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SHORT COMMUNICATION

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Trials of direct detection and identification of *Rhizoctonia solani* AG 1 and AG 2 subgroups using specifically primed PCR analysis

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Abstract Specifically primed polymerase chain reaction (PCR) analysis was used for direct detection and identification of *Rhizoctonia solani* isolates belonging to AG 1 subgroups (IA, IB, and IC) and AG 2 subgroups (2-1 and 2-2). A rapid DNA extraction method with a solution of sodium hydroxide was conducted to extract PCR templates. PCR-specific primer sets for each group were designed from sequences in the regions of the 28S ribosomal DNA of this fungus. The results of specifically primed PCR analysis showed that AG 1-IA, AG 1-IB, AG 1-IC, AG 2-1, and AG 2-2 primers sets contributed detection from the same AG isolates and could escape detection from different AG isolates at a high level of frequency. In this experiment, we suggested that our synthesized primer sets might provide a method for the direct detection and identification of AGs of *R. solani*.

Key words Anastomosis group · *Rhizoctonia solani* · Specifically primed PCR analysis

Plant pathogenic species of *Rhizoctonia* are widely distributed in the world and consist of large and complex groups in fungi (Burpee et al. 1980; Ogoshi 1987). *Rhizoctonia solani* Kühn (teleomorph *Thanatephorus cucumeris* (Frank) Donk), a soil-borne fungus 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). Currently, a classification method based on anastomosis behavior of vegetative hyphal fusion has been used for identifying many intraspecific variations of *R. solani* isolates (Carling 1996; Ogoshi 1976). At least 14 anastomosis groups (AGs) (Carling 1996; Carling et al. 1999; Sneh et al. 1991) have been reported, although the mechanisms of anastomosis behavior are not fully understood (Anderson 1982; Ogoshi 1987).

In our previous study, we attempted direct detection of causal agents of rice sheath diseases by *R. solani* AG 1-IA using specifically primed polymerase chain reaction (PCR) analysis by a rapid DNA extraction method (Matsumoto and Matsuyama 1998), and we successfully detected *R. solani* AG 1-IA isolates from paddy field soils and diseased plant tissues. Therefore, we concluded that this method could become a convenient tool for direct detection and identification of such pathogens on diseased plant tissues and field soils infested with AG 1-IA.

The objective of this study was to classify AGs and AG subgroups of *R. solani* without depending on hyphal anastomosis tests. The hyphal anastomosis test is now becoming the standard method for classifying not only the *R. solani* isolate but also other *Rhizoctonia* spp. isolates. In this experiment, we attempted to use specifically primed PCR analysis for direct detection and identification of AGs and AG subgroups in *R. solani.* It is known that isolates of AG 1 and AG 2 are subdivided into several subgroups and are not easy to classify by hyphal anastomosis tests. Therefore, as the primary examination, we carried out direct detection and identification of isolates belonging to AG 1-IA, AG 1-IB, AG 1-IC, AG 2-1, and AG 2-2, including AG 2-2 IIIB, AG 2-2 IV, and AG 2-2 LP.

Isolates of *R. solani* used in this study are listed in Table 1. All isolates were initially grown on potato-dextrose agar (Difco) in a petri dish. Plugs (4-mm·diameter) taken from the edge of the colonies were placed in 50ml of liquid nutrient-broth medium (Difco) in a 100-ml flask and cultured at 25°C for 7 days. The mycelium was harvested, washed repeatedly with distilled water, lyophilized, and stored at −20°C until use.

PCR amplifications were conducted with DNA from pure fungal cultures. Total genomic DNA as a PCR template was extracted by a quick alkaline DNA extraction method according to Matsumoto et al. (1997). Before DNA extraction, each sample was soaked for 5min in 1ml sterile

