Marker-Assisted Selection for Resistance to Black Shank Disease in Tobacco

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ABSTRACT

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Bulked segregant (BSA) and random amplified polymorphic DNA (RAPD) analyses were used to identify markers linked to the dominant black shank resistance gene, *Ph*, from flue-cured tobacco (*Nicotiana tabacum*) cv. Coker 371-Gold. Sixty RAPD markers, 54 in coupling and 6 in repulsion phase linkage to *Ph*, were identified in a K 326-derived BC₁F₁ (K 326-BC₁F₁) doubled haploid (DH) population. Thirty RAPD markers, 26 in coupling and 4 in repulsion phase linkage to *Ph*, were used to screen 149 K 326-BC₂F₁ haploid plants. Complete linkage between the 26 coupling phase markers and *Ph* was confirmed by screening 149 K 326-BC₂F₁ DH lines produced from the haploid plants in black shank nurseries. RAPD markers OPZ-5₇₇₀ in coupling and OPZ-7₃₇₀ in repulsion phase linkage were used to select plants homozygous for the *Ph* gene for further backcrossing to the widely grown flue-cured cultivar K 326. Black shank disease nursery evaluation of 11 K 326-BC₄S₁ lines and their testcross hybrids to a susceptible tester confirmed linkage between *Ph* and OPZ-5₇₇₀. The results demonstrated the efficiency of marker-assisted selection for *Ph* using a RAPD marker linked in coupling and repulsion. Complete linkage between 26 RAPD markers and the *Ph* gene was confirmed in the K 326-BC₅ generation, and RAPD phenotypes were stable across generations and ploidy levels. These RAPD markers are useful in marker-assisted selection for *Ph*, an important black shank resistance gene in tobacco.

Additional keywords: bulked segregant and RAPD analyses, disease resistance genes, *Phytophthora parasitica* var. *nicotianae*

Soilborne oomycete *Phytophthora parasitica* Dastur var. *nicotianae* (Breda de Haan) Tucker is the causal agent of tobacco black shank. This disease can devastate both flue-cured and burley tobacco types in many producing areas worldwide and results in millions of dollars in lost revenue annually.

In North Carolina, *P. parasitica* var. *nicotianae*-infested fields can contain races 0 and 1 of the pathogen; race 0 is more fit and is the predominant pathogenic race (15). Races 2, 3, and isolate 63 of *P. parasitica* var. *nicotianae* have also been reported to cause black shank in South Africa, Connecticut, and the Hunsur tract in Karnataka, India, respectively (16,23,25). Black shank severity on susceptible genotypes is increased under high stress conditions such as a hot, dry growing season and the presence of root-knot nematodes (14,20,22). An integrated approach involving the use of resistant cultivars, multipurpose soil fumigants, and crop rotation is recommended for control of black shank in flue-cured tobacco (17).

Host resistance is the most economical form of black shank control. Due to the allopolyploid origin of the species, disease resistance genes from *N. tabacum* sources are under oligogenic control but are simply inherited. Conversely, disease resistance genes of interspecific origin are usually monogenic and express complete dominance in tobacco (29).

Most flue-cured tobacco cultivars possess resistance to black shank conferred by additive genes from cigar tobacco cultivar Florida 301 (Fla 301). These genes confer partial resistance to both races 0 and 1 of *P. parasitica* var. *nicotianae* (3,15,28). Dominant, monogenic black shank resistance has been transferred to tobacco from *Nicotiana plumbaginifolia* Viv. and *N. longiflora* Cav. (2,27). Resistance to *P. parasitica* var. *nicotianae* race 0 in the highly resistant flue-cured cultivar Coker 371-Gold (C 371-G) is controlled by a single dominant gene, *Ph*, pyramided upon the Fla 301 system (7).

