Theor Appl Genet (1995) 91: 1184–1189

D. Bai · R. Reeleder · J. E. Brandle

Identification of two RAPD markers tightly linked with the *Nicotiana debneyi* gene for resistance to black root rot of tobacco

Received: 24 January 1995 / Accepted: 23 June 1995

Abstract Linkage of randomly amplified polymorphic DNA (RAPD) markers with a single dominant gene for resistance to black root rot (*Chalara elegans* Nag Raj and Kendrick; Syn. *Thielaviopsis basicola* [Berk. and Broome] Ferraris) of tobacco (*Nicotiana tabacum* L.), which was transferred from *N. debneyi* Domin, was investigated in this study. There were 2594 repeatable RAPD fragments generated by 441 primers on DNAs of 'Delgold' tobacco, a BC_5F_8 near isogenic line (NIL) carrying the resistance gene in a 'Delgold' background, and 'PB19', the donor parent of the resistance gene. Only 7 of these primers produced eight RAPD markers polymorphic between 'Delgold' and 'PB19', indicating there are few RAPD polymorphisms between them despite relatively dissimilar pedigrees. Five of the eight RAPD markers were not polymorphic between 'Delgold' and the NIL. All of these markers proved to be unlinked with the resistance gene in F_2 linkage tests. Of the remaining three RAPD markers polymorphic between 'Delgold' and the NIL, two were shown to be strongly linked with the resistance gene; one in coupling and the other in repulsion. Application of the two RAPDs in the elimination of linkage drag associated with the *N. debneyi* resistance gene and marker-assisted selection for the breeding of new tobacco cultivars with the resistance gene is discussed.

Key words *Nicotiana tabacum* · *Chalara elegans* · Black root rot · Resistance gene · RAPD · Linkage

Communicated by G. E. Hart

D. Bai[1] (✉)
Imperial Tobacco Ltd, P.O. Box 6500, Montreal, Quebec, Canada, H3C 3L6

R. Reeleder · J. E. Brandle
Pest Management Research Centre, Agriculture and Agri-Food Canada (Delhi Farm), P.O. Box 186, Delhi, Ontario, Canada, N4B 2W9

Present address:
[1] Pest Management Research Centre, Agriculture and Agri-Food Canada (London), 1391 Sandford Street, London, Ontario, N5V 4T3, Canada

Introduction

Black root rot, caused by *Chalara elegans* Nag Raj and Kendrick (Syn. *Thielaviopsis basicola* [Berk. and Broome] Ferraris), is a common soil-borne fungal disease found in Canadian tobacco (*Nicotiana tabacum* L.)-growing areas. The disease, characterized by black lesions on the roots and hypocotyl, results in stunt and late-maturing, uneven stands, all of which reduce the yield and quality of flue-cured tobacco. Black root rot is also an important pathogen of numerous other crops, including carrot (Punja et al. 1992), cotton (Rothrock 1992), soybean (Lockwood, et al. 1970), and peanut (Hsi, et al. 1965).

Polygenic partial resistance to black root rot is present in the tobacco genomes (Clayton 1969; Wilkinson et al. 1991). However, such resistance may not provide sufficient protection in situations where soil fumigants are not used (Shew and Shoemaker 1993). Soil fumigation is used routinely and in part assists in minimizing the damage caused by black root rot in Canada. Although crop loss associated with black root rot is small, the cost of control with soil fumigants is very high in both economic and environmental terms. A single gene conferring complete resistance to a wide spectrum of black root rot isolates was first transferred to burley tobacco from *N. debneyi* Domin by interspecific hybridization and backcrossing (Hoffbeck *et al.* 1965; Clayton 1969). However, the gene has not been widely used in flue-cured tobacco breeding because of negative agronomic and chemical traits (later maturity, reduced yield, reduced total nitrogen content, and lower total alkaloids) associated with the resistance gene (Legg et al. 1981; unpublished data).

