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DNA cloning and screening of a partial genomic library from an arbuscular mycorrhizal fungus, *Scutellospora castanea*

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Abstract. A technique has been developed to efficiently extract purified, restrictable genomic DNA from spores of different arbuscular mycorrhizal fungi in order to begin detailed investigations of the genome of the Glomales. The protocol yielded variable amounts of DNA depending on the fungal species; for *Scutellospora castanea* and *Gigaspora rosea* it reached values of 1.5–2 ng/spore. *Eco*RI digests of DNA from *S. castanea* were cloned into pUC18 and about 1000 recombinant DNA clones were obtained. Of those screened, 50 contained inserts of 500–7000 bp. Selected inserts detected DNA sequences from *S. castanea* spores or roots infected by this fungus, but not from nonmycorrhizal roots. This is the first report of a partial genomic library from an arbuscular mycorrhizal fungus.

Key words: *Scutellospora castanea* – Arbuscular mycorrhiza – Genomic library

Introduction

Fungi belonging to the Glomales (Zygomycetes) reproduce by the formation of large, multinucleate spores on mycelium developing from an arbuscular mycorrhiza of a preceding culture. These spores, which are considered asexual except for one species (Tommerup 1988), have been estimated to contain several thousand nuclei (Cooke et al. 1987; Burggraaf and Beringer 1989; Viera and Glenn 1990; Bécard and Pfeffer 1993). Thus they are efficient in maintaining and transmiting genetic information to subsequent generations and no doubt contribute to the ubiquity of the Glomales, as well as to their apparent stability during coevolution in association with many different plants (Morton 1990a,b). Numerous attempts to asymbiotically culture axenic mycelium of Glomales have been unsuccessful (Hepper 1984; Williams 1991) and consequently the genetics of these fungi remains a matter of speculation. Taxonomic diagnosis of the Glomales has relied entirely on the morphological characteristics of spores (Walker 1992), but molecular approaches through recombinant DNA or PCR techniques now offer alternative strategies for detailed studies of their genetic variability and phylogenetic relationships (Hewitt et al. 1991). They also open up possibilities of appreciating polymorphic variability between isolates of a same species, and of obtaining specific probes for rapid identification of arbuscular mycorrhizal (AM) fungi within host tissues, which is virtually impossible at present.

Extraction of genomic DNA from spores of the Glomales is possible (Burggraaf and Beringer 1989; Cummings and Wood 1989; Simon et al. 1992; Waterman and Dales 1993), and amplification of DNA by PCR using oligonucleotide primers has recently been achieved (Simon et al. 1992; Waterman and Dales 1992; Wyss and Bonfante 1993). Up to now, no genes have been cloned from AM fungi, nor have RFLP analyses been published, although DNA has been isolated in a form digestible by restriction enzymes using a procedure requiring a very large number of spores (600000) (Cummings and Wood 1989). In order to begin detailed investigations of the fungal genome in the Glomales, we have: (1) developed a protocol for efficient extraction of restrictable DNA from spores; (2) cloned DNA from *Scutellospora castanea* Walker (Walker et al. 1993) to obtain a partial genomic library; (3) characterized several DNA inserts for their size and specificity vis-à-vis fungal DNA from spores or mycorrhizal roots.

Material and methods

DNA of several AM fungi [*Gigaspora margarita* Becker & Hall (LPA2), *S. castanea* Walker (LPA4), *G. rosea* Nicolson & Schenck (LPA23), two strains of *Acaulospora laevis* Gerdemann & Trappe (LPA1, LPA26)] was isolated from spores which were wet-sieved from pot cultures with *Allium porrum* L., collected individually and surface sterilized (Gianinazzi-Pearson et al. 1989).

