Detection and properties of plasmid-like DNA in isolates from nine anastomosis and intraspecific groups of *Rhizoctonia solani*

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Plasmid-like DNA (pDNA) was found in 48 out of 114 field isolates of *Rhizoctonia solani*. These 48 isolates were distributed as follows among the nine anastomosis groups (AG) and intraspecific groups (ISG) recognized among Japanese isolates of *R. solani*: 0 in AG-1 (sasakii type), 1 in AG-1 (web-blight type), 0 in AG-2-1, 11 in AG-2-2 (rush type), 10 in AG-2-2 (root rot type), 10 in AG-3, 8 in AG-4, 4 in AG-5 and 4 in AG-6. Each isolate carried one, two or three pDNAs identified by gel electrophoresis. Electron microscopic analysis revealed that all these pDNAs were linear molecules. The sequence homology among pDNAs found in representative isolates was examined by Southern blot analysis, using nick-translated pDNAs as probes. Considerable sequence homology was observed among pDNAs obtained from isolates within the same AG and ISG. The pDNAs occurring in the isolates of AG-2-2 were classified into two groups on the basis of the sequence homology.

Introduction

The phytopathogenic fungus *Rhizoctonia solani* Kühn causes damping off in some 200 plant species, and has a wide host range. Vegetative incompatibility in this fungus is determined by anastomosis (AG) and intraspecific (ISG) groups. Japanese isolates have been divided into 9 AGs and ISGs. Of these, AG-1, AG-2-1, AG-2-2, AG-3 and AG-4 correspond to sasakii type (IA) and web-blight type (IB), winter crop type (II), rush type (IIIB) and root rot type (IV), potato type (IV), and praticola type (IIIA), respectively, based on pathogenicity to field crops and cultural behaviour (Watanabe & Matsuda, 1966).

There have been various reports of the presence of plasmid-like DNAs (pDNAs) in phytopathogenic fungi (Garber *et al.*, 1984; Giasson & Lalonde, 1987; Hashiba *et al.*, 1984; Honeyman & Currier, 1986; Kim *et al.*, 1988; Kistler & Leong, 1986; Rubidge, 1986; Samac & Leong, 1988; Tudzynski & Esser, 1986). In each case the pDNAs were linear molecules. Most of the linear pDNAs in phytopathogenic fungi have terminal proteins covalently attached to the 5' termini (Kistler & Leong, 1986). Recently, we showed that the pRS64 plasmids found in one isolate of AG-4 in *R. solani* have hairpin loops at both termini (Miyashita *et al.*, 1990).

Little is known about the function of these linear pDNAs in phytopathogenic fungi. The unique mitochondrial plasmids found in *Fusarium oxysporum* were correlated with different pathotypes that attack crucifers (Kistler & Leong, 1986; Kistler *et al.*, 1987) and the pRS64 plasmids were found in a weakly pathogenic isolate of *R. solani* (Hashiba *et al.*, 1984; Hashiba, 1987*a, b*).

In this report we describe the properties of pDNAs found in 48 isolates from nine AGs and ISGs of *R. solani* isolated from various plants and soils in Japan. A preliminary account of this work has appeared elsewhere (Hashiba, 1988; Miyasaka *et al.*, 1988).

Methods

Fungal isolates. Fungal isolates used in the study (Table 1) were maintained on potato sucrose agar medium at ambient laboratory temperature. Cultures for DNA extraction were grown to the stationary phase at 28 °C in a potato/sucrose liquid medium as described previously (Hashiba *et al.*, 1984). After 1–2 weeks, mycelia were harvested, washed, frozen at –70 °C, and lyophilized.

Detection and preparation of pDNA. pDNA was detected using a slight modification of the procedure of Hirt (1967). Ten grams of lyophilized mycelium was ground to powder and suspended in 160 ml 0.1 M-citrate/phosphate buffer (pH 6.0) containing 0.1 M-EDTA and then lysed by adding 20 ml 10% (w/v) SDS solution. After the addition of 5 M-NaCl to a final concentration of 1 M, this mixture was kept on ice overnight and then centrifuged at 15000 *g* for 30 min. The mixtures were precipitated with 0.6 volumes of 2-propanol at 0 °C, and the precipitates were collected by centrifugation. The total DNA precipitated was resuspended in 7 ml TE (20 mM-Tris, 0.25 mM-EDTA, pH 8.0) buffer containing 0.4% Sarkosyl, and purified by CsCl density-

Abbreviations: AG, anastomosis group; ISG, intraspecific group; pDNA, plasmid-like DNA.

