from a single hypha, or does fusion of different hyphae occur, resulting in a population of genetically different nuclei within the spore? What is the real biological meaning of a spore in the VAM fungi? Is it representative of a single genotype, or is it a population of nuclei equivalent to a multikaryotic hypha packaged as a survival structure? The question to be addressed is whether all nuclei in a spore are identical or whether they are genetically different. The genetic analysis of spore populations from a single-spore inoculation is needed to answer this question.

In this paper, the genetic variation between individual spores obtained from populations of *Gigaspora margarita* was revealed by minisatellite-primed PCR with the M13 core sequence. Tandem repetitive DNA sequences are a common feature of eukaryotic genomes. Minisatellite repeats are hyper-variable regions. These regions are highly informative and can sometimes exhibit allelic forms. The use of the minisatellite M13 as a probe can allow fingerprinting of isolates and species among filamentous fungi (7). This approach has recently been used to reveal genetic variation in the fungus *Heterobasidion annosum* (6). Minisatellites also have been used as primers for genome fingerprinting by PCR amplification. With the M13 DNA as a primer, intraspecific variation in the fungus *H. an-*

nosum also was demonstrated (12). This technology therefore offers an ideal tool for differentiation within clones or species. Using the minisatellite-primed PCR technique, we show evidence of genetic variation between spores within a species.

MATERIALS AND METHODS

Spore production. The VAM fungus *G. margarita* (BEG 34) was used in this study. The fungus was grown in pot cultures with *Trifolium subterraneum* L. (clover). Two-day-old seedlings were incubated with spores and grown in an appropriate soil-sand mix (Kuipto soil [75% Kuipto soil–25% sand, pH 5.79; 2.3 ppm of phosphate]). These are referred to as multisporous cultures.

Single-spore inoculations were carried out as follows. A single spore from a population of *G. margarita* spores obtained from pot culture prepared as described above was surface sterilized (9), and 2-day-old clover seedlings were planted individually in multicompartment trays (50 g of soil mix per compartment) and inoculated with single spores. At 4 to 6 weeks, plants were transferred into 400-g plastic pots containing the same soil mix. These pots were kept in a greenhouse, watered three times per week, and fertilized with Long Ashton nutrient solution minus phosphate once a week. The presence of spores and mycorrhizal colonization were confirmed at 6 to 8 weeks.

DNA extraction from single spores. Spores of *G. margarita* obtained from single- and multisporous cultures were wet sieved from pot cultures and collected individually. Spores were then washed twice in sterile water, sonicated (14), and washed again in sterile water. A single spore was crushed with a minipestle over ice in a 1.5-ml Eppendorf tube containing 50 μ l of 100 mM Tris–100 mM NaCl–2 mM MgCl₂–2% Triton X-100. Samples were then centrifuged at 1,000 rpm for 2 min. The supernatant was transferred to another Eppendorf tube containing 50 μ l of sterile water.

PCR amplification using M13 primer. A 25- μ l reaction mixture contained 0.5 U of *Taq* polymerase (Promega), 25 pM M13 primer (5' GAGGGTGGCGGT TCT 3', synthesized by DNA Express, Macromolecular Resources, Colorado State University), 250 μ M deoxynucleoside triphosphates, and 1.5 mM MgCl₂. Twelve microliters of the diluted DNA from a single spore was used. For the amplification reaction, the thermal cycling parameters were an initial denaturation at 95°C for 4 min, followed by 45 cycles of 94°C for 1 min, 48°C for 2 min, and 72°C for 2 min. A control in which no DNA was added was used. The PCR was performed with a Programmable Thermal Controller (MJ Research Inc.). DNA from each spore was amplified at least twice to check for reproducibility. The amplification product was then analyzed after electrophoresis on a 1.2% agarose gel.