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About 2000 spores were gently crushed in 50 mM Tris pH 8.0, 25 mM EDTA, 50 mM NaCl (TE) and 2% mercaptoethanol. The suspension was centrifuged for 1 min at 1000 *g* to separate the cloudy, lipid-rich supernatant from the broken spore walls with which many nuclei remained associated. The spore walls and nuclei were suspended in a lysis buffer (50 mM Tris pH 8.0, 25 mM EDTA, 2% SDS, 1% lauryl sarcosinate, 1 mg ml^{-1} Proteinase K, 2% mercaptoethanol) for 30 min at 65° C to extract and lyse the nuclei. The lysate was centrifuged for 1 min at 1000 *g* and the supernatant recuperated. Spore walls were treated again with the lysis buffer and the two supernatants combined. Proteins were precipitated for 15 min on ice with 0.5 M potassium acetate and centrifuged down for 15 min at 13000 *g*. Nucleic acids contained in the supernatant were treated with DNAse-free RNAse A (3 mg ml^{-1}) for 10 min at 37° C then purified twice with phenol, once with 1:1 (v:v) phenol:chloroform, and once with chloroform. The purified DNA was precipitated in 2 volumes of ethanol at −20° C or −70° C, centrifuged, washed with 70% ethanol, air-dried and resuspended in TE buffer or sterile water. The quality of DNA obtained from spores of the five different arbuscular mycorrhizal fungi was tested by digestion with different endonucleases (*Bam*HI, *Eco*RI, *Hind*III) following the protocol indicated by the supplier (Boehringer-Mannheim).

For cloning, DNA from *S. castanea* was completely digested with *Eco*RI and digests were ligated overnight at 15° C with *Eco*RI dephosphorylated pUC18 plasmid in the ratio 10:1. Competent *E. coli* DHα5mcrAB cells were prepared according to Hanahan's (1985) procedure using $RbCl_2$ and a 200-µl cell suspension was mixed with 3 µl (0.1 µg) of pUC18-ligated DNA. Transformation was achieved by a 90-s heat shock at 42° C and cell expression induced by shaking (200 rpm) for 1 h at 37° C. Aliquots (100 µl) were plated out on Luria Broth agar containing 50 µg ml^{-1} ampicillin, 0.1 M isopropyl-β-D-thiogalactopyranoside and 0.1 M Xgal. Recombinant clones were screened for inserts: plasmids were extracted by mini-preparation using the boiling procedure described by Maniatis et al. (1982) and, after *Eco*RI digestion, inserts were separated from the pUC18 on a 0.8% agarose gel.

Several inserts were isolated by the freeze-sequeeze technique (Maniatis et al. 1982) and investigated for their correspondence to fungal DNA sequences by Southern analysis. DNA was extracted from root systems of 8- to 10-week-old mycorrhizal and nonmycorrhizal *Allium porrum* using the CTAB method (Rogers and Bendich 1985). Aliquots (15 µl = 1 µg) of insert or of total DNA from *S. castanea* spores, mycorrhizal or nonmycorrhizal roots of *Allium porrum* were labeled with digoxigenin according to the protocol of the Boehringer Research Center (Kit no. 1093657), modified for the washing step after antibody binding. Extracted inserts or digested DNA from spores or roots were separated on 0.8% agarose gel and after depurination in 0.25 M HCl, DNA was transferred to a Bioprobe Biohylon Z^+ membrane in 0.4 M NaOH and 0.6 M NaCl using a TE 80 Transvac vacuum blotting unit (Hoefer). The membrane was briefly washed in 6× SSC buffer (instead of 2× SSC as indicated by the supplier) and hybridized overnight at 68° C with 10–50 ng ml^{-1} digoxigenin-labelled DNA in 5× SSC containing 10% blocking solution, 2% SDS and 0.1% N-lauryl sarcosinate. Unbound probe was removed by washing the membrane twice for 5 min at room temperature in 2× SSC, 0.1% SDS and twice for 15 min at 68° C in 0.1× SSC, 0.1% SDS. Hybridized DNA was revealed using chemiluminescent detection as described by the supplier (Boehringer Research Centre Kit no. 1093657), except that the membrane was washed twice for 3 h in 0.1 M maleic acid, 0.15 M NaCl, 0.3% Tween 20 at room temperature, before incubating in the AMPPD substrate. The membrane was then exposed for 1 h on an Amersham Hyperfilm.