Table 1. Detection of pIDNA in total DNA of *R. solani* isolates studied

AG	Pathological type	Isolate	Host species or locality	pIDNA	Size of pIDNA (kb)
1	Sasakii type (IA)	C-54	<i>Oryza sativa</i>	—	5.5
		C-154		—	
		C-418		—	
		C-501		—	
		Cs-4	<i>Sorghum sudanense</i>	—	
		Cs-Ni		—	
		No. 00		—	
		RI-2-2		—	
	Web-blight type (IB)	131	<i>Oryza sativa</i>	—	
		C-527		+	
		CB-74-22		—	
		No. 15		—	
		RI-2-1	<i>Lactuca sativa</i> Soil <i>Trifolium subterraneum</i> <i>Robinia pseudoacacia</i> var. <i>umbraculifera</i> <i>Raphanus sativus</i> <i>Beta vulgaris</i> var. <i>saccharifera</i>	—	
		Rs-chiba-1		—	
		F3		—	
				—	
2-1	Winter crop type (II)	BO-1	<i>Brassica oleracea</i>	—	
		C-121	<i>Juncus effusus</i> var. <i>decipiens</i>	—	
		HV-1	<i>Hordeum vulgare</i>	—	
		PS-2	<i>Pisum sativum</i>	—	
		RI-2-8	<i>Fragaria ananassa</i>	—	
		RI-28	<i>Brassica campestris</i>	—	
2-2	Rush type (IIIB)	BV-30	<i>Beta vulgaris</i> var. <i>saccharifera</i>	—	
		BV-43		—	
		C-6	<i>Juncus effusus</i> var. <i>decipiens</i>	+	5.2, 3.2
		C-94		+	4.0, 3.0
		C-100		+	2.2
		C-113		+	3.0, 2.2
		C-116	Soil	+	3.0, 2.2
		C-125		+	4.3, 2.9
		C-127		+	3.4
		C-148		+	2.2
	Root rot type (IV)	C-155	<i>Oryza sativa</i>	—	4.2, 2.9 4.7, 2.9 2.2 4.7 4.1 5.3, 4.4, 2.4 4.0, 2.4 4.0, 2.7 3.0 5.5, 2.9 5.4, 4.7 4.4 — 5.0, 4.2
		C-328		+	
		C-329		+	
		C-330		+	
		A-10	<i>Beta vulgaris</i> var. <i>saccharifera</i>	—	
		A-14-1		+	
		B-13		+	
		B-52		+	
		BV-3	<i>Zoysia tenuifolia</i>	—	
		BV-6		+	
		C-34		+	
		C-616		+	
		C-623	<i>Beta vulgaris</i> var. <i>saccharifera</i>	+	
		H-16		+	
		PC-1	Not clear	+	
		SA65-26	Soil	—	
		SH-17-24-1	Not clear	+	

3	Potato type (IV)	C-564	<i>Solanum tuberosum</i>	-	
		NR-3		+	7.0
		P-5		+	6.2
		P-7-1		+	6.2
		R-468		+	6.2, 4.2
		R-526		-	
		R-529		-	
		R-530		-	
		R-542		-	
		ST1-1		+	6.6
		ST-3		+	6.2
		ST3-1		-	
		ST4-1		+	6.6, 4.4
		ST-6		-	
		ST-9		+	6.4, 4.4
		ST11-6		+	6.4, 4.4
		ST-21		+	6.4, 4.4
4	Praticola type (IIIA)	AH-1	<i>Arachis hypogaea</i>	-	
		AH-3		-	
		BO-3	<i>Brassica oleracea</i> (botrytis group)	+	2.7
		C-549	Soil	-	
		C-656	<i>Arachis hypogaea</i>	-	
		Chr-3	<i>Chrysanthemum morifolium</i>	+	2.7
		GM-7	<i>Glycine max</i>	+	2.7
		GM-11		+	2.7
		HI521-21	Soil	-	
		L-1	<i>Vigna sinensis</i>	-	
		P-10	<i>Solanum tuberosum</i>	-	
		R97	<i>Beta vulgaris</i> var. <i>saccharifera</i>	+	2.7
		R101	<i>Brassica oleracea</i>	+	2.7
		Rh-131	<i>Beta vulgaris</i> var. <i>saccharifera</i>	-	
		RI-64	<i>Raphanus sativus</i> (daikon group)	+	2.7
		SH-34	Soil	+	2.7
		U-6	<i>Dahlia</i> spp.	-	
		1271C	<i>Raphanus sativus</i>	-	
5		AL-1	<i>Arctium lappa</i>	-	
		C-672	<i>Solanum tuberosum</i>	-	
		GM-1	<i>Glycine max</i>	-	
		GM-10		-	
		L-12	<i>Phaseolus radiatus</i> var. <i>aurea</i>	-	
		R-35	<i>Phaseolus vulgaris</i>	-	
		SD-521-18	Soil	-	
		SH-30		-	
		SH-31		-	
		ST2-1	<i>Solanum tuberosum</i>	+	3.2
		ST6-1		-	
		ST-8		+	3.2
6		W-3	<i>Triticum aestivum</i>	+	3.2
		WD-1		-	
		WD-23		-	
		501	<i>Convallaria keiskei</i>	+	3.2
		Br-28	Soil	-	
		HAM-1-1		-	
		HI-5-11		+	5.2
		HI-5-16		+	5.2
		HN1-1		-	
		IS1-1		-	
		KNB2-2		+	2.6
		NAT-3-1		-	
		NKN-2-1		-	
		OHT1-1		-	
		SH-13	<i>Orchis aristata</i>	-	
		Shi741		-	
		SO2-1	Soil	-	
		TSH1-1		-	
		UBU1-A		+	5.6