Space limitations in disease nurseries and variable climatic conditions which influence epidemic development retard progress in breeding for resistance to soilborne pathogens in tobacco. Gametophytic selection for resistance in an F₁ *Nicotiana africana*-derived haploid tobacco population has proven to be a rapid method for developing pure line, black shank resistant genotypes. Segregating haploid populations are screened in disease nurseries, and

subsequently the chromosomes are doubled by an in vitro tissue culture procedure to produce doubled haploid (DH) lines. However, selected genotypes can be lost during tissue culture due to the presence of vascular bacterial and fungal endophytes that are not completely controlled with antibiotics (6).

Molecular markers provide a possible solution to this problem. Haploids could be screened for markers, indirectly selected for disease resistance, chromosome doubled, and seed of homozygous lines produced before initiating field trials. In the resulting DH population, genes at the black shank resistance locus would be fixed for the resistance allele, but the gene frequency would be less than one at unselected loci for the rest of the genome. Testing would occur among DH lines in disease nurseries to confirm resistance and eliminate recombinants. Further, the limited space in nurseries could be more efficiently utilized as haploids lacking disease resistance would be eliminated prior to chromosome doubling.

The random amplified polymorphic DNA (RAPD) procedure (31) in combination with bulked segregant analysis (BSA) provides an efficient method for identifying markers linked to specific genes or genomic regions (18). BSA and RAPD analyses have been used in several crop species to identify markers linked to disease resistance genes. The objectives of this study were to identify RAPD markers linked to the *Ph* gene from C 371-G and to assess their utility in marker-assisted selection (MAS) in a backcross breeding program for the development of black shank resistant tobacco cultivars.

MATERIALS AND METHODS

Plant material. Flue-cured tobacco breeding line NCTG 60, a DH line produced from the F₁ of C 371-G × K 326 and homozygous for the *Ph* gene, was crossed to K 326. Haploid plants were produced from this BC₁F₁ hybrid by crossing it to *N. africana* (5), their chromosome numbers were doubled by the in vitro leaf midvein culture method (12), and the DH lines were self-pollinated. Seventeen DH lines from the BC₁F₁ hybrid were classified for black shank resistance in replicated black shank nursery trials (6) and used in BSA and RAPD analyses to identify markers linked to the *Ph* gene.

Three BC₁F₁ DH lines, DH92-2770-40, DH92-3397-2, and DH92-3397-13, homozygous for *Ph*, were crossed to K 326. A total of

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164 BC₂F₁ haploid plants was produced and screened with RAPD markers identified in the BC₁F₁ population bulks as being linked to the *Ph* locus. The chromosomes of each BC₂F₁

haploid plant were doubled, and each resulting DH line was self-pollinated and crossed to a black shank susceptible tester, DH94-1577-1. DH lines and their testcross hybrids were

evaluated for black shank resistance in a field disease nursery (Fig. 1).

RAPD markers OPZ-5₇₇₀ and OPZ-7₃₇₀ linked in coupling and repulsion phase,

Fig. 1. Flow chart of the production and testing of plant materials used in this study.

respectively, to the *Ph* gene were used in MAS to the K 326-BC₅ generation. In the K 326-BC₄S₁ generation, lines were screened with RAPD markers OPZ-5₇₇₀ and OPZ-7₃₇₀, and *Ph/Ph*, *Ph/ph*, and *ph/ph* genotypes were selected. Each line was self-pollinated and hybridized with black shank susceptible KY 14 to produce testcross hybrids. Eleven K 326-BC₄S₁ lines and their testcross hybrids were screened in replicated trials in a black shank nursery to determine the precision of RAPD markers OPZ-5₇₇₀ and OPZ-7₃₇₀ in predicting genotypes (Fig. 1).

Black shank disease evaluation in the field and statistical analyses for the K 326-BC₂F₁ DH and K 326-BC₄S₁ lines. A sample of 149 K 326-BC₂F₁ DH lines and their testcross hybrids were screened in randomized complete block design (RCBD) experiments in black shank nurseries at the Upper Coastal Plain Research Station (UCPRS) in Rocky Mount and the Lower Coastal Plain Tobacco Research Station at Kinston, NC, in 1997. The 11 K 326-BC₄S₁ lines and their testcross hybrids were screened in an RCBD at the UCPRS in Rocky Mount, NC, in 1998.