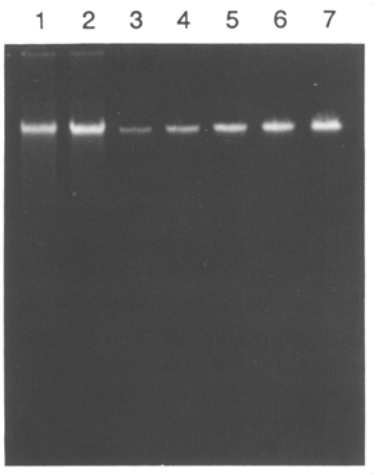

Fig. 1. DNA extracted from spores of *Scutellospora castanea* (*lane 1*) and *Gigaspora rosea* (*lane 2*) and electrophoresed through a 0.8% agarose gel. Adjacent lanes of the gel were loaded with 20, 40, 60, 80 and 100 ng lambda DNA (*lanes 3–7*)

Results and discussion

The simplified protocol described here for obtaining genomic DNA from spores yielded variable amounts of DNA, depending on the species of AM fungus. When extracts from spores of *S. castanea* or *G. rosea,* for example, were compared with DNA from lambda phage (Fig. 1), values were estimated to reach approximately 1.5–2 ng DNA/spore. Although there are indirect estimates suggesting that large spore Glomales species can contain tens of thousands of nuclei per spore (Burggraaf and Beringer 1989; Viera and Glenn 1990), numbers obtained by direct observation of stained nuclei give more realistic values ranging from 1700 to 3850 (Cooke et al. 1987; Bécard and Pfeffer 1993). Taking an average value of 2800 together with fluorocytometric measurements of nuclear DNA contents for *G. margarita* (up to 0.77 pg per nucleus, Bianciotto and Bonfante 1992), this gives approximately 2 ng DNA/spore. This is close to the amount of DNA extracted from *G. rosea* or *S. castanea,* underlining the efficiency of the extraction procedure described here. The high DNA yield obtained after lysis is no doubt due to a large proportion of the nuclei being associated with, or adhering to, the inner surface of the spore wall, as suggested by previous light and electron microscope observations (Sward 1981; Bianciotto and Bonfante 1992).

The DNA obtained from the different fungi was digested by three restriction enzymes; an example is given in Fig. 2 of *Eco*RI digests for five fungi. Spores of the Glomales are rich in lipids, polysaccharides and proteins (Sward 1981) which can contaminate DNA during extraction and make for difficulties in obtaining restrictable DNA. In the two-step procedure we have developed, low-density spore components are eliminated after crushing the spores. Moreover, the protocol requires far fewer spores than that published by Cummings and Wood (1989) and the restricted DNA

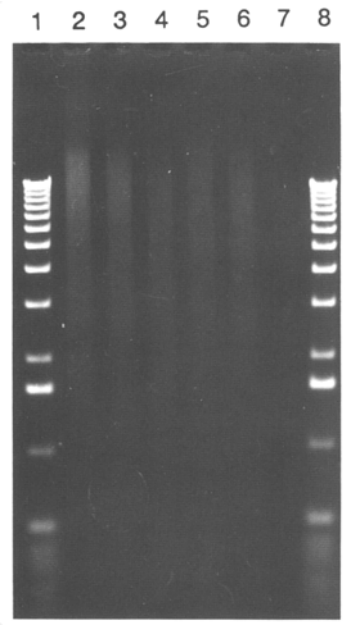

Fig. 2. *Eco*RI digests of 300 ng total DNA from spores of different arbuscular mycorrhizal fungi electrophoresed through a 0.8% agarose gel: *lane 2, S. castanea*; *lane 3, G. rosea*; *lane 4, G. margarita*; *lane 5, Acaulospora laevis* isolate LPA1; *lane 6, A. laevis* isolate LPA26; *lanes 1, 8,* 1-kb DNA ladder

