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FULL PAPER

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Use of single-protoplast isolates in the study of the mating phenomena of *Rhizoctonia solani* (*Thanatephorus cucumeris*) AG-1 IC and IA

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Abstract This study evaluates the effectiveness of using single-protoplast isolates (SPIs) to study the mating phenomena of *Rhizoctonia solani* AG-1 IC and IA. SPIs obtained from three field isolates (F-1, Rh28, and RO2) of AG-1 IC were paired with representative single-basidiospore isolate (SBI)-M1/-M2 testers, each from their own field isolates, or paired in all possible combinations. Tufts were formed between SPIs and SBI-M1/-M2 testers and between SPIs-M1 and -M2. The separation ratios of SPIs-M1 and -M2 were approximately 1:1, which were similar to the results obtained with SBIs. SPIs obtained from three isolates (GNSD, R59, and Tr8) of AG-1 IA, which failed to form basidiospores, were paired in all possible combinations. Although no tufts formed among SPIs from Tr8 and R59, tufts did form between SPIs from GNSD. SPIs from GNSD were separated into homokaryotic (-M1 or -M2) and heterokaryotic isolates, and the separation ratio of -M1 and -M2 was also around 1:1. Amplified fragment length polymorphism (AFLP) phenotypes of the tuft isolates formed between GNSD SPIs-M1 and -M2 suggested that these tuft isolates were all heterokaryotic. These results indicate that all three isolates of AG-1 IC and one isolate GNSD of AG-1 IA are heterokaryotic, and that the other two isolates of Tr8 and R59 of AG-1 IA are homokaryotic. Single-protoplast isolates are effective for studies of the mating phenomena of isolates belonging to different AGs of *R. solani* that could not form a perfect stage.

Key words Basidiomycete · Heterokaryon formation · Single-protoplast · *Thantephorus cucumeris*

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Introduction

Rhizoctonia solani Kühn [teleomorph: *Thanatephorus cucumeris* (A.B. Frank) Donk] is known as a soil-borne plant pathogen and causes economically important diseases to a large variety of vegetables, crop plants such as cereals, turfgrasses, ornamentals, fruits, and forest trees worldwide (Anderson 1982; Adams 1988; Sneh et al. 1996). *R. solani* consists of 13 anastomosis groups (AGs) based on the occurrence of hyphal fusion (anastomosis) (Sneh et al. 1991; Carling et al. 1999, 2002, 2003). Some AGs of *R. solani* have been further divided into subgroups based on cultural morphology, host range, pathogenicity, and molecular techniques (Ogoshi 1976, 1987; Carling and Kuninaga 1990; Sneh et al. 1991; Carling 1996). The sexuality of *R. solani* remains somewhat controversial, perhaps because generalizations from studies of one AG are often applied to the species (Adams 1988). It has been suggested that the species is basically homothallic but that some isolates behave heterothallically (Whitney and Parmeter 1963; Stretton et al. 1967). So far, the mating phenomena of *R. solani* have been investigated only in AG-1 IC, -2-2 IV, -3, -4, and -8. Research shows that isolates of AG-2 and AG-3 are thought to have presumed homothallic mating system (Cubeta and Vilgalys 1997), and isolates of AG-1 IC, AG-4, and AG-8 are heterokaryotic with bipolar mating systems (Anderson et al. 1972; Adams and Butler 1982; Yang et al. 1992; Julian et al. 1996, 1997; Cubeta and Vilgalys 1997). In contrast, Toda and Hyakumachi (2006) reported that both homothallic and heterothallic mating systems are observed in *R. solani* AG-2-2 IV.

Several studies on the genetics of *R. solani* reported that observation of the tufts produced from the zone of contact between two different homokaryotic isolates based on pairing on potato-dextrose charcoal agar (PDCA) medium is a good indicator of heterokaryon formation (Whitney

and Parmeter 1963; Adams and Butler 1982; Anderson 1982; Julian et al. 1996). Tuft formation indicates that the paired homokaryotic isolates belong to different mating groups and that these isolates have a heterothallic mating system, whereas no tuft formation observed between homokaryotic isolates paired in all possible combinations indicates that these isolates have a homothallic mating system. Homokaryotic isolates are normally obtained from single basidiospores. However, whether isolates of *R. solani* AGs or their subgroups have a heterothallic or homothallic mating system has not been fully determined because it is difficult to observe sexual forms of these isolates either in nature or under artificial conditions. On the other hand, single-protoplast isolates were also used to obtain homokaryotic isolates in *R. solani*, as an alternative method (Phillips 1993). Single-protoplast isolates could be used for the studies of the mating systems in species in which a sexual stage is absent or difficult to induce in pure culture, such as *R. solani*.

