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Visualization of ribosomal DNA loci in spore interphasic nuclei of glomalean fungi by fluorescence in situ hybridization

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Abstract Fluorescence in situ hybridization (FISH) was applied to interphasic nuclei isolated from spores of four species of AM fungi : *Scutellospora castanea, Glomus mosseae, Glomus intraradices* and *Gigaspora rosea*. Ribosomal DNA loci were visualized using digoxigenin-labeled 25 S rDNA probes obtained by nested PCR. Several hybridization sites were detected per nucleus and an internuclear variability was observed in the number of loci. This is the first report of successful application of FISH to analyse the genomes of glomalean fungi.

Key words Arbuscular mycorrhiza · Spore nuclei · Fluorescence in situ hybridization · Ribosomal DNA loci

Introduction

Little is known about the make-up or organization of the genomes of arbuscular mycorrhizal (AM) fungi belonging to the order Glomales. Estimated DNA contents of nuclei from spores of different glomalean species indicate relatively large genomes, ranging from 0.23 to 1.08 pg DNA per nucleus (Bianciotto and Bonfante 1992; Hosny et al. 1998). Furthermore, as in other eukaryotic genomes, they possess extensively repeated DNA sequences (Hosny et al. 1997; Zézé et al. 1994, 1998). A few genes have been identified in AM fungi, such as those encoding for nitrate reductase, ATPase, GAPDH, β-tubulin, a phosphate transporter and a DNA-binding protein (Kaldorf et al. 1994; Harrison and van Buuren 1995; Franken et al. 1997; Burleigh and Harrison 1998). Ribosomal RNA genes (rDNA) have been cloned from *Scutellospora castanea* and *Glomus mosseae* (Franken and Gianinazzi-Pearson 1996), and rDNA sequences have been used in phylogenetic studies (Simon 1996) or as molecular markers to monitor fungal diversity (Sanders et al 1995; van Tuinen et al. 1998b).

In eukaryotes, the 18S–5.8S–25 S rRNA genes are organized in clusters of repeated copies (Perry 1976). In *S. castanea* and *Gigaspora margarita*, rRNA genes are present in approximately 70 and 90 copies, respectively (Passerieux 1994). Although ribosomal DNA polymorphisms have been reported among and within spores of the Glomales (Sanders et al. 1995), no cytogenetic information is as yet available for these fungi. Fluorescence in situ hybridization (FISH) has been used to localize genes on chromosomes of mitotic and meiotic nuclei in fungi and plants (Taga and Murata 1994; Prado et al. 1996). We report here a procedure developed to investigate the organization of genes in the AM fungal genome based on FISH of loci in interphasic nuclei from spores. The location of rDNA loci was visualized in nuclei of four fungi : *S. castanea, G. mosseae, G. intraradices* and *Gig. rosea*. As far as we know, this is the first successful application of FISH to localize loci in the nuclei of AM fungi.

Material and methods

Biological material

The arbuscular mycorrhizal fungi *S. castanea* Walker (isolate BEG1), *G. mosseae* (Nicol. & Gerd.) Gerdemann & Trappe (isolate BEG12), *G. intraradices* Schenck & Smith (isolate LPA8) and *Gig. rosea* Nicolson & Schenck (isolate BEG9) were cultured by inoculating spores into pot cultures containing γ-irradiated soil and seedlings of *Allium porrum* L. or *A. cepa* L. New spores produced after 6 months were harvested by wet sieving of the soil.

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Preparation of nuclei

Two or three spores of all four species were hand picked from sievings of cultures, washed with water containing 1% Tween 20 (Sigma), rinsed twice with sterilized water and fixed for 1 h at room temperature either in 4% paraformaldehyde in PIPES (Sigma) buffer or in a methanol:acetic acid [3:1 100% methanol:glacial acetic acid (v/v)]. Nuclei were spread by gently crushing spores in this solution on slides treated with Vectabond reagent (Vector) in order to enhance the adherence of nuclei, and slides were air-dried 1 h at 37 °C.

