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RFLP analysis of the PCR-amplified 28S rDNA in *Rhizoctonia solani*

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RFLP analyses of a portion of the 28S rDNA gene region were conducted by using four restriction endonucleases for 57 isolates of 13 intraspecific groups (ISGs) representing 7 anastomosis groups (AGs) of *Rhizoctonia solani*. Variations in the PCR-amplified rDNA products and the polymorphisms on digestion with restriction enzymes (*Bam*HI, *Hae*III, *Hha*I and *Hpa*II) were observed among three AGs, AG 1, 2 and 4. These differences were also conserved among some ISGs of AG 1 and AG 2. Among ISGs of AG 1, the pattern of rDNA fragments of AG 1-IA obtained by digestion with *Hpa*II was significantly different from those of AG 1-IB and IC. Such difference in the fragment pattern was also observed among AG 2-1, 2-2 IIIB and 2-2 IV by the digestion with *Hha*I and *Hpa*II. A dendrogram derived from the restriction enzyme data showed that ISGs from AG 1 and AG 2 can each be subdivided into distinct groups, those are distantly related to the majority isolates of the other AGs.

Key Words——genetic variation; intraspecific groups; PCR-amplified 28S rRNA; *Rhizoctonia solani*.

Rhizoctonia solani Kühn (teleomorph *Thanatephorus cucumeris* (Frank) Donk), a soil-borne saprophyte with peculiar versatility, has been considered as a species complex because of its many intraspecific variations in pathogenicity, host range, and physiological and morphological characteristics (Adams, 1988). Although researchers have studied the classification of *R. solani* based on these characteristics, the relationships among them are not clear. Recently, a classification method based on anastomosis behavior of vegetative hyphal fusion has been used for identifying many intraspecific variations, and at least, 11 anastomosis groups (AGs) have been reported, although the mechanisms of anastomosis behavior are not fully understood (Anderson, 1982; Carling et al., 1987; Homma et al., 1983; Kuninaga et al., 1978; Ogoshi, 1987; Ogoshi et al., 1990).

Many AGs have now been described and genetic relatedness among groups of *R. solani* based on AG concepts has been investigated by various measures, though no direct correlation has been found between AGs and the groups based on the conventional criteria (Adams, 1979; Bharthan and Tavantzis, 1991; Jabaji-Hare et al., 1990; Matsuyama et al., 1978; Reynolds et al., 1983; Vilgalys and Gonzalez, 1990). Genetic variation within AGs of *R. solani* was detected by comparative study of the evolutionally conserved molecular markers (Liu et al., 1990, 1992, 1993; Liu and Sinclair, 1993). In this context, ribosomal genes are phylogenetically and taxonomically informative and their analysis supported the results from previous attempts to clarify AG relationships by various molecular biological studies (Bruns et al., 1991; Liu et al., 1990, 1992, 1993; Liu and Sinclair, 1993; Michelmore et al., 1987; Vilgalys and Gonzalez, 1990). In this investigation an attempt was made to characterize AGs based on the results of amplification of the polymerase chain reaction (PCR), followed by analysis of restriction fragment length polymorphisms (RFLPs) of nuclear-encoded rDNA from isolates of *R. solani*.

Materials and Methods

Fungal isolates and maintenance Fifty-seven isolates of *R. solani* used in this study are listed in Table 1. The isolates were maintained on potato-dextrose agar (PDA, Funakoshi Chem.) slants amended with 50 mg/L streptomycin sulfate at 25°C in the dark. To prepare DNA samples for restriction analysis, three thin mycelial disks of each isolate cultured on PDA, 4 mm in diam, were gently floated on 50 ml of a liquid nutrient-broth medium (Difco) amended with 50 mg/L of streptomycin sulfate in 100-ml flask and incubated at 25°C for 7 d. The mycelial mat was harvested, washed repeatedly with water, lyophilized and then stored at -20°C until use.

