Unique DNA plasmid pRS64 associated with chromosomal DNAs of the plant pathogenic fungus *Rhizoctonia solani*

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Unique DNA sequences homologous to the linear DNA plasmid pRS64 were investigated in chromosomal DNAs of isolates belonging to anastomosis group 4 (AG-4) of the plant pathogenic fungus *Rhizoctonia solani*. Chromosome-sized DNAs of isolates RI-64 and 1271 of AG-4 were separated into six bands by orthogonal-field-alternation gel electrophoresis and hybridized to a cloned segment of pRS64. A small chromosome-sized DNA band of approximately 1.1 Mb carried the sequences homologous to pRS64 DNA. Sequences homologous to pRS64 were also maintained within the chromosomal DNA of isolate 1271 of AG-4 which does not possess the plasmid. The plasmid showed no homology to the mitochondrial DNA of isolate 1271. The possibility that the linear plasmid pRS64 may act as a transposable genetic element is discussed.

Introduction

Rhizoctonia solani Kühn is a plant pathogenic fungus that has a wide host range and vegetative incompatibility in this fungus is determined by anastomosis (AG) and intraspecific (ISG) groups. Japanese isolates have been divided into nine AGs and ISGs. Plasmid DNAs designated pRS64-1, -2, and -3 were detected in isolate RI-64 of AG-4. These were linear DNA plasmids of similar size (2.7 kb) (Hashiba, 1988; Miyashita *et al.*, 1990). Linear plasmids have been detected in a wide variety of higher plants and fungi, including plant pathogenic fungi (as reviewed in Esser *et al.*, 1986; Meinhardt *et al.*, 1990; Sakaguchi, 1990), but knowledge as to the function of these genetic elements is still poor. Most linear plasmid DNAs have terminal proteins covalently attached to their 5' termini. The terminal proteins have been shown to function as a primer for DNA replication. The pRS64 plasmid DNAs of *R. solani* have unique terminal structures: hairpin loops at both termini (Miyashita *et al.*, 1990). Recently, we have found similar plasmid-like DNAs (plDNAs) in 48 isolates from nine AGs and ISGs of *R. solani* and showed that the plDNAs have considerable sequence homology between isolates within the same AGs and ISGs. However, no sequence homology was found among plDNAs from

different AGs and ISGs. This suggested that the distribution of plDNAs correlated with the distinct host range of AGs and ISGs in *R. solani* (Miyasaka *et al.*, 1990).

The mitochondrial DNA sequence of maize is known to share sequence homology with the linear plasmids S-1 and S-2. Moreover, sequences homologous to S-1 have been reported to occur in the maize nuclear genome (Kemble *et al.*, 1983). However, in the plant pathogenic fungus *Claviceps purpurea* it has been reported that sequences homologous to plasmid pCIK1 were found to be integrated into high-molecular-mass mitochondrial DNA (mtDNA) (Tudzynski & Esser, 1986). Oeser & Tudzynski (1989) considered that the linear plasmid pCIK1 and the S plasmids belonged to a new class of ubiquitous mitochondrial plasmids with virus-like properties.

Here, we report the existence of chromosomal DNA sequences homologous to plasmid DNAs in isolates from AG-4 of *R. solani*.

Methods

Fungal isolates. *R. solani* isolates RI-64 and 1271, belonging to AG-4 were used in this study. The isolate RI-64 containing the linear plasmid pRS64, was obtained from soil infected by the strongly pathogenic isolate 1271, which carries no plasmid (Hashiba *et al.*, 1984). Cultures were grown and maintained on potato/sucrose agar medium (g l⁻¹: potato, 200; sucrose, 20; agar, 20).

Abbreviations: AG, anastomosis group; ISG, intraspecific group; mtDNA, mitochondrial DNA; OFAGE, orthogonal-field-alternation gel electrophoresis; plDNA, plasmid-like DNA.