The objective of this study is to determine whether single-protoplast isolates (SPIs) are as effective as single-basidiospore isolates (SBIs) in the study of mating phenomena in *R. solani* based on (i) the evaluation of heterokaryotic tuft formation by pairing SPIs on PDCA medium and (ii) the confirmation of heterokaryon formation by phenotype through amplified fragment length polymorphism (AFLP).

Materials and methods

Isolates

Three isolates (F-1, Rh28, and RO2) of *R. solani* (*Thanatephorus cucumeris*) AG-1 IC and three isolates (GNSD, R59, and TR8) of AG-1 IA were used for the production of single-basidiospore isolates (SBIs) and single-protoplast isolates (SPIs). Isolate RO2 was obtained from carrot in Tokushima, Japan; isolates Rh28 and F-1 were from sugar beet in Hokkaido, Japan; isolates of AG-1 IA were from rice; isolates GNSD and Tr8 were from Gifu, Japan; and isolate R59 was from Tochigi, Japan. All isolates were maintained on 9-cm Petri dishes containing 12 ml potato dextrose agar (PDA; Becton Dickinson, Sparks, MD, USA) or in test tube slants at 25°C.

Production of single-basidiospore isolates (SBIs)

The soil-over-culture method was used to produce single-protoplast isolates of each field isolate (Ogoshi 1972). A single 3- to 5-mm agar plug of each isolate from stock culture was inoculated onto a 9-cm Petri dish containing 10 ml PDA at 25°C in the dark. After 3 days, a 5-mm agar plug with fresh mycelium was cut from the edge of the hyphal colony and placed in the center of a new 9-cm Petri dish containing 30 ml PDA with 2.5% yeast extract (Bacto Yeast Extract; Becton Dickinson) (PDYA) at 25°C in the dark. After 4–5 days of incubation, when the mycelium overspread the PDYA medium, sterilized soil blocks that are used for seedling culture of rice (Kureha Chemical Industry, Tokyo, Japan) were placed on PDYA to cover the mycelium. Mycelia covered with soil blocks were incubated in a growth chamber at 28°–30°C for 5–7 days in the dark. Sporulation occurred readily at high humidity conditions. Distilled water was added twice per day to maintain soil humidity. When evidence of sporulation showed on the surface of the soil blocks, a piece of soil containing sporulation was picked up with sterile forceps and placed on a glass slide. A piece of soil was stained with 0.05% cotton blue to examine basidia and basidiospore formation by microscopy at 400× magnification. When basidia and basidiospores were observed, a plate with soil blocks was inverted over Petri dishes containing acidified water agar amended with 3% lactic acid (pH 4.0; AWA). After 2–6 days, hyphae that germinated from basidiospores were observed with a stereoscopic microscope. A hyphal tip germinated from each spore was picked up with a sterilized spatula and transferred onto a Petri dish containing 10 ml PDA; this was saved as a single-basidiospore isolate (SBI). Approximately 50 SBIs obtained from each isolate were numbered and saved for further study.