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

Isolate	AG/ISG	Source	Origin	Isolate	AG/ISG	Source	Origin
Cs-Ka	1-IA	Rice	ATCC 76121[a]	H4-38-S-1	2-3	Soybean	MAFF 235449
C-325	1-IA	Rice	IFO 30935[b]	H5-307	2-3	Soybean	MAFF 235450
C-326	1-IA	Rice	IFO 30936	H5-354	2-3	Soybean	MAFF 235452
Cs-2	1-IA	Rice	AHU[c]	ST-11-6	3	Potato	ATCC 76167
Cs-Gi	1-IA	Rice	AHU	ST-2	3	Potato	IFO 30983
CB-515-3	1-IB	Soil	AHU	ST-9	3	Potato	IFO 30985
B 54	1-IB	Sugar corn	AHU	NR-3	3	Potato	MAFF 305251
CF-93-18	1-IB	Soil	IFO 30938	P-5	3	Potato	MAFF 305224
B-19	1-IB	Sugar beet	ATCC 76122	AH-1	4	Peanut	S. Kuninaga
HK-616-23	1-IB	Soil	IFO 30939	Chr-3	4	Sugar beet	S. Kuninaga
P-1	1-IC	Potato	AHU	R97	4	Sugar beet	S. Kuninaga
RH-28	1-IC	–	AHU	RR-5-2	4	Sugar beet	S. Kuninaga
F-2	1-IC	Flax	AHU	Rh-165	4	Sugar beet	S. Kuninaga
BV-7	1-IC	Sugar beet	ATCC 76123	UHBC	4	Sugar beet	S. Kuninaga
F-2	1-IC	Flax	AHU	SH-29	5	Soil	IFO 30954
PS-4	2-1	Pea	ATCC 76124	SH-30	5	Soil	MAFF 305256
SH-3	2-1	Soil	IFO 30940	SH-34	5	Soil	MAFF 305253
F-15	2-1	Flax	IFO 30941	GM-10	5	Soybean	ATCC 76128
TG-1	2-1	Tulip	AKU	SH-25	5	Soil	IFO 30952
R1-2-4	2-1	Barley	MAFF 305203[d]	NKN-2-1	6	Soil	S. Kuninaga
C-96	2-2 IIIB	Mat rush	ATCC 76124	NH1-1	6	Soil	S. Kuninaga
C-100	2-2 IIIB	Mat rush	MAFF	OHT-1-1	6	Soil	S. Kuninaga
C-116	2-2 IIIB	Mat rush	MAFF	NAT-3-1	6	Soil	S. Kuninaga
C-321	2-2 IIIB	Mat rush	IFO 30943	HAM-1-1	6	Soil	S. Kuninaga
C-328	2-2 IIIB	Mat rush	IFO 30944	HO-1556	7	Soil	ATCC 76131
RI-64	2-2 IV	Sugar beet	MAFF	MAFF 305551	7	Soil	MAFF
B-70	2-2 IV	Sugar beet	AHU	MAFF 305552	7	Soil	MAFF
Pf-28	2-2 IV	Sugar beet	MAFF	MAFF 305553	7	Soil	MAFF
Rh-46	2-2 IV	Sugar beet	MAFF	MAFF 305554	7	Soil	MAFF
Rh 509-S-1	2-2 IV	Sugar beet	IFO 30796	W-565	8	Wheat	ATCC 76106
48R	2-2 LP	Zoysia grass	AGU[e]	S21	9	Soil	ATCC 62804
G1	2-2 LP	Zoysia grass	AGU	W-395	10	Barley	ATCC 76107
G3	2-2 LP	Zoysia grass	AGU	TE-2-4	BI	Soil	ATCC 76132
K1-9	2-2 LP	Zoysia grass	AGU	AI-1-4	BI	Soil	MAFF 305263
RGR38	2-2 LP	Zoysia grass	AGU	KO-1-1	BI	Soil	MAFF 305266
R-1	2-3	Soybean	MAFF 235446	CH-1-2	BI	Soil	MAFF 305264
R-6	2-3	Soybean	MAFF 235448	KD-1-1	BI	Soil	MAFF 305265

[a] American Type Culture Collection, USA
[b] Institute for Fermentation, Osaka, Japan
[c] Faculty of Agriculture, Hokkaido University, Hokkaido, Japan
[d] Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan
[e] Faculty of Agriculture, Gifu University, Gifu, Japan

0.05 M phosphate buffer, pH 7.0, containing 0.05% NaCl and 0.01% Tween 20 (phosphate-buffered saline, PBS). Then, 30 mg of lyophilized mycelia was dissolved in 300 μl 0.5 N NaOH with 0.5% (w/v) polyvinylpyrrolidone (PVP; Sigma, St. Louis, MO, USA); 5 μl of the lysate sample was transferred to a new tube containing 495 μl 20 mM Tris-HCl, pH 8.0. A 5-μl aliquot was used for individual PCR amplifications as a DNA template. Water was used as a negative control.

For direct detection and identification of isolates belonging to AG 1 and AG 2 subgroups of *R. solani*, nucleotide primers were synthesized from a variable region identified in the 28S ribosomal DNA (rDNA) of the fungi. The primers used in this study are listed in Table 2. In our previous examination, PCR-restriction fragment length polymorphism (RFLP) analysis of *R. solani* based on partial sequences of the 28S rDNA gene was characterized (Matsumoto et al. 1996). The sequence data based on the 28S rDNA gene also revealed that the *R. solani* AG-common alignment was sequenced at about 400 bp and that *R. solani* subgroup-specific alignment was also sequenced at the portion of almost 100 bp from the 5′-end of the 28S rDNA. Therefore, in this experiment, we used the forward primer deduced from the AG-common sequence and the reverse primer deduced from the subgroup-specific sequence.