Preparation of intact chromosomal DNA. Protoplasts were prepared from *R. solani* using a modified method of Hashiba & Yamada (1982). One gram (fresh weight) of the mycelium grown for 30–36 h in potato/sucrose liquid medium containing polypeptone was harvested on a 150 µm steel sieve, washed with distilled water, and suspended in 10 ml pretreatment solution containing 5 mM-EDTA (pH 8.0) and 0.2% 2-mercaptoethanol at room temperature. After 1 h, the mycelia were washed with distilled water, and resuspended in 10 ml 0.6 M-KCl containing an enzyme mixture: 20 mg cellulase ml⁻¹ ('Onozuka' RS; Yakult Biochemical Co.), 10 mg driselase ml⁻¹ (Kyowa Hakko Co.), 0.4 mg Novozyme 234 ml⁻¹ (Novo Enzyme Products) and 0.1 mg chitinase T-1 ml⁻¹ (Asahi Industries Co.). The culture flask was placed on a reciprocal shaker set at 75 strokes min⁻¹ at 34 °C for 4 h. The culture was then filtered through a 150 µm steel sieve to remove the mycelial fragments, and the filtrate was centrifuged at least twice at 700 g for 5 min to remove the enzymes. The pellet was suspended in 0.5 ml 0.6 M-KCl to a final concentration of approximately 2×10^8 protoplasts ml⁻¹. An equal volume of 2% (w/v) low-melting-point agarose in 0.6 M-KCl was added to the sample, and then the agarose plugs were incubated in a solution containing 1 mg proteinase K ml⁻¹, 1% (w/v) Sarkosyl and 0.45 M-EDTA (pH 9.0) for 24 h at 50 °C. These plugs were then stored in 0.5 M-EDTA (pH 8.0) at 4 °C.

Preparation of total DNA. Mycelia were grown without shaking in polypeptone/potato/sucrose liquid medium. After 1–2 weeks, mycelia were harvested on a paper filter and lyophilized. Total cellular DNAs were prepared using the procedure of Hirt (1967) as modified by Miyashita *et al.* (1990).

Isolation of nuclear and mitochondrial DNA. Mitochondrial and nuclear DNAs were separated using a modification of the procedure of Garber & Yoder (1983). CsCl (4.6 g) was dissolved in 4 ml total DNA solution (containing 100–200 µg DNA). Bisbenzimidazole (60 µl) was added from a 10 mg ml⁻¹ stock solution. Tubes were centrifuged at 165 000 g for 23 h at 20 °C, and following slow deceleration, at 100 000 g for 48 h. Bands of DNA were gently removed by side puncture of the tube with a needle. After centrifugation, the bisbenzimidazole was removed from each DNA band by extracting several times with isopropanol. DNA fractions were precipitated with ethanol and dissolved in Tris/EDTA (TE) buffer.

Isolation of mitochondria. The mycelium was harvested and ground with glass beads at 4 °C in 3 vols 0.5 M-mannitol, 50 mM-Tris/HCl (pH 8.0), 5 mM-EDTA, 1 mM-2-mercaptoethanol and 0.1% BSA until a fine suspension was formed. Mitochondria were isolated from the suspension as described by Day & Hanson (1977). The isolated mitochondria were treated with DNAase and RNAase at room temperature for 30 min to remove extra-mitochondrial DNA and RNA.

Cloning of fragments of pRS64 plasmids. pRS64 plasmid DNAs prepared as described previously (Miyashita *et al.*, 1990) were digested with *Xho*I. Fragments of 2.2 and 1.5 kb from pRS64-1 and -2, respectively, were ligated into pUC19. The ligation mixture was used to transform *Escherichia coli* JM109. Plasmid DNAs of Amp^r Lac⁻ transformants were used as probes for Southern hybridization.