Probes

Nested PCR products of the 5' end of the large ribosomal subunit regions of all four species (van Tuinen et al. 1998b) were digoxigenin-labeled and used as probes as follows. Three spores of each species were crushed in 40 µl of TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA) and heated at 95 °C for 10 min in the presence of 10 µl of 20% Chelex-100 (BioRad). The crude DNA suspension was separated from cellular fragments by centrifugation at 12 000 *g* for 5 min and 5 µl was used for the first amplification reaction using LR1 and FLR2 primers (Table 1). PCR reactions were performed in a final volume of 25 µl containing 10 mM Tris-HCl pH 9, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mg/ml BSA (bovine serum albumin) or gelatin (Appligène), 200 µM dNTP, 500 nM of each primer and 0.5 U *Taq* polymerase (Appligène). Each reaction was overlayered with mineral oil and amplification was performed in a thermal cycler (MJ Research PTC-100) for 35 cycles: denaturation at 93 °C for 1 min, annealing at 58 °C for 1 min and finally extension at 72 °C for 1 min. 5 µl of the first PCR amplification, diluted 1/1000, served as template for a second reaction using the taxon-specific primers 4.24 (*S. castanea*), 5.25 (*G. mosseae*), 8.22 *(G. intraradices*) and 23.46 (*Gig. rosea*) (Table 1) in combination with the LR1 or FLR2 primer (van Tuinen et al. 1998b). Amplification conditions were as above except for the dNTP mixture and annealing temperature: digoxigenin (DIG) DNA Labeling Mix of dNTPs (Boehringer) was used at 1/10 dilution and the annealing temperature was increased to 60 °C to enhance the specificity of the primers. After labeling, 20 µl of PCR product was precipitated with 2 µl of 4 M LiCl and 75 ml of 96% ethanol at –80 °C for 30 min, centrifuged (15 min at 14 000 *g*) and the pellet was washed in 70% ethanol and then dried.

Southern blot hybridization

PCR product aliquots (40 ng), from the first amplification (LR1-FLR2) were separated by electrophoresis in 1.4% agarose gels and Southern blotted to nitrocellulose (Hybond-N+, Amersham). DIG labeled PCR products (200 ng) from the second amplification were used as probes for DNA-DNA hybridizations performed according to standard procedures (Boehringer-Mannheim Biochemica 1995).

Fluorescence in situ hybridization (FISH)

Nuclei on slides were incubated with 100 µl DNase-free RNase A (100 µg/ml) and 5 U/ml RNase T (Boehringer) in 2×SSC (1×SSC: 150 mM NaCl, 15 mM sodium citrate pH 7.0) for 1 h at 37 °C under a plastic coverslip. Coverslips were removed and slides were washed in 2×SSC at room temperature for 5 min, dehydrated in a graded ethanol series (70%, 90%, 100% 5 min each) and air dried. Hybridization mixture (15 µl), containing 50% (v/v) deionized formamide, 1 mM EDTA, 10% (w/v) dextran sulfate, 1× Denhardt's solution (0.1% polyvinylpyrrolidone, 0.1% w/v BSA, 0.1% w/v Ficoll), 600 µg/ml herring sperm DNA, 0.1% SDS (sodium dodecyl sulphate) and 10 ng/µl DIG-labeled probe was loaded onto each slide, then sealed with a vinyl coverslip and rubber cement. Samples were denatured at 75 °C for 10 min. Slides were incubated overnight at 37 °C in a closed plastic box on tissue paper saturated with 2×SSC to create a humid atmosphere. Two controls were carried out: (1) no probe was added to the nuclei; (2) primary antibody was omitted.