PCR amplification was performed in a reaction mixture containing 5 μl DNA template in 20 mM Tris-HCl, pH 8.0, 2.5 units of Taq DNA polymerase (Promega, Madison, WI, USA), 10 μl of 10 × Taq PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100), 100 μM of each deoxynucleoside triphosphate (dNTP) (dATP, dCTP, dGTP, and dTTP), 50 pmol each of forward and reverse primers, 2.5 mM $MgCl_2$, and sterile water in a total volume of 100 μl in a 0.5-ml microfuge tube; 50 μl of mineral oil (Katayama) was added to each tube to prevent evaporation. The program for amplification consisted of 30 cycles of denaturation at 94°C for 1 min, primer annealing at 54°C for

Table 2. Designed primers for direct detection and identification of AG 1-IA, AG 1-IB, AG 1-IC, AG 2-1, and AG 2-2 isolates in *R. solani*

Organism or sequence	Nucleotide sequence
R. solani AG-common primer (forward)	5′-CTCAAACAGGCATGCTC-3′
R. solani subgroup-specific primer (reverse)	
AG 1-IA	5′-CAGCAATAGTTGGTGGA-3′
AG 1-IB	5′-AAGGTCCTTTGGGGTTGGGG-3′
AG 1-IC	5′-CTTTTTTTGGGGGGCCTTGC-3′
AG 2-1	5′-AGGCAATAGGTTATTGGACC-3′
AG 2-2	5′-CATGGATGGGAGAACTTTTA-3′

2 min, and DNA extension at 72°C for 3 min. Aliquots (10 µl of the amplification products) were subjected to electrophoresis using a 3.0% agarose gel with Tris-borate ethylenediaminetetraacetic acid (EDTA) buffer at 100 V for 1 h, and the gel was stained with ethidium bromide.

Twelve representative isolates of *R. solani* shown in Table 1 were tested with AG 1-IA, AG 1-IB, AG 1-IC, AG 2-1, and AG 2-2, including AG 2-2 IIIB, AG 2-2 IV, and AG 2-2 LP, using the primer pairs shown in Table 2 (Figs. 1, 2). The primer pairs, *R. solani* AG-common primer and each of the subgroup-specific primers for AG 1-IA, AG 1-IB, AG 1-IC, AG 2-1, and AG 2-2 produced a single band of 265, 300, 230, 250, or 300 bp with DNA from the same group isolates, respectively. However, the specific primers for AG 1-IB also produced another size of 500-bp fragments (see Fig. 1). No PCR amplification product was obtained from representative isolates of AG 3, AG 4, AG 5, AG 6, AG 7, and AG BI. These results suggest that the detection and identification primer sets, i.e., AG 1-IA, AG 1-IB, AG 1-IC, AG 2-1, and AG 2-2-specific primers, become one of the convenient molecular tools for an easy and rapid AG detection method without hyphal anastomosis tests.

A total of 74 isolates in *R. solani* was further investigated for effectiveness of these primer sets (Table 3). The AG 1-IA primer set gave no PCR product from the other AG isolates. However, the AG 1-IB, AG 1-IC, AG 2-1, and AG 2-2 primer sets obtained fragments from some isolates (Table 3). In the case of AG 1, the AG 1-IB primer sets amplified DNA from isolates NR3 of AG 3 and GM-10 of AG 5. The AG 1-IC primer set also had an amplification product from P-5 isolates of AG 3. In the case of AG 2, the AG 2-1 detection primer also produced a fragment from RR-5-2 of AG 4 and KD-1-1 of AG BI. The AG 2-2 primer set amplified DNA from all tested isolates of AG 2-2 IIIB, AG 2-2 IV, AG 2-2 LP, GM-10 of AG 5, and MAFF 305553 of AG 7. In our experiment, isolates of AG 6, AG 8, AG 9, and AG 10 could not be detected by our designed primer sets. These results indicated that our synthesized specific primer sets for detecting and identifying isolates from AG 1-IA, AG 1-IB, AG1-IC, AG 2-1, and AG 2-2 succeeded only partially. Excepting the AG 1-IA-specific primers, the other AG-specific primer sets detected the other AG isolates at a low frequency. However, our results revealed that these primer sets based on the 28S rDNA gene would provide a possibility of useful methods for detection and identification of these subgroups.