Extraction of DNA and amplification with PCR Genomic DNA was extracted by the procedure of Vilgalys and Gonzalez (1990) with slight modifications. The lyophilized and ground mycelia (30 mg) were homogenized in 450 μl of extraction buffer (50 mM Tris-HCl, pH 7.2, 50 mM EDTA, 1% sodium *N*-lauroyl sarcosinate, 1% 2-mercaptoethanol) and incubated at 65°C for 30 min. The homogenate was thoroughly shaken using 450 μl of chloroform/phenol/isoamyl alcohol (24 : 25 : 1, v/v/v) mixture. After centrifugation for 15 min at 15,000 rpm, the water phase was collected and ammonium acetate was added to a concentration of 2.5 M. DNA was precipitated with 225 μl of isopropanol, then collected by

Table 1. Isolates of *Rhizoctonia solani* used in this study.

Isolate	AG/ISG	Source	Origin
Cs-Ka	1-IA	Rice	AHU[a]
Cs-2	1-IA	Rice	AHU
Cs-Gi	1-IA	Rice	AHU
Cs-IW	1-IA	Rice	AHU
R-1-2-2	1-IA	Rice	NIAS[b]
Shiba-2	1-IB	Soil	AHU
B 54	1-IB	Sugar cane	AHU
Rs-chi-2	1-IB	Radish	AHU
CB-515-3	1-IB	Soil	AHU
HK-616-23	1-IB	Soil	AHU
BV-7	1-IC	Sugar beet	AHU
F-2	1-IC	Flax	AHU
P1	1-IC	Potato	AHU
P10	1-IC	Potato	AHU
RH-28	1-IC	—	AHU
SH-3	2-1	Soil	AKU[c]
SH-5	2-1	Soil	AKU
F-15	2-1	Flax	AKU
TG-1	2-1	Tulip	AKU
BO-1	2-1	Cabbage	MAFF[d]
C-96	2-2 IIIB	Mat rush	AHU
C-100	2-2 IIIB	Mat rush	MAFF
C-116	2-2 IIIB	Mat rush	MAFF
C-321	2-2 IIIB	Mat rush	AKU
C-323	2-2 IIIB	Mat rush	AKU
B-50	2-2 IV	Sugar beet	AHU
B-70	2-2 IV	Sugar beet	AHU
RI-64	2-2 IV	Sugar beet	AHU
B-2	2-2 IV	Sugar beet	AHU
BV-26	2-2 IV	Sugar beet	AHU
ST-2	3	Potato	AKU
ST-7	3	Potato	AKU
ST-9	3	Potato	AKU
P-5	3	Potato	MAFF
NR-3	3	Potato	MAFF
AH-1	4 HG-I	Peanut	S. Kuninaga
Chr-3	4 HG-I	Chrysanthemum	S. Kuninaga
R97	4 HG-I	Sugar beet	S. Kuninaga
RR-5-2	4 HG-II	Sugar beet	S. Kuninaga
Rh-165	4 HG-II	Sugar beet	S. Kuninaga
UHBC	4 HG-II	Sugar beet	S. Kuninaga
SH-25	5	Soil	AKU
SH-26	5	Soil	AKU
SH-29	5	Soil	AKU
SH-30	5	Soil	MAFF
ST-8	5	Potato	MAFF
NTA 3-1	6	Soil	S. Kuninaga
OHT 1-1	6	Soil	S. Kuninaga
HAM 1-1	6	Soil	S. Kuninaga
MAFF 305552	7	Soil	MAFF
MAFF 305553	7	Soil	MAFF
MAFF 305554	7	Soil	MAFF
MAFF 305555	7	Soil	MAFF
MAFF 305263	BI	Soil	MAFF
MAFF 305264	BI	Soil	MAFF
MAFF 305265	BI	Soil	MAFF
MAFF 305266	BI	Soil	MAFF

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d) Ministry of Agriculture, Forestry and Fisheries, Ibaraki, Japan.

centrifugation at 13,000 rpm for 15 min. The pellet was washed with 70% aqueous ethanol solution, dried *in vacuo*, dissolved in 120 μl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and incubated at 37.5°C for 30 min for treatment with ribonuclease A (20 μg/ml, DNase-free; Sigma, USA).