Hybridization using M13 DNA. M13 minisatellite-primed PCR products were separated in a 1.2% agarose gel. After electrophoresis, the gel was rinsed briefly in distilled water, incubated in denaturing solution (0.5 M NaOH and 1.5 M NaCl) for 30 min, neutralized in 1.0 M Tris (pH 8.0)–1.5 M NaCl for 30 min, and rinsed in sterile water. The DNA was transferred according to the method of Southern (11). The membrane was rinsed in 5 $\times$ SSC and DNA fixed by UV light (312 nm, 4 min). The membrane was then briefly rinsed in 2 $\times$ SSC and prehybridized for 2 h at 48°C in 10 ml of the following mixture: 3 ml of 5 $\times$ HSB (3 M NaCl, 100 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] [Sigma], 25 mM Na₂-EDTA, pH 6.8)–2 ml of Denhardt's solution III (2% gelatin, 2% Ficoll, 2% polyvinylpyrrolidone, 10% sodium dodecyl sulfate [SDS], 5% tetrasodium

* Corresponding author. Present address: Laboratoire de Phytoparasitologie, INRA/CNRS BV 1540, 21034 Dijon Cedex, France.

FIG. 1. (A) Single-spore amplification using M13 primer. Lanes 2 to 19 represent spores from *G. margarita* obtained by multispore inoculation, and lanes 1 and 20 represent *Hind*III-digested lambda DNA. (B) Reamplification of DNA from six spores of those used in panel A: spore 1 (lane 2), spore 13 (lane 3), spore 11 (lane 4), spore 17 (lane 5), spore 2 (lane 6), and spore 5 (lane 7). Also shown are negative control (lane 8) and *Hind*III-digested lambda DNA (lane 1).

pyrophosphate)—3 ml of 25% dextran sulfate—250 μ l of salmon sperm (5 μ g/ml)—3 ml of sterile water.

The M13 DNA was labelled at the 3' end with 32 P by the terminal transferase method according to information from Boehringer Mannheim. The unincorporated nucleotides were removed by column chromatography with Sephadex G-100 (Sigma). The M13 purified probe was added to the hybridization buffer, and hybridization was carried out overnight at 48°C in fresh prehybridization buffer. After hybridization, membranes were washed for 20 min in 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)—0.1% SDS at 48°C, 20 min in 1 $\times$ SSC—0.1% SDS at 48°C, and 20 min in 0.5 $\times$ SSC—0.1% SDS at 48°C. The hybridization signals were detected by X-ray exposure of membranes to Amersham autoradiographic films.

RESULTS AND DISCUSSION

A total of 15 and 18 spores of *G. margarita* originating from single- and multispore cultures were analyzed. The sizes of fragments amplified reproducibly ranged from 360 to 1,180 bp in both populations (Fig. 1 and 2). Spores from both multispore and single-spore inoculations exhibited a high level of variation. Twelve bands were found in the 18 spores originating from a multispore inoculation, whereas at least 15 were recorded in the spores from single-spore cultures. The analysis of the genetic structure of each type of spore showed that, of the 18 spores from the multispore culture, 13 different fingerprints could be identified, whereas there were 14 in those from the single-spore cultures. From the multispore culture, spores 2, 3, 4, and 5 on one hand and spores 6, 7, and 8 on the other have similar fingerprints; the others exhibited different fingerprints (Fig. 1). From the spores produced by single-spore inoculation (Fig. 2), only spores N and P have similar fingerprints, while the others are different. In comparing the structures of the two populations, it was possible to find similar fingerprints in both of them. For example, spores 11 and F; 9 and H; 15 and G; and 2, 3, 4, and 5 and N and P have the same fingerprints. A fragment of 360 bp is present in most spores in

FIG. 2. Single-spore amplification using M13 primer. Lanes B to P represent spores of *G. margarita* obtained by single-spore inoculation. Lanes A and Q represent *Hind*III-digested lambda DNA.

both cases. The two types of populations have a similarity in all fingerprints where the 1,100- and 1,180-bp bands occur. Both fragments occur in some spores (16 and D). In some spores (6, 7, 8, 9, 10, 11, 13, 15, 17, 19, C, E, F, G, H, I, and J), only the 1,180-bp band was present, while in others (spores 12 and 14), only the 1,100-bp band was present; in some spores, both these bands were apparently absent. This region may be a locus with two alleles with the possibility of having a null allele where nothing could be seen after ethidium bromide staining. When we hybridized the gel shown in Fig. 1 with labelled M13 DNA, a faint signal could be seen at approximately 1,180 bp, for two additional spores (spores 4 and 18), although this band had not appeared with ethidium bromide staining (Fig. 3). This suggests the possibility that this fragment was amplified but that the product obtained was insufficient to be detected by staining.