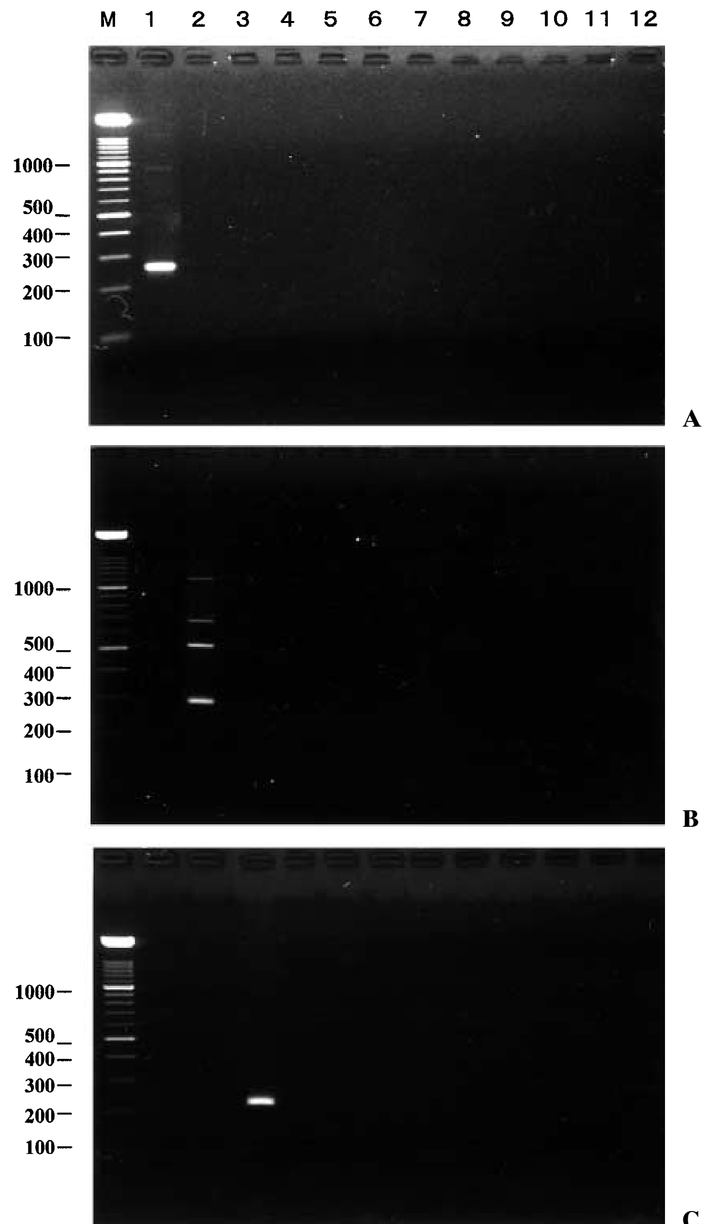

Fig. 1. Polymerase chain reaction (PCR) amplification of total genomic DNA from fungal tissues using the *Rhizoctonia solani* subgroup-specific primer sets of AG 1-IA (**A**), AG 1-IB (**B**), and AG 1-IC (**C**). *M*, molecular marker (100-bp ladder marker); *lane 1*, Cs-Ka (AG 1-IA); *lane 2*, CB-515-3 (AG 1-IB); *lane 3*, P-1 (AG 1-IC); *lane 4*, PS-4 (AG 2-1); *lane 5*, C-96 (AG 2-2 IIIB); *lane 6*, RI-64 (AG 2-2 IV); *lane 7*, ST-11-6 (AG 3); *lane 8*, AH-1 (AG 4); *lane 9*, SH-29 (AG 5); *lane 10*, NKN-2-1 (AG 6); *lane 11*, HO-1556 (AG 7); *lane 12*, TE-2–4 (AG BI)

Table 3. Polymerase chain reaction (PCR) amplification test of *R. solani* isolates with AG 1-IA, AG 1-IB, AG 1-IC, AG 2-1, and AG 2-2 specific primers