Production of protoplasts

The methods used for protoplast production and regeneration in this study followed those described by Peberdy (1980) and Hashiba and Yamada (1982). Small (5-mm) agar plugs bearing mycelia of each isolate were inoculated onto 9-cm Petri dishes containing 12 ml PDA medium at 25°C in the dark. After 2 days of incubation, ten to fifteen 5-mm agar disks with mycelia, which were cut from the edge of the colony, were further plated onto 9-cm Petri dishes containing 10 ml CM liquid medium (0.5% glucose, 0.5% malt extract, 0.5% yeast extract, per liter), and then incubated at 25°C for 1 day in the dark. Fresh mycelia were collected and washed with AMM liquid medium (1% glucose, 0.03% ammonium tartrate, 50 ml 50× SSC, per liter) twice, pulverized by a homogenizer at 1500 rpm for 90 s, transferred to a 500-ml flask containing 150 ml AMM medium, and then incubated on a shaker at 130 rpm at 25°C for 12–16 h. Before incubation of the mycelia in lytic enzyme solution, the lytic enzyme combination [80 mg Novozyme 234 (Calbiochem, La Jolla, CA, USA), 80 mg cellulase Onozuka Rs (Yakult Pharmaceutical, Tokyo, Japan), 40 mg zymolyase (Seikagaku, Tokyo, Japan), 40 mg β-glucuronidase (Sigma Chemical, St. Louis, MO, USA), 5 mg chitinase (Sigma Chemical, USA), and 4 ml 1.2 M $MgSO_4$ (pH 5.8) was mixed using a magnetic stirrer in a 10-ml flask. The mixed lytic enzyme solution was transferred into a 10-ml glass tube and then centrifuged at 14000 rpm for 10 min. The supernatant liquor was decanted in a 10-ml flask, then passed through a 0.22-μm Millipore syringe driven filter (Millipore, Bedford, MA, USA) to sterilize the mixed enzymes. The grown fresh mycelia were transferred into a 10-ml flask containing the lytic enzyme mixture by filtration with a suction filter and kept in a shaker at 60 rpm and 30°C. After

a 4-h reaction time, the cell wall was digested and the protoplasts were released.

Isolation of regenerated single-protoplast isolates (SPIs)

The enzyme solution containing released protoplasts was filtrated through a double layer of sterile Kimwipe (Kureshia, Tokyo, Japan) into a 10-ml glass tube for purification. The undigested tissues were thus eliminated. The filtrate solution was centrifuged for 10 min at 2000 *g* after the addition of 3 ml SE osmotic stabilizer solution [SE: 18.2% D-sorbitol, 50 ml 0.5 M ethylenediaminetetraacetic acid (EDTA) (pH 8.0) per liter). Then, 4 ml supernatant of SE fluid containing the protoplasts was transferred to a new tube containing 5 ml STC osmotic stabilizer (STC: 24% D-sorbitol, 0.3% $CaCl_2$, 25 ml Tris-HCl, pH 7.0, per liter), and centrifuged for 10 min at 2000 *g*. Protoplasts, with a density of about 10^7/ml, were collected in the lower half portion of the STC solution. The protoplast suspension was diluted to 10^4 protoplasts/ml, and 0.2 ml suspension was then spread on the surface of Petri dishes containing 10 ml PDA amended with $MgSO_4 \cdot 7H_2O$ (2% agar, 2% glucose, 10.8% $MgSO_4 \cdot 7H_2O$). After incubation for 2 days at 25°C in the dark, hyphal tips from regenerating protoplasts were observed under the microscope and marked. The periphery (about 1.5 mm^2) around the marked regenerated single protoplast was cut with a sterile needle, transferred to a fresh PDA slant, and saved at 25°C for further study.

Heterokaryon formation between SBIs or SPIs or between SPIs and their representative SBI testers

Pairing incubations between SBIs or SPIs or between SPIs and their representative SBI testers were performed using PDA with 0.5% activated charcoal (PDCA) and incubated for 1 week at 25°C in the dark, a modified method of Julian et al. (1996). All the SBIs were divided into SBI-M1 and SBI-M2. SPIs from each isolate of AG-1 IC were paired with their own SBI-M1 and SBI-M2 testers or were paired in all possible combinations. Isolates of AG-1 IA did not produce a perfect stage, so their SPIs could not be paired with their own SBIs. Instead, their SPIs were paired in all possible combinations. When the fibrous tufts formed at the junction of two SBI colonies, paired SBIs were estimated to be sexually compatible and were distributed into two different mating groups, respectively. Although paired SBIs could not form tufts, they were assumed to be sexually incompatible and were distributed into the same mating group. Based on this knowledge, when fibrous tufts formed between SPI and either SBI-M1 tester or SBI-M2 tester, the SPI was considered to be a homokaryon and the mating type was either -M2 or -M1. When no tuft was formed with either -M1 or -M2, the SPI was considered to be a heterokaryon. Similarly, when fibrous tufts were formed between two SPIs, these two SPIs were considered to belong to two different mating groups, respectively. When the SPI could not form tufts with any other SPIs, this SPI was considered to be a heterokaryon. Hyphae from each tuft (putative heterokaryon) were picked up with sterilized forceps and transferred to Petri dishes containing 10 ml AWA (water agar amended with 3% lactic acid). Hyphal tips that appeared from the tuft were cut and transferred on PDA plates and incubated at 25°C in the dark. The colonies formed from those hyphae were saved as tuft isolates.