Koebner and Shepherd (1986, 1988) and Rogowsky et al. (1991) induced allosyndetic recombination in wheat translocation lines 1BL/1RS and 1DL/1RS by removing or suppressing the *ph1b* gene and selected several interstitial wheat-rye translocation lines having no linkage drag from rye with the aid of DNA markers. If the negative traits in the tobacco containing the *N. debneyi* resistance gene are caused by the nearby deleterious genes in the *N. debneyi*

Table 1 RAPD polymorphisms among the recurrent parent 'Delgold', the resistance gene donor parent 'PB19', and the near iso-genic line (NIL) possessing the *N. debneyi* gene for resistance to black root rot in a 'Delgold' background

Primer	Primer sequence (5′ to 3′)	Size of polymorphic RAPD marker (bp)	Designation of the RAPD locus	'Delgold'	'PB19'	NIL
UBC 83	GGGCTCGTGG	550	$UBC83_{550}$	+[1]	–	–
UBC 108	GTATTGCCCT	400	$UBC108_{400}$	–	+	–
UBC 308	AGCGGCTAGG	670	$UBC308_{670}$	–	+	–
UBC 336	GCCACGGAGA	450	$UBC336_{450}$	–	+	–
UBC 348	CACGGCTGCG	800	$UBC348_{800}$	–	+	–
UBC 417	GACAGGCCAA	420	$UBC417_{420}$	+	–	–
UBC 418	GAGGAAGCTT	700	$UBC418_{700}$	+	–	+
		1050	$UBC418_{1050}$	–	+	+

[a] +, Presence of the RAPD marker; –, absence of the RAPD marker

transfer, they may be similarly eliminated by reducing the size of the introgressed segment. DNA markers linked with the *N. debneyi* gene will be useful in identifying the smallest *N. debneyi* segments among the induced recombinants.

DNA markers associated with the resistance gene can be used to determine the genotype of tobacco seedlings before transplanting. The cost of transplanting and cultivation could be reduced, and the breeding efficiency of tobacco cultivars with the resistance gene could be increased. In the present article, we report two RAPD (randomly amplified polymorphic DNA, Williams et al. 1990) markers that are tightly linked with the *N. debneyi* resistance gene.

Materials and methods

Plant materials

The black root rot resistance gene from *N. debneyi* was first transferred to burley tobacco, cv 'Burley 49' (Clayton 1969; Hoffbeck et al. 1965). A backcrossing program was conducted at the Pest Management Research Centre (Delhi Farm), Agriculture and Agri-Food Canada, to introgress the gene into a flue-cured tobacco breeding line, 'PB19' ('Burley 49'/'Virginia Gold'//'Yellow Gold'/'3'/'McNair 20'). A near-isogenic line (NIL) with the black root rot resistance gene in a 'Delgold' background was developed by crossing 'PB19' with tobacco cv 'Delgold' ('Hicks broadleaf'/'3'/'*N. rustica*' cv 'Babor'/'Virginia 115'//'Virginia 115'/'4'/'Virginia 115'), followed by five backcrosses to 'Delgold', and eight generations of selfing (BC_5F_8). 'Delgold' is the current dominant flue-cured tobacco cultivar grown in Canada. It is moderately susceptible to black root rot. 'Delgold', the NIL, and the donor parent of the resistance gene, 'PB19', were all used in the initial screening for polymorphic RAPD markers. The reason for including the donor parent in the initial screening for polymorphic RAPD markers was to detect the partially linked RAPD markers with the resistance gene, if any. Inheritance of the black root rot resistance and polymorphic RAPD markers from the initial screening and their linkage tests were carried out with an F_2 population (85 plants) from the cross: 'PB19'/'Delgold'.

Evaluation of black root rot resistance

'Delgold', the NIL, 'PB19', and the F_2s from the cross 'PB19'/'Delgold' were tested for black root rot resistance in a tank using a modification of the system proposed by Litton (1983). A virulent culture of *C. elegans* isolated from tobacco plants grown in Southern Ontario was used as the inoculum source in all of our evaluations. Root systems of the inoculated plants were washed free of silica sand and examined macroscopically 2 weeks after inoculation. Plants were rated for severity of black root rot on a 0 to 9 scale, where 0=root systems free of symptoms and 9=severely damaged roots. The F_2 plants were then transplanted to pots and held in the greenhouse for selfing to produce F_3 progeny. Genotypes of F_2 plants for black root rot resistance were confirmed with F_3 progeny tests carried out as above.