AG	Amplification productivity with subgroup-specific primers set[a]				
	AG 1-IA	AG 1-IB	AG 1-IC	AG 2-1	AG 2-2
AG 1-IA	+	−	−	−	−
AG 1-IB	−	+	−	−	−
AG 1-IC	−	−	+	−	−
AG 2-1	−	−	−	+	−
AG 2-2 IIIB	−	−	−	−	+
AG 2-2 IV	−	−	−	−	+
AG 2-2 LP	−	−	−	−	+
AG 2-3	−	−	−	−	−
AG 3	−	NR3[b]	P-5	−	−
AG 4	−	−	−	RR-5-2	−
AG 5	−	GM-10	−	−	GM-10
AG 6	−	−	−	−	−
AG 7	−	−	−	−	MAFF 305553
AG 8	−	−	−	−	−
AG 9	−	−	−	−	−
AG 10	−	−	−	−	−
AG BI	−	−	−	KD-1-1	−

[a] +, all tested isolates produced the expected fragment by PCR amplification; −, no PCR product was present in any tested isolate
[b] Isolate produced the fragment; the other isolates did not

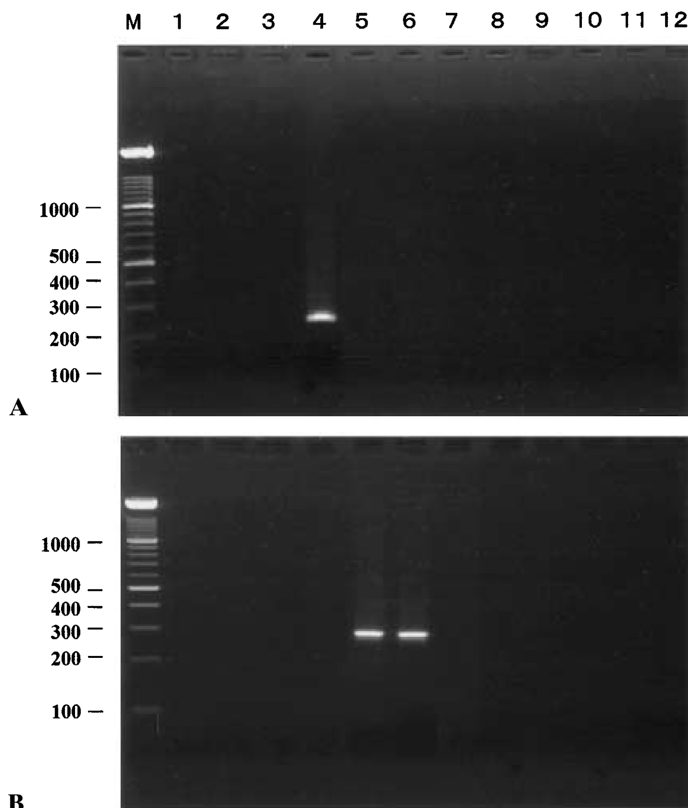

Fig. 2. PCR amplification of total genomic DNA from fungal tissues using the *R. solani* subgroup-specific primer sets of AG 2-1 (**A**) and AG 2-2 (**B**). *M*, molecular marker (100-bp ladder marker); *lane 1*, Cs-Ka (AG 1-IA); *lane 2*, CB-515-3 (AG 1-IB); *lane 3*, P-1 (AG 1-IC); *lane 4*, PS-4 (AG 2-1); *lane 5*, C-96 (AG 2-2 IIIB); *lane 6*, RI-64 (AG 2-2 IV); *lane 7*, ST-11-6 (AG 3); *lane 8*, AH-1 (AG 4); *lane 9*, SH-29 (AG 5); *lane 10*, NKN-2-1 (AG 6); *lane 11*, HO-1556 (AG 7); *lane 12*, TE-2-4 (AG BI)

In recent taxonomic studies, the detection and identification of *Rhizoctonia* species using oligonucleotide primers designed from their specific ribosomal DNA regions for PCR amplification have contributed to enhanced diagnostic capability to differentiate *R. solani* AGs and *Rhizoctonia* species (Brisbane et al. 1995; Mazzola et al. 1996; Johanson et al. 1998). In our experiment, this simple PCR-based technology allows rapid and accurate identification and detection of *R. solani* isolates in AG 1-IA, AG 1-IB, AG 1-IC, AG 2-1, AG 2-2, and the other *R. solani* AGs. The alkaline DNA extraction method also allowed the easy and rapid preparation of DNA for a series of PCR amplifications and electrophoresis analyses (Matsumoto et al. 1997). However, we could not synthesize an AG 2-2 subgroup-specific primer set because of the difficulty in finding sequences to match their specific region in the 28S ribosomal DNA. Therefore, we would suggest that an AG 2-2 subgroup-specific primer set be synthesized from another region, including ITS and 18S ribosomal DNA. Moreover, we could not apply synthesis of the specific primer sets for detection and diagnosis of the other AGs including AG 3, AG 4, AG 5, AG 6, AG 7, and AG BI specific primer sets; these will be tested next.

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