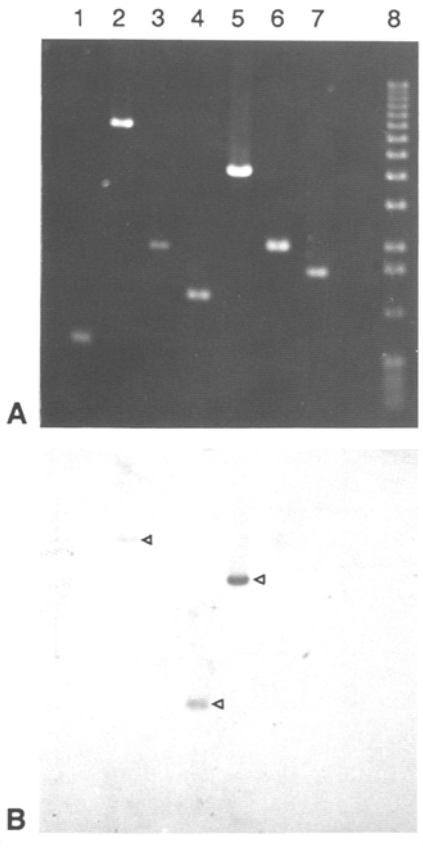

Fig. 3. A Electrophoresis through a 0.8% agarose gel of different-sized squeeze-freezed inserts (200 ng DNA) from the genomic DNA library of *S. castanea.* **B** Southern hybridization of **A** with digoxigenin-labeled total DNA from *S. castanea* spores. *Lane 1,* 500 bp; *lane 2,* 6400 bp; *lane 3,* 1800 bp; *lane 4,* 1300 bp; *lane 5,* 4000 bp; *lane 6,* 2000 bp; *lane 7,* 1500 bp; *lane 8,* 1-kb DNA ladder. The signals obtained (*arrows*) correspond to the 6400-bp, 1300-bp and 4000-bp inserts

obtained has been successfully used to clone genomic DNA from *S. castanea.*

A random library of about 1000 recombinant DNA clones was obtained from the *Eco*RI-digested DNA of *S. castanea,* and of 70 that were screened, 50 contained inserts ranging from 500 to 7000 bp (results not shown). Since spores can be contaminated by bacteria, seven of the clones with inserts between 500 and 6400 bp (Fig. 3A) were investigated for their correspondence to fungal sequences. In a first approach, the inserts shown in Fig. 3A were hybridized with total digoxigenin-labeled nuclear DNA from *S. castanea* spores. The inserts gave different hybridization intensities and three showed a significant signal (1300 bp, 4000 bp, 6400 bp; Fig. 3B), suggesting that they could represent repeated DNA sequences present in a high copy number in the fungal genome. In a second approach, two of these inserts (1300 bp, 6400 bp), and a third (1800 bp) which also strongly hybridized with total spore DNA (results not shown), were labeled with digoxigenin and hybridized with an *Eco*RI digest of DNA from either *S. castanea* spores, nonmycorrhizal roots or *S. castanea*-infected roots of *Allium porrum.* The 1300-bp (pSC1), 1800-bp (pSC2) and 6400-bp (pSC3) inserts specifically hybridized with *Eco*RI fragments of corresponding sizes extracted from spores or mycorrhizal roots (Figs. 4, 5). None of the inserts gave a hybridization signal with *Eco*RI-digested DNA from nonmycorrhizal roots, even though a significant amount (3.5 μg) was loaded onto the gel (Fig. 4). The similar hybridization patterns obtained with pSC1, pSC2 and pSC3 using both spores or mycorrhizal roots clearly shows that they represent DNA sequences from *S. castanea.* The fact that none of the selected inserts hybridized with sequences of *Eco*RI-digested DNA from nonmycorrhizal roots of *Allium porrum* suggests that they can be used to specifically detect the presence of the fungal genome in roots.

In conclusion, this is the first report of cloning of genomic DNA from an AM fungus and its use to probe for fungal tissue within roots. Further screening of other recombinant DNA clones from the partial genomic library is presently under way. Preliminary results indicate that some of the inserts are specific to *S. castanea* whilst others hybridize with sequences from other genera of the Glomales. Species- or isolate-specific DNA sequences should allow the identification of fungi in planta, whilst aspecific fungal DNA clones offer the possibility of applying RFLP analyses directly to mycorrhizal tissues. Since fungi in the Glomales are unculturable organisms, the use of mycorrhizal roots will eliminate problems associated with spore analyses (production, isolation, quantity) and represents a potential new tool for analyzing polymorphism in order to define the taxonomic or phylogenetic relationship within this fungal group.