RAPD analyses

A total of 700 decamers (single-stranded 10-base oligonuleotides) were used as primers in the RAPD analyses. They were obtained from the Oligouncleotide Synthesis Laboratory of the University of British Columbia (Primer UBC1 – UBC700). Genomic DNA was extracted from 'Delgold', the NIL, 'PB19', and the F_2 plants from the cross 'PB19'/'Delgold' using a modification of the rapid leaf disc method of Edwards et al. (1991). The first two true leaves of 4-to 5-week-old tobacco seedlings grown in the temperature-controlled tanks were excised prior to inoculation with the black root rot conidia and placed in a 1.5-ml plastic tube. Sterile silica sand was added for better grinding, and 2 ml of RNAase was added to the extraction solution to remove RNA.

Each 25 ml of polymerase chain reaction (PCR) mixture contained 20 ng DNA, 50 m*M* KCl, 10 m*M* Tris-HCl (pH 9.0 at 25°C), 0.1% Triton X-100, 0.2 m*M* each of dATP, dCTP, dGTP and dTTP, 0.2 m*M* of random primer, 2.5 m*M* $MgCl_2$, and 0.625 units of Taq polymerase. Amplification was performed in a Perkin Elmer DNA Thermal Cycler 480 for 45 cycles. The starting cycle was 2 min at 94°C, 10 min at 35°C, and 2 min at 72°C. Subsequent cycles were 45 s at 94°C, 2 min at 72°C, and 45 s at 35°C, followed by 10 min at 72°C. The RAPD fragments were separated electrophoretically on 1.5% agarose gels, stained with ethidium bromide, and visualized on an UV transilluminator.

Chi-square (χ^2) tests were used in analyses of the inheritance of black root rot resistance, the polymorphic RAPD markers, and their linkage. The nomenclature used by Michelmore et al. (1991) was employed in describing RAPD loci.

Results

Screening of RAPD polymorphisms among the original parents and the NIL

'Delgold', the NIL, and 'PB19' were screened with 700 decamers, only 63% of which generated repeatable RAPD fragments. A total of 2594 discernible DNA fragments ranging from 100 to 2000 bp were produced, correspond-

Table 2 Segregation of RAPD markers and the gene for resistance to black root rot resistance and their linkage tests in the F_2 population from the cross 'PB19'/'Delgold' (*BRR* black root rot, resistant)

Locus	Expected ratio	Observed frequency	χ^2	*P*
BRR	3:1	60:25	0.88	0.25–0.50
$UBC83_{550}$	3:1	64:21	0.004	0.90–0.95
$UBC108_{400}$	3:1	63:22	0.035	0.75–0.90
$UBC308_{670}$	3:1	68:17	1.13	0.25–0.50
$UBC336_{450}$	3:1	60:25	0.88	0.25–0.50
$UBC348_{800}$	3:1	62:23	0.19	0.50–0.75
$UBC417_{420}$	3:1	57:28	2.86	0.05–0.10
$UBC418_{700}$	3:1	59:26	1.42	0.10–0.25
$UBC418_{1050}$	3:1	60:25	0.88	0.25–0.50
$BRR/UBC83_{550}$	9:3:3:1[a]	39:21:25:0[a]	13.70	<0.005
$BRR/UBC108_{400}$	9:3:3:1	46:17:14:8	1.55	0.50–0.75
$BRR/UBC308_{670}$	9:3:3:1	50:19:11:5	2.24	0.50–0.75
$BRR/UBC336_{450}$	9:3:3:1	43:17:14:11	6.88	0.05–0.10
$BRR/UBC348_{800}$	9:3:3:1	38:24:19:4	7.01	0.05–0.10
$BRR/UBC417_{420}$	9:3:3:1	40:16:19:10	1.88	0.50–0.75
$BRR/UBC418_{700}$	9:3:3:1	46:13:16:10	4.75	0.10–0.25
$BRR/UBC418_{1050}$	9:3:3:1	60:0:0:25	107.94	<0.005

[a] The phenotype order is: black root rot resistant and presence of the RAPD marker, black root rot resistant and absence of the RAPD marker, black root rot susceptible and presence of the RAPD marker, black root rot susceptible and absence of the RAPD marker

ing to an average of 5.9 fragments per primer. Seven of these primers generated eight polymorphic RAPD markers between the original parents, 'Delgold' and 'PB19', and only three of the eight RAPD markers were polymorphic between 'Delgold' and the NIL (Table 1).

