SHORT COMMUNICATION

ASCRIPTION OF Nt-ISOLATES OF *RHIZOCTONIA SOLANI* TO ANASTOMOSIS GROUP 2-1 (AG-2-1) ON ACCOUNT OF rDNA-ITS SEQUENCE SIMILARITY

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SUMMARY

A homogeneous group of *Rhizoctonia solani* isolates from tobacco, which showed intermediate features between anastomosis groups BI and 2-1 (AG-BI and AG-2-1), was recently characterized. It was not conclusively assigned to any previously known AG and was provisionally named 'Nt-isolates'. The rDNA sequence similarity in the ITS1 region of the Nt-isolates was evaluated and a high similarity (> 90%) was found with AG-2-1 tester isolates, while it was remarkably lower with tester isolates from AG-BI and other AG-2 subgroups. Therefore, despite a low anastomosis frequency, Nt-isolates are to be considered members of AG-2-1.

Key words: Rhizoctonia solani, anastomosis groups, tobacco, rDNA sequence similarity.

Among plant pathogenic fungi, *Rhizoctonia solani* Kühn [teleomorph: *Thanatephorus cucumeris* (Frank) Donk] is conspicuous on account of its infraspecific taxonomy based on the anastomosis group (AG) concept (Parmeter *et al.*, 1969). Isolates of the fungus are classified according to their ability to anastomose with tester isolates belonging to establishe AGs. Anastomosis frequency as well as some morphological, physiological and biomolecular features, may vary within a given AG; such a variability has led to the characterization of subgroups which are currently considered the evolutionary units of the species. Moreover, several ecological types have been described on account of variation in pathogenicity on different hosts. So far 12 anastomosis groups (AG-1 through 11, and AG-BI) and a number of subgroups and ecological types have been characterized (Sneh *et al.*, 1991; Carling, 1996).

A homogeneous group of *R. solani* isolates from tobacco (*Nicotiana tabacum* L.) fields was recently characterized by means of anastomosis behaviour, as well as polygalacturonase-isozyme patterns and restriction fragment length polymorphisms (RFLPs) of ribosomal DNA internal transcribed spacers (rDNA-ITS) (Nicoletti *et al.*, 1999). As the cluster presented some peculiar features it was provisionally denominated 'Nt-isolates' (Nt = *N. tabacum*) and was not conclusively assigned to any known anastomosis group. Actually Nt-isolates showed a higher frequency of anastomosis with tester isolates from AG-BI while biomolecular assays were indicative of a closer relatedness with AG-2-1. Owing to such contradictory features, Nt-isolates seemed to represent a puzzle within the known frame of *R. solani* AGs.

Both polygalacturonase isozymes and DNA-based RFLPs allow an indirect appraisal of the genetic affinity among taxonomic entities and have been pointed out as the most useful genetic markers for analyzing population structure in *R. solani* (Cubeta and Vilgalys, 1997). However, a direct, and therefore more significant measure, may be provided by the evaluation of DNA sequence similarity of restricted portions of the genome. For this purpose, genes coding for ribosomal RNA (rDNA) are the most suited since they are highly conservative and allow a better understanding of the genetic affinity between taxonomic entities (Vilgalys and Gonzalez, 1990). Since rDNA of *R. solani* has shown more variation in the ITS1 region, the relationships among taxonomic groups can be determined on the basis of the sequence data of this region only (Kuninaga *et al.*, 1997).

Evaluation of rDNA-ITS1 sequence similarity of 4 selected Nt-isolates and tester isolates belonging to AG-BI and most of the subgroups and ecological types so far described in AG-2 (Table 1) was carried out for a more conclusive classification.

Each isolate was grown on potato dextrose broth (Difco) for 4 days at 25°C. Mycelial mats were harvested by filtration, blotted dry, lyophilized, ground to a fine powder in liquid nitrogen, then stored at -20°C prior to DNA purification. Genomic DNA was extracted as described in a previous paper (Kuninaga *et al.*, 1997). The mycelial powder was suspended in NTE buffer (100 mM NaCl, 30 mM Tris-HCl, 10 mM EDTA,

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Table 1. *R. solani* isolates used in the present study.