Variability between spores related to their biological characters has been reviewed elsewhere (5), but genetic variation between spores of an individual species of VAM fungus has received little attention. Nucleotide variation of an ITS-amplified fragment from a single spore of *Glomus mosseae* has been reported elsewhere (10). Moreover, the same authors found that the restriction enzyme digest of the initial amplified product of the ITS region totaled more than the original size, suggesting the likelihood of more than one sequence in the ITS region. The results of our analysis of spores from multispore cultures of *G. margarita* show a variation between the fingerprints obtained, which in general confirms previous observations (10). Variation between spores from multispore cultures

FIG. 3. Hybridization of the blot in Fig. 1A with labelled M13 DNA. Faint bands can also be seen at position 1180 for spores 4 and 18.

is most likely to have arisen from differences between the spores originally used to make the cultures. However, it could also arise if the nuclei in the individual spores were different. In this work, spores originating from single-spore cultures were used for the first time. The fingerprints from analysis of the spores of *G. margarita* which originated from single-spore cultures by M13 minisatellite-primed PCR showed clear variations.

VAM spores are generally considered to be asexual, implying that they reproduce by mitosis. During this type of reproduction, dissimilar sorting of nuclei can occur, leading to a low level of variation within progeny. If this is the mechanism underlying the variation, the level of diversity observed between spores originating from a single-spore inoculation is surprisingly high. The complexity of the structure found in both populations is what would be expected if there were a sexual phenomenon (a genetic reassortment between either dissimilar haploid nuclei or uniform diploid nuclei). However, we cannot clearly distinguish which of these mechanisms occurs. The possible presence of a simple locus at the position 1,100 to 1,180 bp could provide a marker for research on heritable characters for genetic analysis in the species because this locus is present within most spores produced from either multispore or single-spore cultures.

A spore from *G. margarita* contains about 2,000 nuclei (1). If one speculates that this is a population derived from a single nucleus, the progeny of this spore might be expected to have similar fingerprints regardless of whether the mechanism of character transmission is parasexual or asexual. This means that a high level of variation within a spore population produced by a single-spore inoculation would not be expected, an expectation contrasting markedly with our observations. Recently, with the probe (CAT)₅, it was demonstrated that progeny (single-basidiospore strains) from a homokaryotic dikaryon exhibited a single fingerprint (3). However, analysis of the progeny from synthesized heterokaryotic dikaryons showed variability. The case of a spore containing 2,000 nuclei is not strictly comparable. Nevertheless, assuming that the nuclei are haploid, our results suggest that the presence of two or more nuclear types within a spore increases the possibility of a high level of variation within spores produced in single-spore cultures. The analysis of the first generation of spores produced by single-spore cultures of different VAM fungi should allow us to determine whether heterokaryosis is a general phenomenon in the Glomales. When the randomly amplified polymorphic DNA technique was used on spores from *Glomus intraradices* obtained by single-spore inoculation, a variation was observed (unpublished data). This supports the hypothesis that spores of other Glomales may be heterokaryotic.

Evidence of repeated DNA sequences in genomes of VAM fungi has been reported elsewhere (15), and the possibility of using a tandemly repeated DNA sequence as a diagnostic probe for detection in colonized roots has been demonstrated previously (16). The successful use of the M13 minisatellite-primed PCR also opens the possibility of using a common band in fingerprints of different spores from a single species to type this species in mixed populations.

Among the outstanding questions remains the following involving nuclear division: is nuclear division selective, with selection of nuclei by the fungus to achieve some specialized functions such as the establishment of symbiosis so that nuclei in spores and hyphae are different? Again, the possible presence of a simple locus (1,100 to 1,180 bp) opens the possibility of monitoring the behavior of nuclei in different hyphae by *in situ* hybridization using fragments representing alleles at this locus as probes. It also opens the possibility of analyzing progeny of a single spore from an axenic culture in order to study the genetics of these fungi.