Two primers used in this study were synthesized for the amplification of a portion of the *R. solani* 28S rDNA repeat homologous to positions 17-1,448 within the *Saccharomyces cerevisiae* 25S rRNA according to the procedure of Vilgalys and Hester (1990). Ten μl of 100-fold dilution of DNA solution prepared by the above procedure was added to the reaction mixture as recommended by the manufacturer using *Tth* DNA polymerase (Toyobo Biochemicals, Japan) in 100 μl volume. The thermal cycles were conducted 30 times, with parameters of 1 min at 94°C, 2 min at 50°C, 1 min at 50-72°C for gradual increase and 3 min at 72°C. After amplification, each sample was subjected to electrophoresis using a 1% agarose gel in TBE buffer (100 mM Tris-HCl, 20 mM EDTA, 100 mM boric acid, pH 8.3).

DNA restriction and data analysis After preparation of PCR-amplified rDNA, each PCR product was digested with four restriction enzymes (*Bam*HI, *Hae*III, *Hha*I and *Hpa*II) and the sample was subjected to electrophoresis in a 3.5% agarose gel (NuSieve, FMC Bioproducts) with TBE buffer.

Relative similarities among isolates were estimated by the cluster analysis. A resemblance matrix based on Euclidean distance coefficients was computed from pairwise comparison of each isolate with every other isolate based on the number and size of digested fragments with each restriction enzyme. A dendrogram was constructed by an unweighted paired group method with arithmetic averages (UPGMA) using the statistics software package SYSTAT 5.2.1.

Results

The 28S rDNA products, a single 1.4-kbp or 1.8-kbp fragment, were obtained by agarose gel electrophoresis following amplification with PCR (Fig. 1). Variations in sizes of rDNA products were detected for the test isolates belonging to different ISGs within each of AG-1, 2 and 4. A single 1.4-kbp fragment was obtained from five test isolates (AG 1-IB, AG 1-IC, AG 2-2 IV, AG 4 HG-II and AG 7) and a 1.8-kbp fragment was obtained from the remaining isolates (AG 1-IA, AG 2-1, AG 2-2 IIIB, AG 3, AG 4 HG-I, AG 6 and AG BI). Isolates of AG 5 produced both 1.4- and 1.8-kbp fragments. No qualitative variation of PCR-amplified products of 28S rDNA was observed among isolates of each ISG in *R. solani*.

RFLP profiles obtained after digestion of PCR-amplified 28S rDNA represented specific fragment patterns by electrophoresis (Figs. 2, 3; Table 2). Three enzymes, *Bam*HI, *Hha*I and *Hpa*II, were used in dividing different ISGs including the AGs of *R. solani*. Among AGs, isolates of subgroups within each of AG 1, 2, and 4 showed specific fragment patterns as well as the variation in size of amplified 28S rDNA among different ISGs. RFLP pat-

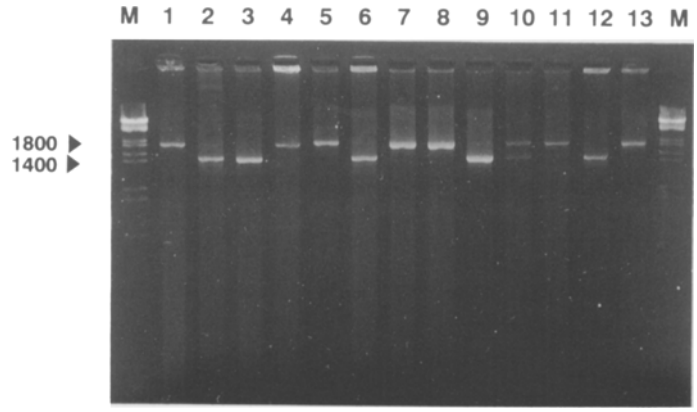

Fig. 1. Electrophoretic pattern of PCR-amplified 28S rDNA of 13 ISGs of *R. solani* in a 1.0% agarose gel.
M, Molecular marker (λ-DNA digested with *Bam*HI and *Hind*III); lane 1, Cs-Ka; lane 2, B-54; lane 3, BV-7; lane 4, BO-1; lane 5, C-96; lane 6, RI-64; lane 7, ST-2; lane 8, AH-1; lane 9, RR-5-2; lane 10, SH-25; lane 11, OHT 1-1; lane 12, MAFF 305552; lane 13, MAFF 305263. Sizes of DNA fragments (kbp) are indicated on the left.