Fig. 1. Detection of plDNAs and Southern hybridization analysis of total DNAs from isolates of different AGs and ISGs. (a) Total DNA from different *R. solani* isolates was separated by electrophoresis and stained with ethidium bromide. (b) Total DNA from the gel in (a) was blotted onto nitrocellulose and hybridized to the entire plDNA of isolate RI-64 (AG-4) labelled with ^{32}P . Lane 1, λ DNA digested with *Hind*III; 2, isolate C-527; 3, C-94; 4, C-616; 5, H-16; 6, BV-6; 7, SH-17-24-1; 8, P-7-1, 9, P-5; 10, GM-7; 11, GM-11; 12, RI-64; 13, B-52; 14, HI-5-16.

gradient centrifugation [7 g CsCl and 0.4 ml ethidium bromide (10 mg ml^{-1})]. The samples were centrifuged at $95000 g$ for 48 h. The bands removed from the gradients were extracted four times with butanol, precipitated with ethanol, and dissolved in TE buffer. A portion of the DNA sample thus obtained was subjected to agarose gel electrophoresis in 0.7% agarose. The plDNA band(s) was (were) excised and electroeluted. All isolates were independently analysed three times by gel electrophoresis for the presence of plDNA.

Electron microscopy. A DNA-protein monolayer for electron microscopy was prepared by the formamide and cytochrome *c* technique as described by Kleinschmidt (1968). Double-stranded pBR322 (4361 kb) served as an internal length standard. Specimens were observed in a JEM100B type electron microscope.

Southern transfer and hybridization conditions. Total cellular DNAs were fractionated by electrophoresis in a 0.7% agarose gel, and transferred to nitrocellulose filters. Each plDNA isolated from the

representative isolates of AG-1 to AG-6 was labelled with [α - ^{32}P]dCTP by nick-translation and the radioactively labelled product was hybridized for 24 h at 42°C to DNA in nitrocellulose filters in 50% (v/v) formamide, $6 \times \text{SSC}$, $100 \mu\text{g}$ denatured salmon sperm DNA ml^{-1} , $5 \times \text{Denhardt's solution}$ and 0.5% (w/v) SDS ($1 \times \text{SSC}$ is 0.15 M-NaCl , $0.015 \text{ M-sodium citrate pH } 7.0$; $1 \times \text{Denhardt's solution}$ is 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone). Hybridization patterns were visualized by autoradiography. All these procedures were carried out as described by Maniatis *et al.* (1982).

Results

Detection of plDNA by agarose gel electrophoresis

Total DNA was extracted from 114 field isolates of *R.*

solani (15 isolates of AG-1, 6 isolates of AG-2-1, 14 isolates of AG-2-2 (IIIB), 13 isolates of AG-2-2 (IV), 17 isolates of AG-3, 18 isolates of AG-4, 16 isolates of AG-5, and 15 isolates of AG-6) (Table 1), and purified by CsCl density-gradient centrifugation. These DNA samples were subjected to agarose gel electrophoresis. The pDNA was separated as one, two or three bands stained with ethidium bromide as illustrated in Fig. 1 (a). pDNA bands were observed in 48 isolates (Table 1), which were distributed among the nine Japanese AGs and ISGs as follows: 0 in AG-1 (IA), 1 in AG-1 (IB), 0 in AG-2-1, 11 in AG-2-2 (IIIB), 10 in AG-2-2 (IV), 10 in AG-3, 8 in AG-4, 4 in AG-5, and 4 in AG-6. All isolates of AG-1 (IA) and AG-2-1 contained no detectable pDNA.