Orthogonal-field-alternation gel electrophoresis (OFAGE). OFAGE was performed using a 1% (w/v) agarose gel in 0.5 × TBE buffer (1 × TBE is 90 mM-Tris/HCl, 90 mM-boric acid, 2.5 mM-EDTA), cooled to 8–10 °C, as described by Carle & Olson (1984). Gels were run for about 120 h at 3 V cm⁻¹ with a 60 min switching interval, followed by 48 h at 3 V cm⁻¹ with a 2 min switching interval. The gels were then stained with 1 µg ethidium bromide ml⁻¹ for 20 min and destained in 0.5 × TBE for 20 min.

Southern hybridization. Agarose gels containing chromosomal DNAs were hydrolysed by acid depurination prior to alkali denaturation, then transferred to nylon membrane filters. DNA probes were prepared by

labelling with [α -³²P]dCTP using the multipriming labelling system (Amersham). Nylon membranes were hybridized for 16–24 h at 42 °C in 25% (v/v) formamide, 6 × SSC, 100 µg denatured salmon sperm DNA ml⁻¹, 5 × Denhardt's solution and 0.5% (w/v) SDS (1 × SSC is 0.15 M-NaCl, 0.015 M-sodium citrate, pH 7.0; 1 × Denhardt's solution is 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone). Hybridization patterns were visualized by autoradiography. All procedures were carried out as described by Maniatis *et al.* (1982).

Results

Mitochondrial location of plasmid DNAs

To investigate the location of plasmid DNAs of *R. solani* AG-4, total DNAs or DNA isolated from intact mitochondria were separated by CsCl-bisbenzimidazole centrifugation. Plasmid bands were found only in the mtDNA fractions (Fig. 1). After DNAase treatment of intact mitochondria, the plasmids were still present, indicating their mitochondrial location.

Separation of chromosomal DNAs by OFAGE

We have modified the procedure of Hashiba & Yamada (1982) to prepare high yields of protoplasts from *R. solani*. This procedure yields 3×10^7 protoplasts per g (fresh weight) of mycelia. Chromosomal DNAs of both isolates, RI-64 and 1271, were efficiently released from protoplasts and subjected to OFAGE. Using 1% agarose gels and pulse intervals of 60 min and 2 min for periods of 120 and 48 h, respectively, both the RI-64 and 1271 genomes were resolved into six distinct bands (Fig. 2a).

Fig. 1. Electrophoretic analysis of nuclear and mtDNA fractions from CsCl-bisbenzimidazole-purified DNA and DNA isolated from intact mitochondria of isolate RI-64 from *R. solani* AG-4. Lanes: 1, λ DNA digested with *Hind*III; 2, DNA from nuclear fraction; 3, DNA from mitochondrial fraction; 4, DNA isolated from intact mitochondria.

Fig. 2. Southern blot of *R. solani* chromosomal DNA separated by OFAGE. (a) Maximal separation of the largest DNA fragments; initial run of 120 h with 60-min pulse at 3 V cm^{-1} followed by 48 h with 2-min pulse at 3 V cm^{-1} . (b) Southern blot of chromosomal DNA and probed with pRS64 DNA. Lanes: 1, isolate 1271; 2, isolate RI-64. Arrow heads indicate chromosomal DNA bands of *R. solani*. The estimated sizes of the chromosomes in the electrophoretic bands from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are indicated on the left.

The sizes of these six bands were 0.8, 1.1, 1.6, 1.8, 2.5 and 3.8 Mb, respectively, as estimated by reference to the chromosomal DNAs of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*.

Sequence homology of pRS64 DNA to chromosomal DNAs

As the *R. solani* isolate RI-64 carried the pRS64 plasmids, but 1271, the isolate from which it was derived,

did not, the relationship between plasmid DNA and chromosomal DNAs in mycelia of these isolates was investigated. Cloned DNA fragments of pRS64 were hybridized to electrophoretically separated chromosomal DNAs. As can be seen in Fig. 2(b), hybridization was restricted to a single band of 1.1 Mb, suggesting the presence of a unique chromosomal position of the integrated plasmid DNA in both isolates.