DNA extraction

DNA was extracted from isolate GNSD, SPIs, and tuft isolates of *R. solani* AG-1 IA using the method reported by Yoder (1988) with modifications. Three small fresh PDA agar plugs of each isolate were removed from the growing margin of 3-day-old cultures on PDA and transferred onto a 9-mm-diameter Petri dish containing 10 ml potato-dextrose broth (PDB). After 5–6 days of incubation at 25°C in the dark, mycelial mats were collected by filter paper, washed with sterile distilled water, blotted dry, and frozen at –80°C until use. The DNA from each mycelium was extracted using the methodology described by Toda and Hyakumachi (2006).

AFLP analysis

AFLP analysis was performed using extracted genomic DNA. The genomic DNA was diluted to 10 ng/µl, following quantification of DNA using a spectrophotometer (Amersham Biotech, Piscataway, NJ, USA). The principle of AFLP analysis is described in detail by Vos et al (1995). DNA digestion and ligation reactions for AFLP were performed according to the manual supplied with the AFLP kit (Applied Biosystems, Foster City, CA, USA). Select primer combination (*Eco*RI-AG/*Mse*I-CA) was used for selective amplifications. Next, 8 µl AFLP product was subjected to electrophoresis in a 15% polyacrylamide gel. The gel was stained serially with 0.1% $AgNO_3$ and 1.5% NaOH containing formaldehyde for 10 min. AFLP phenotypes were visualized by acryl board light. The reproducibility of the AFLP analysis results was tested by repeating the entire AFLP procedure three times using the same genomic DNA.

Results

Production of SBIs and SPIs from *R. solani* AG-1 IC F-1, Rh28, and RO2, and SPIs from AG-1 IA GNSD, Tr8, and R59

Forty-seven SBIs and 200 SPIs were obtained from isolate F-1 of AG-1 IC; 55 SBIs and 103 SPIs were obtained from isolate Rh28; 50 SBIs and 99 SPIs were obtained from isolate RO2; isolates GNSD, Tr8, and R59 of AG-1 IA did not produce SBIs; 85 SPIs were obtained from isolate GNSD; and 30 SPIs were obtained from each isolate Tr8 and T59.

Table 1. Appearance of tuft formation between SBIs or SPIs or between SPIs and their representative SBI testers obtained from each field isolate F-1, Rh28, and RO3 of *Rhizoctonia solani* AG-1 IC, and tuft formation between SPIs from GNSD, TR8, and R59 of AG-1 IA, and the ratio of mating type 1, mating type 2, and heterokaryotic isolates of these SBI and SPI progeny

AG/subgroup	Isolate name	Pairing combination	Appearance of tuft formation	Ratio of M1:M2:hetero[e]
AG-1 IC	F-1	SBIs × SBIs[a]	+[d]	27:20:0
		SBIs × SPIs[b]	+	45:48:107
		SPIs × SPIs[c]	+	45:48:107
	Rh28	SBIs × SBIs	+	27:28:0
		SBIs × SPIs	+	24:27:48
		SPIs × SPIs	+	24:27:48
	RO2	SBIs × SBIs	+	26:24:0
		SBIs × SPIs	+	39:43:17
		SPIs × SPIs	+	39:43:17
AG-1 IA	GNSD	SPIs × SPIs	+	19:22:44
	Tr8	SPIs × SPIs	–[f]	–
	R59	SPIs × SPIs	–	–