ACKNOWLEDGMENTS

We are grateful to Debra Miller for producing and maintaining VAM fungi cultures, especially those from single-spore inoculation. We thank Paul Harvey for helpful discussions.

This work was supported by the Cooperative Research Centre for Soil and Land Management, Australia.

REFERENCES

1. Bécard, G., and P. E. Pfeffer. 1993. Status of nuclear division in arbuscular mycorrhizal fungi during *in vitro* development. *Protoplasma* **194**:62–68.
2. Burggraaff, A. J. P., and J. E. Beringer. 1989. Absence of nuclear DNA synthesis in vesicular arbuscular mycorrhizal fungi during *in vitro* development. *New Phytol.* **111**:25–33.
3. Descenzo, R. A., and T. C. Harrington. 1994. Use of (CAT)₅ as a fingerprinting probe for fungi. *Phytopathology* **84**:534–540.
4. Gerdemann, J. W., and J. M. Trappe. 1974. Endogonaceae in the Pacific Northwest. *Mycol. Mem.* **5**:1–76.
5. Giovannetti, M., and V. Gianinazzi-Pearson. 1994. Biodiversity in arbuscular mycorrhizal fungi. *Mycol. Res.* **98**:705–715.
6. Karlsson, J. O. 1993. Genetic variation in *Heterobasidion annosum* detected with M13 fingerprinting and ribosomal DNA probes. *Exp. Mycol.* **18**:48–56.
7. Meyer, W., A. Koch, C. Niemann, B. Beyersmann, J. T. Epplen, and T. Boe. 1991. Differentiation of species and strains among filamentous fungi. *Curr. Genet.* **19**:239–242.
8. Morton, J. B., and G. L. Benny. 1990. Revised classification of arbuscular mycorrhizal fungi (Zygomycetes): a new order, Glomales, two new suborders, *Glomineae* and *Gigasporineae*, and two new families, Acaulosporaceae and Gigasporaceae, with an emendation of Glomaceae. *Mycotaxon* **37**:471–491.
9. Rovalinirina, F., V. Gianinazzi-Pearson, and S. Gianinazzi. 1987. Preliminary studies on *in vitro* endomycorrhizal inoculation of micropropagated tree species of nutritional value, p. 91–101. In F. S. P. Ng (ed.), *Trees and mycorrhiza: proceedings of the Asian Seminar IFS* (Malaysia).
10. Sanders, I. R., M. Alt, K. Groppe, T. Boller, and A. Wiemken. 1995. Identification of ribosomal DNA polymorphisms among and within spores of Glomales: application to studies on the genetic diversity of arbuscular mycorrhizal fungal communities. *New Phytol.* **130**:419–427.
11. Southern, E. M. 1975. Long range periodicities in mouse satellite DNA. *J. Mol. Biol.* **94**:51–70.
12. Stenlid, J., J. O. Karlsson, and N. Hogberg. 1994. Intraspecific genetic variation in *Heterobasidion annosum* revealed by amplification of minisatellite DNA. *Mycol. Res.* **98**:57–63.
13. Viera, A., and M. G. Glenn. 1990. DNA content of vesicular-arbuscular mycorrhizal fungal spores. *Mycologia* **82**:263–267.
14. Wyss, P., and P. Bonfante. 1993. Amplification of genomic DNA of arbuscular mycorrhizal (AM) fungi by PCR using short arbitrary primers. *Mycol. Res.* **97**:1351–1357.
15. Zézé, A., H. Dulieu, and V. Gianinazzi-Pearson. 1994. DNA cloning and screening of a partial genomic library from an arbuscular mycorrhizal fungus, *Scutellospora castanea*. *Mycorrhiza* **4**:251–254.
16. Zézé, A., M. Hosny, V. Gianinazzi-Pearson, and H. Dulieu. 1996. Characterization of a highly repeated DNA sequence (SC1) from the arbuscular mycorrhizal fungus *Scutellospora castanea* and its use as a diagnostic probe in planta. *Appl. Environ. Microbiol.* **62**:2443–2448.