Hybridization signal detection

Coverslips were delicately removed and nuclei were washed to eliminate excess probe in 2×SSC/50% formamide at 37 °C, then twice in 2×SSC at 37 °C for 5 min each. Hybridization was detected by localization of the DIG using a three-step immunoreaction to amplify the signal (Boehringer). All detection reagents were diluted in PBS buffer containing 1% BSA (Fraction V, Sigma) and 0.1% Tween 20. Slides were incubated with 0.5 µg/ml monoclonal anti-DIG mouse antibody (Molecular Probes) at 37 °C for 1 h. After rinsing in PBS (phosphate-buffered saline) for 2×5 min each, slides were incubated with 10 µg/ml anti-mouse IgG-DIG conjugate or unlabeled rabbit anti-mouse IgG (Molecular Probes) at 37 °C for 1 h. This step was followed by incubation with 2 µg/ml sheep anti-DIG FITC (fluorescein isothiocyanate) conjugate or 100 µg/ml goat anti-rabbit IgG (H+L) Alexa 488 (Molecular Probes) conjugate, at 37 °C for 1 h in a dark, humid atmosphere. All slides were washed thoroughly with PBS buffer. Nuclei were stained with 100 µl of a propidium iodide solution (5 µg/ml in 2×SSC) for 15 min at room temperature and washed with 2×SSC. Slides were coated with a mounting solution (Prolong Antifade Kit, Molecular Probes) containing an anti-fading reagent.

Microscopical observations

Slides were examined with a DMRB epifluorescence microscope (Leica) with a I3 filter (excitation 488, emission 520) and by confocal scanning microscopy (Leica TCS 4D, Heidelberg, Germany) with filters (excitation and emission 488/BP-FITC for FITC or Alexa 488 and 568/LP 590 for propidium iodide). Hybridization experiments were repeated twice for each fungus, and the number of hybridization signals per nucleus was counted from direct observations of 20–25 nuclei for each species in the second experiment.

Table 1 PCR primers used in this study

Primer	5′-Sequence-3′	Direction	TM	Specificity
LR1[a]	GCA TAT CAA TAA GCG GAG GA	Forward	58 °C	Eukaryotes
FLR2[c]	GTC GTT TAA AGC CAT TAC GTC	Reverse	58 °C	Fungi
4.24[b]	TGT CCA TAA CCC AAC TTC GT	Reverse	56 °C	*Scutellospora castanea*
5.25[c]	ATC AAC CTT TTG AGC TCG	Forward	52 °C	*Glomus mosseae*
8.22[b]	AAC TCC TCA CGC TCC ACA GA	Reverse	62 °C	*Glomus intraradices*
23.46[c]	GCT ATC CGT AAT CCA ATA CTG	Reverse	60 °C	*Gigaspora rosea*

[a] van Tuinen et al 1998a
[b] van Tuinen et al. 1998b
[c] this study

Results and discussion

The DIG-labeled probes prepared by nested PCR from spore DNA of *S. castanea, G. mosseae, G. intraradices* and *Gig. rosea* were tested by Southern blot hybridization. The probes hybridized with the corresponding product of the first nested PCR amplification for each fungus (Fig. 1). Base variation in targeted sequences was observed within a single isolate but only of 1.5–2.9% (data not shown), which is not sufficiently high to distinguish between variants under the stringency conditions used for the FISH experiments.

Only interphasic nuclei were observed from spores of the four species. Loci corresponding to the 25 S rDNA DIG-labeled fungal probes were successfully localized in slide preparations of nuclei. Hybridization signals appeared as fluorescent green spots (FITC or Alexa 488) which were dispersed in the red-stained background (propidium iodide) of the whole nuclei (Fig. 2). Similar results were obtained whether spores were fixed in paraformaldehyde/PIPES buffer or in the methanol:acetic acid solution. No hybridization signal