AG, anastomosis group
[a]Single-basidiospore isolates (SBIs) obtained from each field isolate of *Rhizoctonia solani* AG-1 IC paired among each other in all possible combinations
[b]Single-protoplast isolates (SPIs) obtained form each field isolate of *R. solani* AG-1 IC paired with their own representative SBI testers
[c]Single-protoplast isolates (SPIs) obtained form each field isolate of *R. solani* AG-1 IC and AG-1 IA paired among each other in all possible combinations
[d]Tuft formation appeared between paired SBIs and between SPIs
[e]The ratio of mating type 1, mating type 2, and heterokaryotic isolates of SBI and SPI progeny obtained from each field isolate
[f]No tuft formation was observed between paired SPIs; these isolates have a homothallic mating system and could not be divided into mating type 1 and mating type 2

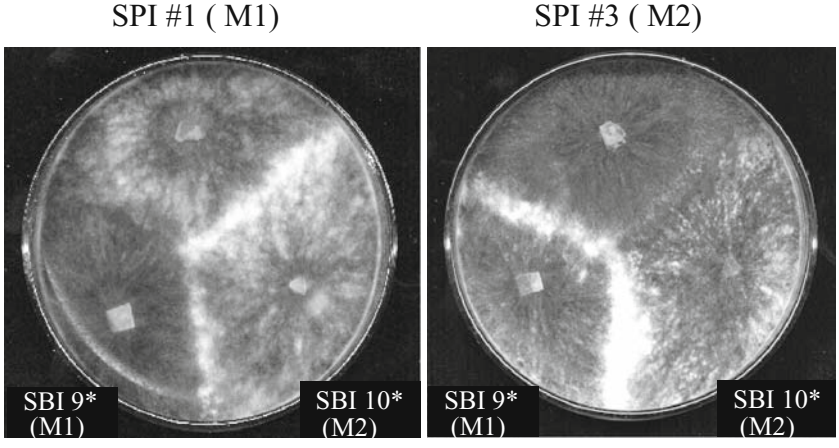

Fig. 1. Tuft formation between single-protoplast isolates (SPIs) and their representative single-basidiospore isolate (SBI) testers. SPI #1, obtained from field isolate Rh28 of *R. solani* AG-1 IC, was determined to be the homokaryotic mating type 1 isolate (SPI-M1), on the basis of tuft formation only with representative SBI tester 10* (M2) but not with 9* (M1); SPI #3 was determined as the homokaryotic mating type 2 isolate (SPI-M2), based on tuft formation only with SBI 9* (M1) but not with 10* (M2)

Tuft formation between SBIs in all possible combinations, between SPIs and their representative SBI testers, and between SPIs in all possible combinations of *R. solani* AG-1 IC F-1, Rh28, and RO2

Among 47 SBIs obtained from isolate F-1 of AG-1 IC, 27 were homokaryotic mating type 1 (M1) and the other 20 SBIs were mating type 2 (M2). Two hundred SPIs from isolate AG-1 IC F-1 were paired with SBI testers of F-1 1* (M1) and 16* (M2) and then paired in all possible combinations. Forty-five SPIs could form tufts only with SBI-M2, 48 only with -M1, and 107 SPIs could not form tufts with either SBI-M1 or -M2. Tufts could also be formed between these groups of 45 SPIs and 48 SPIs. The other 107 SPIs could not form tufts with either the 45 or 48 SPIs. These results indicated that 45 and 48 SPIs of the F-1 were considered as homokaryotic -M1 and -M2, respectively; the other 107 SPIs were classified as heterokaryotic isolates, and isolate F-1 was found to have a heterothallic mating system (Table 1).

Among the 55 SBIs obtained from isolate Rh28 of AG-1 IC, 27 were -M1 and the other 28 were -M2. Ninety-nine SPIs from isolate AG-1 IC Rh28 were paired with SBI testers of Rh28 SBI 9*(M1) and 10*(M2) and then paired in all possible combinations. Twenty-four SPIs could form tufts with SBI-M2, 27 SPIs could form tufts with SBI-M1, and the other 48 SPIs could not form tufts with either -M1 or -M2 SBIs. Tufts were formed between these 24 SPIs and the 27 SPIs. The other 48 SPIs could not form tufts with either the 24 or 27 SPIs. These results indicated that 24 and 27 SPIs were considered to be homokaryotic -M1 and -M2, respectively; the other 48 SPIs were classified as heterokaryotic isolates, and isolate Rh28 was found to have a heterothallic mating system (Table 1, Fig. 1).