The sizes of the pDNAs detected in the *R. solani* isolates of different AGs varied from 2.2 kb to 7.0 kb (Table 1). The pDNAs found in the eight isolates of AG-4 and the four isolates of AG-5 each had the same size, 2.7 kb and 3.2 kb, respectively (Table 1). The sizes of the pDNAs found in AG-2, AG-3 and AG-6 varied widely (Table 1). The same or similar sizes of pDNAs were distributed in different AGs.

Structure of pDNAs

The pDNAs of representative isolates from eight AGs and ISGs were separated from agarose gel and subjected to electron microscopy. All the pDNAs examined consisted of linear DNA molecules, and no circular molecules, as observed for marker pBR322, were detected (Fig. 2). The sizes of the DNAs estimated by measuring the lengths of linear pDNA molecules under the electron microscope were in close agreement with those estimated on the basis of mobility on agarose gel.

Sequence homology among pDNAs found in various *R. solani* isolates

pDNAs of different sizes were found in the same or different isolates of *R. solani*. To determine whether these pDNAs had sequence homology, a Southern hybridization analysis was done. The entire lengths of pDNAs obtained from representative isolates of eight AGs and ISGs were nick-translated and used as hybridization probes. Examples of the resulting autoradiographs are shown in Fig. 1 (b). Considerable sequence homology was observed among pDNAs obtained from isolates within the same AG and ISG, but no sequence homology was found among pDNAs obtained from isolates of different AGs and ISGs (Table 2).

In most isolates of AG-2-2 examined, one of two pDNAs obtained from the same isolate hybridized with

Fig. 2. Electron micrographs of pDNA molecules from two isolates of *R. solani* by the formamide and cytochrome *c* technique. (a) pDNA molecules from isolate P-7-1 (AG-3); (b) pDNA molecules from isolate ST-8 (AG-5). Bars, 1 µm.

the probe, but in some isolates of AG-2-2 both pDNAs obtained from the same isolate hybridized with the same probe (Table 2). Within AG-2-2, the pDNAs of AG-2-2 (IIIB) isolates hybridized with the probe of AG-2-2 (IIIB) (isolate C-94), but not with that of AG-2-2 (IV) (isolate H-16). In contrast, the pDNAs of AG-2-2 (IV) isolates hybridized with the probe of AG-2-2 (IV), but not with that of AG-2-2 (IIIB) (Table 2). The pDNA of one isolate of AG-2-2 (isolate BV-6) hybridized with both probes.

Discussion

Analysis of total DNA from *R. solani* field isolates of different AGs and ISGs showed that about half of the isolates (48 out of 114) contained pDNA. The percentage of isolates carrying pDNA (42%) is much higher than that reported previously (Hashiba *et al.*, 1984). The pDNAs carried by the 48 isolates ranged from 2.2 to 7.0 kb in size. In the case of AG-4 and AG-5, the pDNAs of each isolate in the respective AG had the same size, 2.7 kb and 3.2 kb, respectively. pDNA was

Table 2. Southern hybridization analysis of *plDNA* from *R. solani* AG-1 to -6 isolates probed with *plDNA* from a representative isolate of each AG or ISG

Key: [P], the entire length of a *plDNA* obtained from the representative isolates of one AG or ISG was used as the hybridization probe; [+], considerable sequence homology was observed; -, no sequence homology was observed.