Detection of a unique nuclear sequence

To test the possibility of integration of the plasmid DNAs in either nuclear or mitochondrial DNAs, the labelled fragment of pRS64 was hybridized to nuclear and mitochondrial DNAs of isolate 1271 digested to completion with *Bam*HI and *Eco*RI. Several fragments of the nuclear DNA hybridized with the probe of cloned pRS64 DNA (Fig. 3). The sizes of these fragments obtained by restriction with *Bam*HI and *Eco*RI ranged from 8.8 to 2.8 kb and from 8.4 to 3.6 kb, respectively. When mtDNA of isolate 1271 was digested with *Bam*HI and *Eco*RI, no homology between fragments of mtDNA and pRS64 was observed (Fig. 3).

Discussion

Analysis of total DNA in field isolates of *R. solani* AG-4 showed that half the isolates (8 out of 18) contained pDNA (Miyasaka *et al.*, 1990). Analysis of mtDNA showed that all of the 15 isolates from *R. solani* AG-4 except isolate 1271 contained pDNA (unpublished data). Furthermore, we also detected plasmid bands only in mitochondrial fractions. After DNAase treatment of intact mitochondria, the pDNAs proved resistant, indicating their mitochondrial location. However, these

Fig. 3. Detection of a unique nuclear sequence. (a, c) Gel-electrophoretic analysis of restriction enzyme digests of mitochondrial (a) and nuclear (c) DNAs from isolate 1271. (b, d) Results of hybridization of Southern transfer strips from (a) and (c), respectively, with cloned pRS64 DNA probes. Lanes: 1, λ DNA digested with *Hind*III; 2, nuclear and mitochondrial DNA digested with *Bam*HI; 3, nuclear and mitochondrial DNA digested with *Eco*RI.

experiments do not rule out the possibility that the pDNA exists extra-mitochondrially in the cytoplasm as well as within mitochondria.

To gain information on the chromosomal DNA of *R. solani*, we have applied the technique of OFAGE to separate large DNAs from the filamentous fungus. At least six chromosome-sized DNA bands from *R. solani* could be separated by OFAGE. In this way the total genome size of *R. solani* is estimated to be more than 11 Mb. The assignment of linkage groups to the resolved chromosomal DNA bands may be undertaken using linkage group-specific probes. The physical analysis of fungal genomes by OFAGE has been successful with other plant pathogenic fungi as an aid to karyotyping or genomic mapping (Kinscherf & Leong, 1988; McCluskey & Mills, 1990; Mills & McCluskey, 1990; Rollo *et al.*, 1989; Tzeng *et al.*, 1989). Recently, chromosome length polymorphisms were observed among different races of *Ustilago hordei* (McCluskey & Mills, 1990).

Fungal plasmids have been shown to share homology with either nuclear DNA (Wright & Cummings, 1983) or mtDNA or both (Van den Boogaart *et al.*, 1982; reviewed by Samac & Leong, 1989). Southern hybridization analysis with nearly full-length clones of pRS64-1 and -2 (excluding the terminal regions) identified an homologous sequence within a 1.1 Mb chromosomal DNA band. The cloned fragments of pRS64 shared sequence homology with nuclear DNA but not mtDNA from isolate 1271. This suggests that pRS64 may well be stably integrated into the chromosomal DNA. DNA sequences homologous to plasmid pCIK1 were found to be integrated at the 5' end of the large rRNA gene within the mitochondrial genome of the plant pathogenic fungus *Claviceps purpurea* (Tudzynski & Esser, 1986). Similar cases have been reported in other eukaryotes (Bertrand *et al.*, 1985, 1986), and discussed in terms of linear DNA plasmids acting as transposable genetic elements.

We have shown that there is considerable sequence homology among plasmid DNAs obtained from isolates within the same AG and ISG of *R. solani* (Miyasaka *et al.*, 1990). It is still to be determined whether the plasmids are actively transcribed and translated. Interesting questions arise as to how the plasmid sequences are maintained within the genomic DNA of isolate 1271, and how the plasmid DNAs may be excised and amplified in isolate RI-64. Equally it is possible that the integrated DNA sequences may play a role in plasmid expression.

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