Inheritance of the gene for resistance to black root rot and the polymorphic RAPD markers, and their linkage tests in an F_2 population

In the F_2 population from the cross 'PB19'/'Delgold', the black root rot resistance gene and the eight polymorphic RAPD markers were segregating according to Mendelian rule as dominant genes (Table 2). Only two of the RAPD markers, $UBC83_{550}$ and $UBC418_{1050}$, were linked with the resistance gene. In the F_2 linkage test of $UBC83_{550}$ with the resistance gene, no bands (550 bp) were detected in any of the 21 homozygous resistant F_2 plants, while the bands were present in all of the susceptible and heterozygous F_2 plants (Table 2 and Fig. 1), indicating that $UBC83_{550}$ was tightly linked, in repulsion, with the resistance gene. In the F_2 linkage test of $UBC418_{1050}$ with the resistance gene, all of the 60 resistant F_2 plants had the 1050-bp bands, while none of the 25 susceptible F_2 plants had these bands (Table 2 and Fig. 2), indicating that $UBC418_{1050}$ was completely linked, in coupling, with the resistance gene.

Evidence for the origin of the black root rot resistance gene from *N. debneyi*

Eighteen tobacco cultivars from diverse tobacco growing regions of the world, plus *N. debneyi*, were screened using primers UBC83 and UBC418 (Table 3). $UBC83_{550}$ was present in all susceptible tobacco cultivars, but absent in the cultivars and lines carrying the gene for resistance to black root rot from *N. debneyi*. $UBC418_{1050}$ was present in *N. debneyi* and in all resistant tobacco cultivars carrying the resistance gene from *N. debneyi*, but absent in the susceptible cultivars and lines without the gene, regardless of their geographic origins (Fig. 3). The presence of $UBC418_{1050}$ in *N. debneyi* is strong evidence at the DNA level that the black root rot resistance gene originated from *N. debneyi*.

Discussion

Near-isogenic lines have been used successfully to tag several important disease resistance genes using restriction fragment length polymorphism (RFLP) and RAPD markers (Bentolila et al. 1991; Hartl et al. 1993; Hinze et al. 1991; Penner et al. 1993; Schachermayr et al. 1994; Schuller et al. 1992; Williamson et al; 1994; Young and Tanksley 1989). In this study, five RAPD markers ($UBC108_{400}$, $UBC308_{670}$, $UBC336_{450}$, $UBC348_{800}$, and $UBC418_{700}$) that were polymorphic between 'Delgold' and the donor parent 'PB19', but not between the recurrent parent 'Delgold' and the NIL proved to be segregating at random with respect to the resistance gene in the F_2 linkage tests (Table 2). Three other RAPD markers ($UBC83_{550}$, $UBC417_{420}$, and $UBC418_{1050}$) were found to be polymorphic between the recurrent parent 'Delgold' and the NIL but not between the NIL and the donor parent 'PB19'; two of these three markers proved to be tightly linked with the resistance gene.