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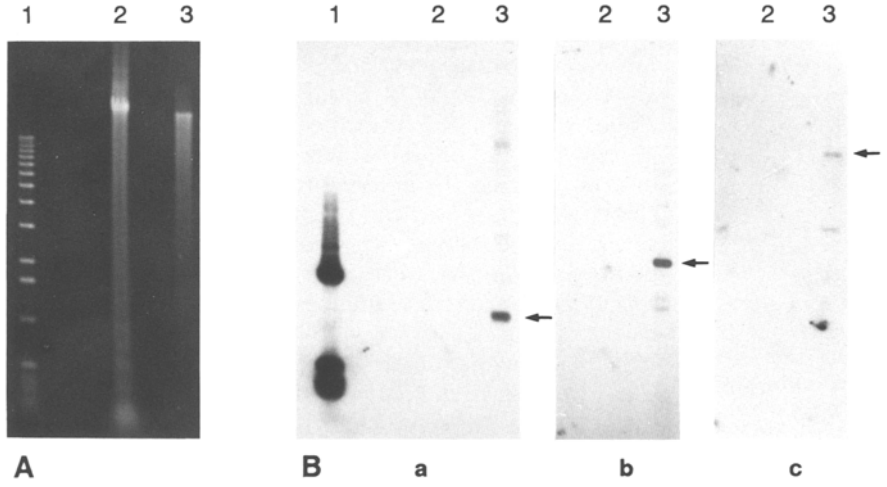

Fig. 4. A Electrophoresis through a 0.8% agarose gel of *Eco*RI-digested DNA from nonmycorrhizal *Allium porrum* roots (3.5 μg, *lane 2*) and *S. castanea* spores (1.5 μg, *lane 3*); *lane 1,* 1-kb DNA ladder. **B** Southern hybridization of **A** with the digoxigenin-labeled pSC1-1300-bp (*a*), pSC2-1800-bp (*b*) and pSC3-6400-bp (*c*) inserts (*arrows*) from the partial genomic library of *S. castanea*

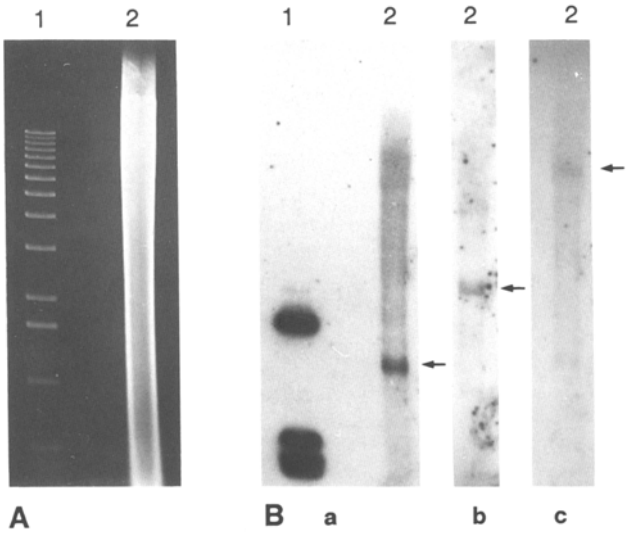

Fig. 5. A Electrophoresis through a 0.8% agarose gel of *Eco*RI-digested DNA (3.5 μg) from *Allium porrum* roots infected with *S. castanea* (*lane 2*); *lane 1,* 1-kb DNA ladder. **B** Southern hybridization of **A** with the digoxigenin-labeled pSC1-1300-bp (*a*), pSC2-1800-bp (*b*) and pSC3-6400-bp (*c*) inserts (*arrows*) from the partial genomic library of *S. castanea*

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