Among the 50 SBIs obtained from isolate RO2 of AG-1 IC, 26 were -M1 and the other 24 were -M2. Ninety-nine

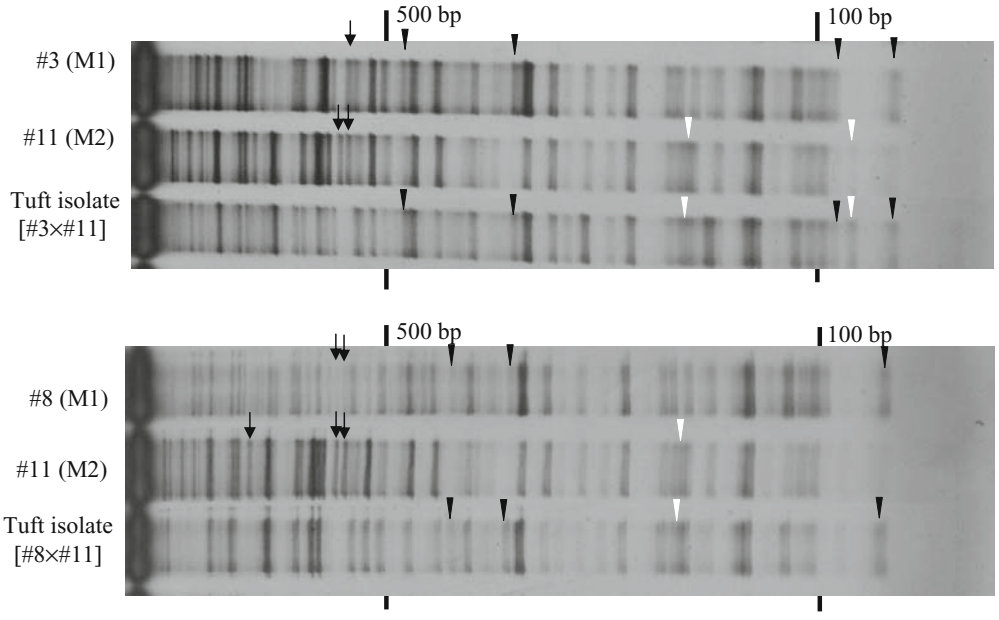

Fig. 2. Amplified fragment length polymorphism (AFLP) phenotypes of tuft isolates obtained from SPIs-M1 and -M2 progeny of field isolate GNSD of *R. solani* AG-1 IA. The primer pair used for selective amplifications was *Eco*MI-AG/*Mse*I-CA. Banding patterns show the difference among tuft isolates and their contributed SPIs. *Triangles* indicate the specific markers of each isolate; *black triangles* indicate the specific markers present in SPI-M1 and tuft isolates; *white triangles* indicate the specific markers present in SPI-M2 and tuft isolates. *Arrows* indicate the specific markers present only in SPI-M2 or both -M1 and -M2 but absent in tuft isolates

SPIs from RO2 were paired with SBI testers of RO2 SBI 1* (M1) and 10* (M2) and paired in all possible combinations. Thirty-nine SPIs could form tufts with SBI-M2, 43 SPIs could form tufts with SBI-M1, and the other 17 SPIs could not form tufts with either 39 or 43 SPIs. Tufts were formed between these 39 and the 43 SPIs. The other 17 SPIs could not form tufts with either 39 or 43 SPIs. These results indicated that 39 and 43 SPIs were considered to be homokaryotic -M1 and -M2, respectively; the other 17 SPIs were classified as heterokaryon isolates, and isolate RO2 was found to have a heterothallic mating system (see Table 1).