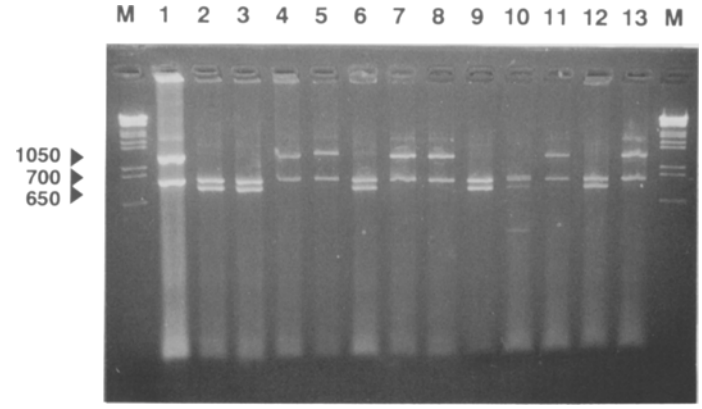

Fig. 2. Electrophoretic pattern of PCR-amplified 28S rDNA of 13 ISGs of *R. solani* digested with *Bam*HI in a 3.5% agarose gel.
M, Molecular marker (λ-DNA digested with *Bam*HI and *Hind*III); lane 1, Cs-Ka; lane 2, B-54; lane 3, BV-7; lane 4, BO-1; lane 5, C-96; lane 6, RI-64; lane 7, ST-2; lane 8, AH-1; lane 9, RR-5-2; lane 10, SH-25; lane 11, OHT 1-1; lane 12, MAFF 305552; lane 13, MAFF 305263. Sizes of DNA fragments (kbp) are indicated on the left.

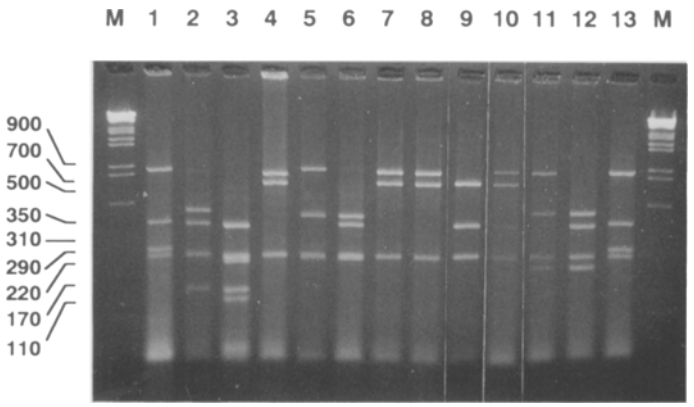

Fig. 3. Electrophoretic pattern of PCR-amplified 28S rDNA of 13 ISGs of *R. solani* digested with *Hpa*II in a 3.5% agarose gel.
M, Molecular marker (λ-DNA digested with *Bam*HI and *Hind*III); lane 1, Cs-Ka; lane 2, B-54; lane 3, BV-7; lane 4, BO-1; lane 5, C-96; lane 6, RI-64; lane 7, ST-2; lane 8, AH-1; lane 9, RR-5-2; lane 10, SH-25; lane 11, OHT 1-1; lane 12, MAFF 305552; lane 13, MAFF 305263. Sizes of DNA fragments (kbp) are indicated on the left.

Table 2. Restriction fragment sizes (bp) of PCR-amplified 28S rDNA for 13 intraspecific groups of *R. solani* by four endonucleases.