Isolate	AG/subgroup	Geographic origin	Host	Source
RT16	Nt-isolates	Campania, Italy	tobacco	R. Nicoletti
RT23	Nt-isolates	Campania, Italy	tobacco	R. Nicoletti
R93.1	Nt-isolates	Dordogne, France	tobacco	D. Blancard
RF4	Nt-isolates	Campania, Italy	broad bean	R. Nicoletti
Ala-Gra	AG-2-1	Alaska, USA	soil	S. Kuninaga
R123	AG-2-1	Aichi, Japan	radish	S. Kuninaga
88-033	AG-2-1	Western Australia	barley	G.C. MacNish
88-822	AG-2-1	Western Australia	barley	G.C. MacNish
F56L	AG-2-1	Alaska, USA	soil	D.E. Carling
R144	AG-2-t	The Netherlands	tulip	G. Dijst
B60	AG-2-2 IIIB	Okayama, Japan	sugar beet	S. Kuninaga
IFO 30942	AG-2-2 IIIB	Kumamoto, Japan	mat rush	IFO[1]
61-D-3	AG-2-2 IIIB	Illinois, USA	soybean	C. Windels
89-21-4	AG-2-2 IIIB	Georgia, USA	corn	C. Windels
BC-10	AG-2-2 IV	Hokkaido, Japan	sugar beet	S. Kuninaga
DRGO1	AG-2-2 IV	Dominican Republic	bean	G. Godoy-Lutz
H13	AG-2-2 IV	Honduras	bean	G. Godoy-Lutz
86-42-4	AG-2-2 IV	Minnesota, USA	sugar beet	C. Windels
G4	AG-2-2 LP	Gifu, Japan	*Zoysia* grass	M. Hyakumachi
48R	AG-2-2 LP	Hyogo, Japan	*Zoysia* grass	M. Hyakumachi
237258	AG-2-3	Miyagi, Japan	soybean	MAFF[2]
A68	AG-8	Southern Australia	wheat	S. Kuninaga
AI 1-4	AG-BI	Hokkaido, Japan	soil	S. Kuninaga
SH1-2	AG-BI	Hokkaido, Japan	soil	S. Kuninaga
SHC81	AG-BI	Hokkaido, Japan	soil	S. Kuninaga
TE2-4	AG-BI	Hokkaido, Japan	soil	S. Kuninaga

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10 mM 2-mercaptoethanol, pH 8.0) containing 0.5% (v/v) Nonidet P-40 and centrifuged at 10,000 rpm for 1 min. The pellet was resuspended in NSE buffer (100 mM NaCl, 200 mM sucrose, 10 mM EDTA, pH 4.4) before adding TES buffer (10 mM Tris-HCl, 250 mM EDTA, pH 9.2, 2.5% SDS). The homogenate was incubated for 30 min at 55°C. The solution was extracted with an equal volume of phenol: chloroform: isoamyl alcohol (50: 48: 2) and centrifuged at 10,000 rpm for 20 min. The upper layer was mixed with 0.1 volume of 3 M sodium acetate, then with 0.54 volume of isopropanol. The DNA pellet was collected, rinsed with 70% ethanol, dried under vacuum, then dissolved in TE buffer. The regions of rDNA repeat were amplified using PCR conditions with the primers, NS7, ITS2, ITS4 and ITS5 (White *et al.*, 1990). The PCR primer also contained the sequences of Rhodamine-labeled primers, RV-M and M4 (TaKaRa, LTD) for cycle sequencing reactions. The PCR amplification reactions were performed in a 50 µl mixture containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 1.5 mM MgCl, 200 µM of each of four deoxynucleoside triphosphates, 5 pmol of each primer, 1.0 µl of template, and 2.5 units of Taq polymerase. The amplification was performed with a thermal cycler MP (TaKaRa, LTD). The cycle parameters were an initial denaturation at 94°C for 2 min, followed by 30 cycles consisting of denaturation at 94°C for 40 s, annealing at 55°C for 1 min and extension at 72°C for 1 min, and a final extension for 5 min at 72°C. Single-stranded DNA template was prepared using the following procedure: one of the PCR primers was biotinylated, the biotinylated PCR products were immobilized on streptavidin-coated paramagnetic beads (Dynabeads M-280 streptavidin, DYNAL), and single-stranded DNA

was prepared according to the manufacturer's instructions. Both strands were then sequenced directly using a Takara Taq cycle sequencing core kit (TaKaRa, LTD) and the above described Rhodamine-labeled primers. The sequencing products were separated in 6% polyacrylamide gel, and the fragments were monitored using a Takara FMBIO-100 system (TaKaRa, LTD). DNA sequence similarity based on the proportion of different nucleotide sites including insertion-deletion was calculated from the data, using the Lipman-Pearson algorithm (Lipman and Pearson, 1985) in DNASIS software package (Hitachi).