Only 0.3% of the 2594 RAPD fragments generated by 441 primers were polymorphic between 'Delgold' and the donor parent 'PB19'. The low levels of polymorphism of RAPD markers detected was probably due to a low diver-

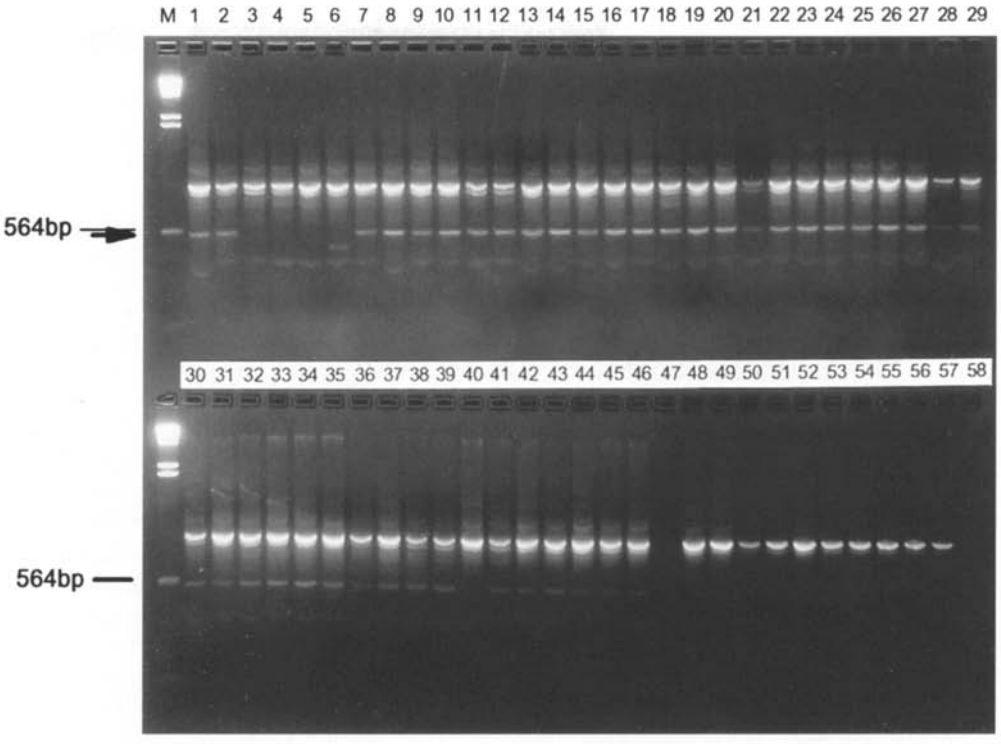

Fig. 1 A RAPD marker (550 bp, *arrow*) generated by primer UBC83 (GGGCTCGTGG) ($UBC83_{550}$) was completely linked with the susceptible "allele" of the black root rot resistance gene (repulsion linkage). The susceptible parent, 'Delgold' (*lanes 1–2*), susceptible F_2 plants (*lanes 7–27*) and heterozygous resistant F_2 plants (*lanes 28–39* and *41–49*) from the cross 'Delgold'/'PB19' all had $UBC83_{550}$, while the resistant parent, 'PB19' (Lanes *3–4*), the NIL with the black root rot resistance gene in 'Delgold' (*lanes 5–6*), and the homozygous resistant F_2 plants (*lanes 40*, *50–57*) were all without $UBC83_{550}$