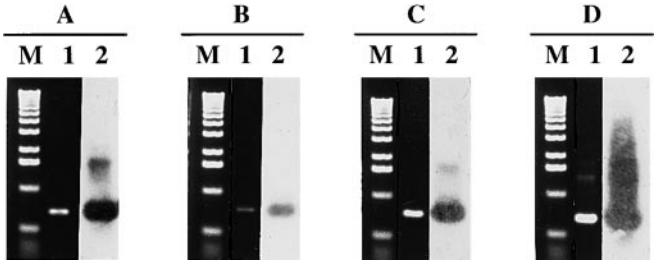

Fig. 1 Southern blot analysis of DIG-labeled 25S rDNA probes for *Scutellospora castanea* (*A*), *Glomus mosseae* (*B*), *G. intraradices* (*C*) and *Gigaspora rosea* (*D*). *Lanes 1 A–D*: Products of PCR amplification with LR1 and FLR2 primers. *Lanes 2 A–D*: DNA-DNA hybridization with DIG-labeled PCR products of the second amplification using the taxon-specific primers 4.24 (*S. castanea*), 5.25 (*G. mosseae*), 8.22 (*G. intraradices*) and 23.46 (*Gig. rosea*) in combination with LR1 or FLR2 (*M* molecular weight marker)

Fig. 2 Fluorescence in situ hybridization of 25S rDNA in interphasic nuclei of *S. castanea* (**A**), *G. mosseae* (**B**), *G. intraradices* (**C**) and *Gig. rosea* (**D**) using DIG-labeled products of the second nested PCR amplification as probes. Fluorescein isothiocyanate (FITC) or Alexa 488 signals are visualized as green spots (*arrows*), *bar* 1 μm