AG	Restriction enzyme			
	*Bam*HI	*Hha*I	*Hpa*II	*Hae*III
AG-1 IA	700, 1050	180, 600, 850	220, 290, 350, 900	150, 550, 650
AG-1 IB	650, 700	180, 250, 600	170, 290, 350, 500	150, 550, 650
AG-1 IC	650, 700	180, 250, 600	110, 170, 220, 290, 350	150, 550, 650
AG-2-1	700, 1050	180, 770, 850	290, 700, 900	150, 550, 650
AG-2-2 IIIB	700, 1050	180, 770, 850	290, 500, 900	150, 550, 650
AG-2-2 IV	650, 700	180, 250, 770, 850	290, 350, 500	150, 550, 650
AG-3	700, 1050	180, 770, 850	290, 700, 900	150, 550, 650
AG-4 HG-I	700, 1050	180, 770, 850	290, 700, 900	150, 550, 650
AG-4 HG-II	650, 700	180, 250, 770	290, 350, 700	150, 550, 650
AG-5	650, 700	180, 770, 850	290, 700, 900	150, 550, 650
AG-6	700, 1050	180, 770, 850	220, 290, 500, 900	150, 550, 650
AG-7	650, 700	180, 250, 770	220, 290, 350, 500	150, 550, 650
AG-BI	700, 1050	180, 770, 850	290, 310, 350, 900	150, 550, 650

terns of the isolates AG 1-IB and AG 1-IC were different from that of AG-1 IA. After digestion with *Hpa*II, a 170-bp fragment was obtained from AG 1-IB and 1-IC, but not from AG 1-IA. Moreover, 110- and 220-bp fragments were obtained from AG 1-IC (Fig. 3). The test isolates AG 2-1 and AG 2-2 also showed similar fragment patterns after digestion with *Hpa*II (Fig. 3). *Hpa*II digestion produced a 700-bp fragment in AG 2-1 and one of 900-bp in AG 2-2. Moreover, *Hha*I digestion revealed a variation between AG 2-2 IIIB and AG 2-2 IV isolates. Isolates of AG 2-2 IV produced a specific 250-bp restriction fragment (Table 2). Moreover, the test isolates of AG 4 HG-1 and HG-II had different RFLP patterns of digestion with *Bam*HI, *Hha*I and *Hpa*II (Figs. 2, 3; Table 2).

After digestion of amplified 28S rDNA with *Bam*HI, all test isolates produced two kinds of fragments, a common 700-bp fragment and a 600- or a 1050-bp fragment (Table 2). In the case of AG 1-IA, other bands than these three bands stated above, were detected. These bands could appear as the resemble of the high concentration of the sample. All isolates produced three different fragments by digestion with *Hha*I: a common 180-bp and two out of four fragments such as 250-, 600-, 770- and 850-bp. Exceptionally, AG 2-2 IV isolates contained the unique set of 250-, 770- and 850-bp fragments. After digestion with *Hpa*II, a common 290-bp and two or three out of 220-, 350-, 500-, 700- and 900-bp fragments were obtained from test isolates of all ISGs. Significantly, AG BI isolates produced a unique set of 310-, 350- and 900-bp fragments. All of the test isolates yielded common 150-, 550- and 650-bp fragments on digestion with *Hae*III (Table 2).

Based on fragment patterns of digests of amplified rDNA with *Bam*HI, *Hha*I and *Hpa*II, the relations among isolates in AGs were investigated (Fig. 4). Two putative groups of *R. solani* isolates could be identified by the

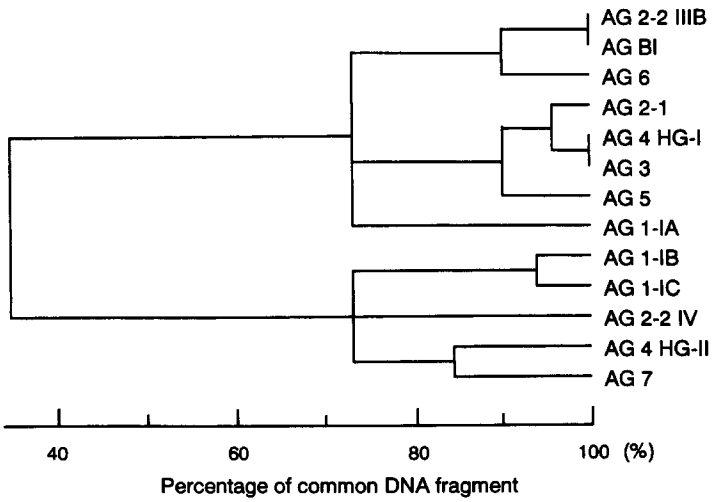

Fig. 4. Dendrogram showing the phylogenetic relationship of 13 ISGs of *R. solani*.

cluster analysis based on their RFLP profiles. These groups were also related with the sizes of PCR-amplified rDNA products. Three groups which are independent of the old ISGs based on pathological, physiological and biochemical properties in AG 2 were significantly dispersed in the dendrogram. Six putative clusters in *R. solani* were detected by The RFLP profiles with up to 70% homology.