Tuft formation between SPIs paired in all possible combinations of *R. solani* AG-1 IA GNSD, R59, and Tr8

Eighty-five SPIs obtained from isolate GNSD of AG-1 IA were paired in all possible combinations. Nineteen SPIs could form tufts with 22 SPIs, but the other 44 SPIs could not form tufts with either 19 or 22 SPIs. As a result, 19 and 22 SPIs were considered to be homokaryotic -M1 and -M2; the other 44 SPIs were heterokaryotic isolates, and isolate GNSD was found to have a heterothallic mating system (Table 1).

No tufts were formed among the 30 SPIs from isolates Tr8 and R59. Both isolates Tr8 and R59 were found to have a homothallic mating system (Table 1).

AFLP analysis of tuft formation between SPI-M1 and -M2 of *R. solani* AG-1 IA isolate GNSD

AFLP phenotypes of tuft isolates contained the common and specific banding patterns contributed by -M1 and -M2 SPIs of AG-1 IA isolate GNSD (Fig. 2). Tuft isolate [#3 × #11] contained four specific banding patterns contributed by SPI #3-M1 and two of #11-M2. Notably, this tuft isolate lost one specific banding pattern that was present in both SPIs and lost one which was present in #11-M2. Tuft isolate [#8 × #11] contained three specific banding patterns of SPI #8-M1 and one of #11-M2, and it lost two specific banding patterns that were present in both SPIs and lost one which was present in #11-M2.

Discussion

It is possible to produce homokaryotic isolates from single protoplasts prepared from a heterokaryotic mycelium in *R. solani* AG-1 IA and IC. Previous work demonstrated that *R. solani* AG-1 IC, -4, and -8 have heterothallic bipolar mating systems based on pairings between single-basidiospore or single-protoplast progeny obtained from isolates (Whitney and Parmeter 1963; Anderson et al. 1972; Yang et al. 1992; Julian et al. 1996, 1997). In this study, three isolates of *R. solani* AG-1 IC were observed to clarify the mating system by using both SBIs and SPIs. Tufts were observed in both SBIs and SPIs, which belong to different mating types (see Table 1). The results demonstrated that these three isolates of AG-1 IC are heterokaryotic and have heterothallic mating systems. The separation ratios of SPIs-M1 and -M2 of each isolate were approximately 1:1, similar to the separation ratios of SBIs-M1 and -M2 (Table 1). These separation ratios are consistent with the results for SBIs obtained from AG-1 IC and AG-2-2 IV (Julian et al. 1996; Toda and Hyakumachi 2006) and with the results for SPIs obtained from AG-4 (Phillips 1993).

Whether the mating system of AG-1 IA is homothallic or heterothallic is not known. In this study, tuft formation was observed between SPI pairings from the AG-1 IA isolate GNSD, but tufts were not observed in SPI pairings of AG-1 IA Tr8 and R59 (see Table 1), indicating that both

homothallic and heterothallic mating systems are present in *R. solani* AG-1 IA, similar to the results obtained with AG-2-2 IV (Toda and Hyakumachi 2006). Julian et al. (1999) and Toda and Hyakumachi (2006) reported that tuft isolates possess all the common and specific AFLP products of paired SBIs obtained from AG-1 IC and AG-2-2 IV. AFLP analysis was used in this study to investigate whether the tuft isolates produced between paired SPIs obtained from isolate GNSD of AG-1 IA were heterokaryons. The results confirmed that these tuft isolates are heterokaryons based on their AFLP phenotypes, because the AFLP phenotypes of tuft isolates possessed common and specific AFLP products exhibited by almost all paired contributed SPIs (see Fig. 2). Furthermore, certain products present in either or both paired SPIs were not found in AFLP phenotypes of tuft isolates; this might have occurred by parasexual recombination in heterokaryotic hyphae. This phenomenon was not observed by Julian et al. (1999) or Toda and Hyakumachi (2006).

Our results indicate that it is possible to use SPIs instead of SBIs to study the mating phenomena of *R. solani*. SPIs are more suitable and reliable than SBIs because all isolates can produce protoplasts easily whereas they cannot all produce basidiospores. Also, it takes only 1 week to produce SPIs compared to 1 month for SBIs in *R. solani.* Single-protoplast isolates will be effective for studying the mating phenomena of isolates belonging to different AGs of *R. solani* that cannot form the perfect stage.

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