AG	Pathological type	Isolate	Size of <i>plDNA</i> (kb)	Source of probe:						
				C-527 (AG-1)	C-94 (AG-2-2 IIIB)	H-16 (AG-2-2 IV)	P-7-1 (AG-3)	RI-64 (AG-4)	ST-8 (AG-5)	HI-5-16 (AG-6)
1	Web-blight type (IB)	C-527	5.5	[P]	-	-	-	-	-	-
2-2	Rush type (IIIB)	C-6	5.2	-	-	-	-	-	-	-
			3.2	-	+	-	-	-	-	-
		C-94	4.0	-	-	-	-	-	-	-
			3.0	-	[P]	-	-	-	-	-
		C-125	4.3	-	-	-	-	-	-	-
			2.9	-	+	-	-	-	-	-
		C-328	4.2	-	-	-	-	-	-	-
			2.9	-	+	-	-	-	-	-
		C-329	4.7	-	-	-	-	-	-	-
			2.9	-	+	-	-	-	-	-
	Root rot type (IV)	A-14-1	4.7	-	-	+	-	-	-	-
		BV-6	4.0	-	+	+	-	-	-	-
			2.4	-	+	-	-	-	-	-
		C-616	3.0	-	-	+	-	-	-	-
		C-623	5.5	-	-	-	-	-	-	-
			2.9	-	-	+	-	-	-	-
		H-16	5.4	-	-	+	-	-	-	-
			4.7	-	-	[P]	-	-	-	-
		SH-17-24-1	5.0	-	-	+	-	-	-	-
			4.2	-	-	+	-	-	-	-
3	Potato type (IV)	P-5	6.2	-	-	-	+	-	-	-
		P-7-1	6.2	-	-	-	[P]	-	-	-
		ST-3	6.2	-	-	-	+	-	-	-
4	Praticola type (IIIA)	GM-7	2.7	-	-	-	-	+	-	-
		GM-11	2.7	-	-	-	-	+	-	-
		RI-64	2.7	-	-	-	-	[P]	-	-
5		ST-2-1	3.2	-	-	-	-	-	+	-
		ST-8	3.2	-	-	-	-	-	[P]	-
		W-3	3.2	-	-	-	-	-	+	-
		501	3.2	-	-	-	-	-	+	-
6		HI-5-16	5.2	-	-	-	-	-	-	[P]

not found in the six isolates of AG-2-1 examined, and *plDNA* was found in only one of the 15 isolates of AG-1. The frequency of distribution of isolates carrying *plDNAs* may be different depending on the AGs and ISGs or host plants.

Each isolate of AG-2-2 and AG-3 that carried *plDNA* contained one, two or three *plDNAs* of different sizes identified by gel electrophoresis, whereas the *plDNA*-carrying isolates of AG-1, AG-4, AG-5 and AG-6 contained only one *plDNA*. Originally we found the pRS64 plasmid as a single band on agarose gel (Hashiba *et al.*, 1984; Hashiba, 1987*a, b*), but subsequent restriction mapping showed that pRS64 actually consisted of three species of plasmids, pRS64-1, -2 and -3

(Miyashita *et al.*, 1990). This suggests that different species of plasmids may well be involved in bands of the same size observed in the present study.

Electron micrographs of purified *plDNAs* revealed that they were linear molecules. Most linear plasmid DNAs, including the linear plasmids found in phytopathogenic fungi, have terminal proteins covalently attached to the 5' termini. These proteins are thought to work as primers. The three linear plasmid DNAs, pRS64-1, -2 and -3, obtained from the isolate RI-64 (AG-4) have hairpin loops at both termini (Miyashita *et al.*, 1990). Southern blotting analysis of total DNA obtained from RI-64 showed the presence of dimeric forms of pRS64 DNA (Miyashita *et al.*, 1990). We suggested that

these dimeric forms of pRS64 may be involved in the replication system of pRS64 DNA, and proposed a possible model for replication of pRS64 plasmids (Miyashita *et al.*, 1990). More work is required to determine whether all pDNAs of *R. solani* have the same hairpin loop structure at both termini.

Linear mitochondrial plasmids have been detected in a wide variety of phytopathogenic fungi (Tudzynski *et al.*, 1986; Garber *et al.*, 1986). In order to determine the location of pDNAs in AG-4 of *R. solani*, mitochondria were isolated by differential centrifugation. After DNAase treatment of mitochondria the pDNAs were still found, indicating their mitochondrial location (unpublished results). However, these experiments do not rule out the possibility that the pDNA exist extramitochondrially in the cytoplasm as well as within mitochondria.

Southern hybridization analysis of various *R. solani* isolates showed that the pDNAs have considerable sequence homology among isolates of the same AG and ISG, but not among those of different AGs and ISGs. This suggests that the distribution of pDNAs correlates with the distinct host range of AG and ISG in *R. solani*, and may be used for the classification of this fungus. The relationship between the pDNAs and AGs or ISGs is in agreement with the results of DNA base sequence homology (Kuninaga & Yokosawa, 1985), serological comparisons (Adams & Butler, 1979), DNA base comparison (Kuninaga & Yokosawa, 1980), ecological and morphological characteristics (Ogoshi, 1976), and electrophoresis of isoenzymes (Matsuyama *et al.*, 1978) and soluble proteins (Reynolds *et al.*, 1983). Since these pDNAs are host-specific, they may carry genes that determine host specialization. We wish to determine whether these pDNAs contain open reading frames that are transcribed and translated. This will require a transformation system for *R. solani* to be established. The relationship between the pDNAs and the nature of AGs may also be indicated by using plasmid-cured strains of *R. solani*. We hope to resolve the question by manipulating plasmid-free, plasmid-containing and cured strains by protoplast fusion.

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