Discussion

Genetic differences among the isolates of different AGs in *R. solani* have been discerned by DNA restriction analysis using PCR-amplified 28S rDNA. One advantage of this analysis in comparison with previous DNA/DNA hybridization studies is the smaller amount of DNA that it requires. Another advantage is that restriction fragment data are usable to estimate genetic distance according to their systematic and genetic similarity (Vilgalys and Gonzalez, 1994). Therefore, genetic relationships among different ISGs of *R. solani* were investigated based on variations in the fragment data on digestion with four restriction endonucleases using a portion of the rDNA repeat coding for 28S rDNA.

Restriction analysis of PCR-generated rDNA fragments showed that the variations in amplified rDNA existed among isolates from three different AGs. Three groups were found in AG 1, three groups in AG 2, and two groups in AG 4. The three groups of the AG 1 isolates related to the three ISGs, IA, IB and IC, based on cultural phenotypes. The isolates from three groups of AG 2 were also correlated with the former three ISGs, AG 2-1, AG 2-2 based on their anastomosis frequencies, and AG 2-2 IIIB and IV based on their pathogenicity and morphology. Liu et al. (1992, 1993) and Liu and Sinclair (1993) reported that patterns of rDNA variation represented at least six groups within AG 1 isolates and five groups within AG 2 isolates based on the restriction analysis for a portion of rDNA repeat coding for the internal transcribed spacer (ITS) sequences including 5.8S rDNA. Furthermore, cellular fatty acid analysis also showed that at least three ISGs existed in AG 1 and five groups within AG 2 isolates (Stevens Johnk and Jones, 1993, 1994). These conclusions suggest that the genetic variations among isolates from each of AG 1 and AG 2 represent phylogenetic diversity in the groups divided by hyphal anastomosis. On the other hand, DNA/DNA hybridization by Kuninaga and Yokosawa (1984a, b) showed that extensive variation occurred within AG 4 and AG 6 according to their DNA similarity. We observed similar variations in the restriction fragment patterns of AG 6 isolates (data not shown), while genetic variation represented two groups within AG 4 isolates (Figs. 2, 3; Table 2). DNA restriction fragment data also showed similar genetic variations among isolates from each of AG 3, 5 and BI.

Cluster analysis of DNA restriction fragment data for isolates of different ISGs in the same AG was conducted to investigate their systematic relationships. AG 1-IA isolates formed a cluster distinct from AG 1-IB and IC. On the other hand, genetic relationships among the three subgroups of AG 2 isolates did not show close systematic positions. Previous attempts at phylogenetic study of several ISGs based on ribosomal DNA sequence data also demonstrated that there were several evolutionary groupings within isolates of *R. solani* (Vilgalys and Gonzalez, 1994). Phylogenetic analysis also indicated that isolates of AG-1, 4 and 6 each had common evolutionary behavior, whereas different ISGs of AG-2 isolates did not closely relate to each other (Liu et al., 1992, 1993). These results, including those of our investigation, indicate that most of AGs would also show phylogenetic evolutionary units, and therefore hyphal anastomosis behavior may not always be the best parameter for studying evolutionary relationships among different ISGs.

We conclude that phylogenetic information is of benefit to characterize the evolutionary relationships among isolates of *R. solani*. Further investigation by comparison of sequence data from PCR-amplified rDNA genes will throw light on evolutionary relationships among different ISGs of *R. solani* isolates or among varied AGs within other *Rhizoctonia* spp. For this purpose, genetic markers have to be identified to distinguish phylogenetically evolutionary groups of *R. solani* more easily and rapidly.

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