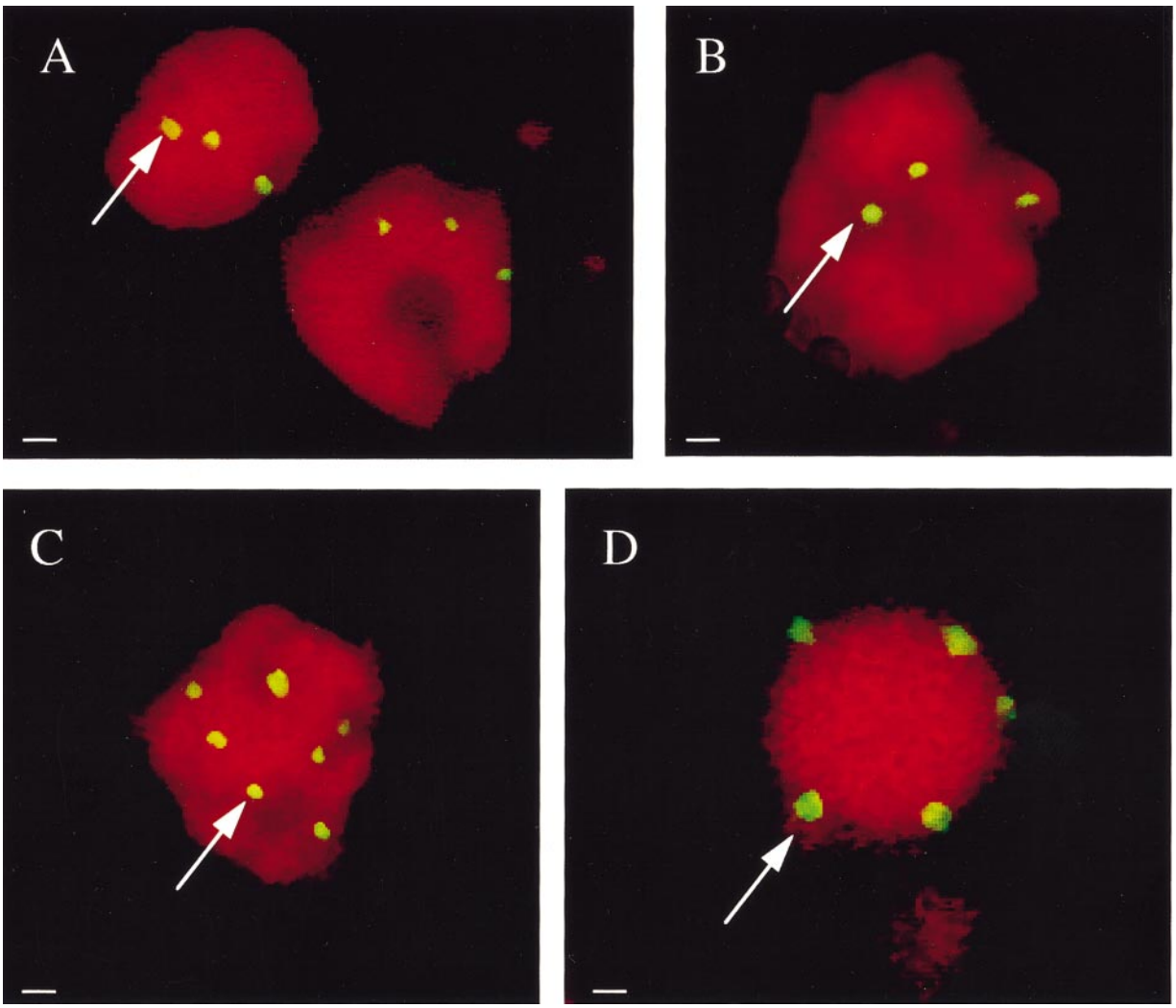

Table 2 Percentage nuclei showing 3, 5 or 7 hybridization sites with 25S rDNA probes detected by FISH in four AM fungi

Species	Number of hybridization sites		
	3	5	7
S. castanea	76.0	24.0	0
G. mosseae	66.7	33.3	0
G. intraradices	44.0	52.0	4.0
Gig. rosea	77.3	22.7	0

was obtained in nuclei from the control treatments. Different numbers of hybridization signals were observed per nucleus in the four species of AM fungi (Table 2). The nuclei of all four fungi were consistently distributed into two populations, having either 3 or 5 hybridization sites per nucleus, suggesting that the ribosomal genes in the different species are present at either 3 or 5 loci. Counting of hybridization site frequency for *S. castanea* and *Gig. rosea* showed that about 75% of nuclei possessed 3 loci and 25% 5 loci. Values were slightly different for *G. mosseae* where 3 and 5 loci were found in two-thirds and one-third of the nuclei, respectively. Approximately equal numbers of nuclei had 3 or 5 loci in *G. intraradices*, and on one occasion 7 loci were observed in this fungus. These results clearly indicate an internuclear variability in the number of rDNA loci in the genome of Glomales, and suggest differences between some species in the distribution of ribosomal genes. However, it cannot be entirely excluded that observed values sometimes underestimate locus frequencies because, for example, of variation in hybridization intensities at sites in some nuclei.

In conclusion, the present data illustrate the first successful application of FISH to detect DNA sequences in the nuclei of AM fungi. FISH of interphasic nuclei from spores made it possible to localize 25 S rDNA loci and estimate their frequency in the genomes of *S. castanea, G. mosseae, G. intraradices* and *Gig. rosea*. Copy numbers for rDNA genes in *S. castanea* and *Gig. rosea* have been evaluated at about 70 and 90, respectively (Passerieux 1994). These values together with frequency of loci detected in the present study suggest that the ribosomal genes are clustered within the genomes of glomalean fungi. The technique of FISH applied to spreads of spore nuclei should offer the possibility of verifying whether different ribosomal sequences are present in a single nucleus (Hijri M, Hosny M, van Tuinen D, Dulieu H, data submitted for publication) and reply to questions concerning the occurrence of genetic polymorphism within a single spore (Sanders et al. 1995). In addition, the technique opens the exciting possibility of visualizing loci of different genes in a wide range of Glomales and of expanding our understanding of the genomes of these fungi.

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