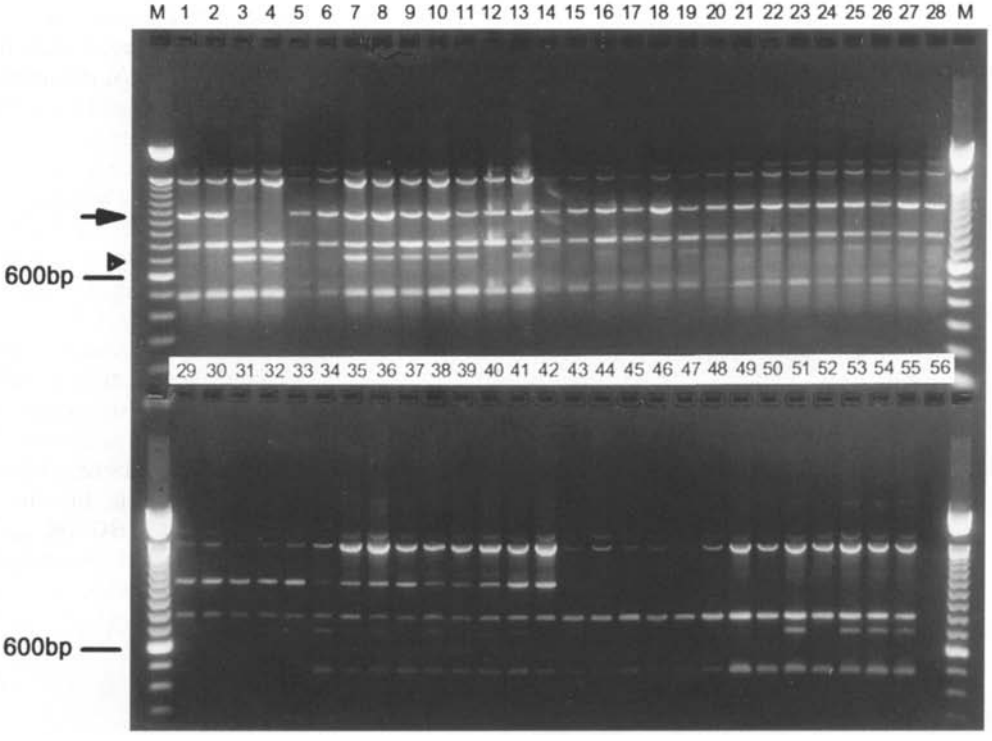

Fig. 2 A RAPD marker (1050 bp, arrow) generated by primer UBC 418 (GAGGAAGCTT) ($UBC418_{1050}$) was tightly linked with the resistant allele of *N. debneyi* black root rot resistance gene (coupling linkage). The resistant parent, 'PB19' (*lanes 1–2*), and the resistant F_2 plants from the cross 'PB19'/'Delgold' (*lanes 5–42*) all had $UBC418_{1050}$, while susceptible parent, 'Delgold' (*lanes 3–4*) and the susceptible F_2 plants (*lanes 43–55*) were all without $UBC418_{1050}$. The *solid triangle* indicates the RAPD marker $UBC418_{700}$, which is segregating randomly with the resistance gene

sity of DNA sequences in tobacco in general, as the pedigrees of these two lines were dissimilar.

The coupling RAPD marker, $UBC418_{1050}$, was present in all resistant tobacco cultivars carrying the *N. debneyi* gene and in *N. debneyi* itself, but was absent in all susceptible cultivars examined. This indicates that $UBC418_{1050}$ may represent a segment of DNA introgressed from *N. debneyi*. Since no recombination was detected in the 85 F_2 plants between $UBC83_{550}$ and the susceptible "allele" of the resistance gene, $UBC83_{550}$ is likely located in the corresponding tobacco chromosomal segment that was replaced by the alien transfer.

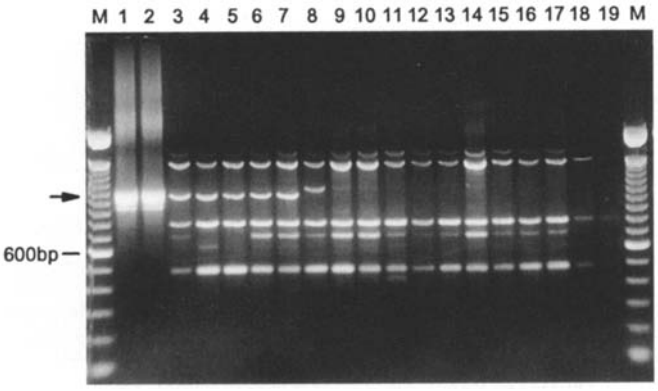

Fig. 3 $UBC418_{1050}$ RAPD marker (arrow) was present in *N. debneyi*, the tobacco cultivars with the black root rot resistance gene, but absent in the tobacco cultivars without the resistance gene. *M* 100 bp ladder markers, *lanes 1–2 N. debneyi*, *lane 3* Burley 49, *lanes 4–5* 'PB19', *lanes 6–7* the NILs, *lane 8* Southedge; *lane 9* 'Delgold', *lane 10* Delfield, *lane 11* Delliot, *lane 12* Candel, *lane 13* AC Cheng, *lane 14* Hicks broadleaf, *lane 15* Delhi 34, *lane 16* Virginia 21, *lane 17* Delhi 76, *lane 18* K326, *lane 19* Wanda