Results of complementary analysis of rDNA-ITS1 are reported in Table 2. Nt-isolates present a sequence similarity with AG-2-1 tester isolates from different geographic origins higher than 90%, which has been considered the minimum level of complementarity for isolates belonging to AG-2-1 (Kuninaga, 1997). Sequence similarity with AG-BI is lower, although the lowest has been recorded with isolates from the three ecological types so far described within AG-2-2 (Ogoshi, 1987; Hyakumachi *et al.*, 1998).

The results herewith reported provide conclusive evidence that Nt-isolates are actually members of AG-2-1, despite a low anastomosis affinity with its representative tester isolates, and emphasize the importance of the biomolecular approach for a correct classification of *R. solani* isolates belonging to AG-2, which is undoubtedly the most heterogeneous grouping (Sneh *et al.*, 1991). Recently, a low anastomosis frequency with AG-2-1 was recorded for some isolates which were part of a homogeneous group recovered from tulip in the Netherlands; this fact, combined with differences in pathogenicity, led to the establishment of a new grouping named AG-2-t (Schneider *et al.*, 1997). As a matter of fact, both observations may lessen the significance of a low anasto-

Table 2. r-DNA sequence similarity (%) in the region of ITS1.

		Nt-isolate			
AG/subgroup	Tester isolate	RT16	RT23	R93.1	RF4
Nt-isolates	RF4	98.6	97.7	97.2	
	R93.1	97.2	99.5		
	RT23	99.1			
AG-2-1	88-033	96.8	97.2	97.7	96.3
	Ala-Gra	96.3	96.8	97.2	95.9
	R123	93.8	93.8	94.2	93.3
	88-822	96.3	95.4	95.4	95.9
	F56L	96.3	96.8	97.2	95.9
AG 2-t	R144	95.9	96.3	96.8	95.4
AG-2-2 IIIB	61-D-3	70.4	68.9	68.9	70.8
	B60	71.6	72.0	71.6	72.0
	89-21-4	71.6	72.0	71.6	72.0
	IFO 30942	73.0	72.9	72.5	73.4
AG-2-2 IV	BC-10	71.6	72.2	71.1	71.2
	H13	71.0	70.6	70.6	71.0
	DRGO1	72.2	72.2	72.2	72.6
	86-42-4	70.5	70.4	69.9	70.9
AG-2-2 LP	G4	65.3	64.9	64.9	65.8
	48R	65.3	64.9	64.9	65.8
AG-2-3	237258	81.1	80.6	81.5	80.6
AG-8	A68	85.1	72.0	72.5	85.1
AG-BI	TE2-4	81.7	82.1	82.6	81.3
	SHC81	80.9	81.3	81.8	80.4
	AI 1-4	81.3	81.8	82.2	80.9
	SH1-2	81.3	81.8	82.2	80.9

mosis frequency as criteria for the classification of isolates belonging to AG-2-1 which may present other distinctive features. It also must be considered that genetic variation within AG-2 may be higher than that recorded in other AGs, as already pointed out by MacNish *et al.* (1994) who described some more groupings from Australian crops on the basis of pectic zymograms.

The original description of AG-BI reported a high anastomosis frequency with isolates from AG 2-2 (Kuninaga *et al.*, 1979). In this note we provide new evidence of high affinity of AG-BI with AG-2-1 which may be favourable to its integration in AG-2. Moreover, isolates of AG-8 are also known to bridge with isolates of AG-BI and AG-2 (Sneh *et al.*, 1991). Therefore the separation of the so-called 'bridging isolates' into a specific anastomosis group seems to have become questionable, and a more thorough investigation for a better understanding of the relationships among AG-BI, AG-8, and the various taxonomic entities within AG-2 based upon their biomolecular features should be carried out.

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