Table 3 Survey of tobacco cultivars for presence of $UBC83_{550}$ and $UBC418_{1050}$ RAPD markers (*BRR* black rot root, *R* resistant, *S* susceptible)

Species or Cultivars	Origin	Reaction to BRR[a]	$UBC83_{550}$	$UBC418_{1050}$
N. debneyi	USA	R[a]	–	+
Burley 49	USA	R	–	+
'PB19'	Canada	R	–	+
NIL	Canada	R	–	+
Coker 319	USA	S	+	–
Hicks broadleaf	USA	S	+	–
Virginia 115	USA	S	+	–
Virginia 21	USA	S	+	–
Delhi 34	Canada	S	+	–
Delhi 76	Canada	S	+	–
'Delgold'	Canada	S	+	–
Candel	Canada	S	+	–
Delliot	Canada	S	+	–
Delfield	Canada	S	+	–
AC Cheng	Canada	S	+	–
K326	USA	S	+	–
K399	USA	S	+	–
NC60	USA	S	+	–
Southedge	Australia	S	+	–
Wanda	Poland	S	+	–
Q-269-S	Australia	S	+	–

[a] +, Presence of marker; –, absence of marker

In most of the studies in which strong linkage has been reported between RFLP or RAPD markers and disease resistance loci, the latter traits were derived from introgression via interspecific crosses (Ishii et al. 1994; Martin et al. 1991; Paran and Michelmore 1991; Ronald et al. 1992; Schachermayr et al. 1994; Williamson et al. 1994). In the present study, two RAPD markers were found to be linked with the *N. debneyi* gene for resistance to black root rot. The ready detection of DNA markers associated with the introgressed genes from other species may be attributed to (1) the high diversity of DNA sequences between the alien transfers and the corresponding host chromosomal segment, and (2) no or low occurrence of recombination between the alien transfer and the corresponding host chromosomal segment. Chungwongse et al (1994) have recently shown that recombination within the region of alien DNA carrying the *hv* gene in tomato can be greatly reduced. Thus, the apparent tight linkage between $UBC418_{1050}$ and the gene for resistance to black root rot may not truly represent their physical distance.

N. debneyi is a tetraploid species (2n=XXYY=48) that has two distinct genomes from those of tobacco (2n=SSTT=48) (Goodspeed 1954). Among the 450 primers screened with tobacco (cv 'Delgold') and *N. debneyi*, 89% generated polymorphic RAPD markers (unpublished data). In this study, however, 441 primers were screened and only one RAPD marker was found to be tightly linked in coupling to the *N. debneyi* transfer containing the gene for resistance to black root rot. This indicates that despite the fact that the map distance may underepresent the physical distance between the gene and the RAPD marker, the size of the alien transfer is probably small.

In order to eliminate the negative agronomic and chemical traits associated with the resistance gene, the size of the alien transfer could be further reduced by induced recombination. The two linked RAPD markers, $UBC83_{550}$ and UBC_{1050}, can be used in monitoring further reduction of the alien introgression. The two markers can also provide a starting point for location, isolation, and cloning of the alien resistance gene in the tobacco genomes. However, enriching of the density of markers in their region will be required.

RAPD marker-assisted selection for black root rot resistance could accelerate the introgression of the resistance gene into current tobacco cultivars. The resistant plants in the backcross population could be easily selected, without disease testing, by detection of the presence of the coupling RAPD marker, $UBC418_{1050}$. Since $UBC83_{550}$ was present in all susceptible tobacco cultivars without the resistance gene, homozygous resistant plants could be further distinguished from heterozygous resistant plants by detecting the absence of the repulsion RAPD marker. The RAPD marker-assisted selection of black root rot resistance can be easily done before the transplanting of tobacco seedlings from greenhouse to field. This will ensure that all plants in field trials are homozygous resistant and eliminates the need for progeny testing selections. Thus, the efficiency of breeding tobacco cultivars with the *N. debneyi* gene for resistance to black root rot can be greatly improved.

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