In experiments conducted in 1997, each RCBD consisted of 22-plant rows of 25 treatments replicated twice. The treatments consisted of 10 K 326-BC₂F₁ DH lines, the BC₂F₁ DH parent as a resistant check and K 326 as a susceptible check arranged in a split-plot design with their testcross hybrids and the tester DH94-1577-1 included as another susceptible check. The 10 K 326-BC₂F₁ DH lines consisted of an approximately equal mixture of lines having and lacking RAPD markers identified in coupling phase linkage with *Ph* in the K 326-BC₂F₁ generation. In 1998, the 11 K 326-BC₄S₁ families and their testcross hybrids were grown in an RCBD experiment with plant row spacing and replications as previously described, with K 326 as the susceptible check.

In tests conducted in both years, all plots were bordered by the black shank susceptible cultivar Hicks to monitor disease pressure and maintain population densities of *P. parasitica* var. *nicotianae*. Black shank was assessed biweekly on an individual plant basis, with the final disease reading taken 98 days after transplanting. Black shank disease incidence (BSDI) was expressed as a percentage of the number of plants killed by the disease over the total number of plants in that row.

Analyses of variance (ANOVA) were performed on days of survival in the field (DIF) and arcsine transformed percent BSDI data using the GLM procedure in SAS (SAS Institute, Inc., Cary, NC). The data for the K 326-BC₂F₁ DH lines with RAPD markers linked in coupling phase to the *Ph* locus were analyzed separately from those of the DH lines lacking these markers, to obtain correct *F* tests for the latter, as the error variance was heterogeneous among the groups.

RAPD analyses of advanced K 326-BC lines. All *Ph/Ph* homozygous plants identified with RAPD markers OPZ-5₇₇₀ and OPZ-7₃₇₀ in the K 326-BC₄S₁ population were crossed to K 326 to produce the BC₅ generation. Five K 326-BC₅ plants were screened with 30 RAPD markers (Table 1) to examine linkage relationships of the introgressed region in this advanced generation produced by MAS.

DNA extraction. Total genomic DNA was extracted from leaves 1 to 1.5 cm long using a modified (11) CTAB method (1). DNA was quantified by fluorometry (Hoefer TKO 100, Pharmacia Biotech, Piscataway, NJ) and standardized to 5 ng µl⁻¹.

Bulk construction and polymerase chain reaction (PCR) protocols. Two contrasting DNA bulks (18) were formed from equal volumes of standardized DNA from black shank resistant and black shank susceptible DH lines. Ten BC₂F₁ DH lines without the *Ph* gene were used to produce a black shank susceptible bulk, and seven BC₂F₁ DH lines with *Ph* formed a black shank resistant bulk. The bulks were screened with 1,237-decamer primers obtained from Operon Technologies (Alameda, CA) and the University of British Columbia (Vancouver, Canada) to identify polymorphisms between the contrasting bulks.

Each RAPD reaction (31) was performed in a 15-µl reaction volume containing 15 ng of genomic DNA, 10 mM Tris-HCl, pH 8.3, and 50 mM KCl (1× Stoffel buffer), 200 µM of each dNTP, 1 unit of AmpliTaq DNA polymerase Stoffel fragment (PE Applied Biosystems Corp., Foster City, CA), 4 mM MgCl₂, 15 µg of bovine serum albumin (Sigma Chemical Co.,

St. Louis, MO), and 20 pM of a decamer primer. For a primer control, template DNA was replaced with sterile water (Sigma Chemical Co.). Each reaction was overlaid with 50 µl of sterile mineral oil (Sigma Chemical Co.).

Amplifications were performed in a 96-well PTC 100 thermal cycler (MJ Research, Watertown, MA); the temperature program used depended on the specific primer (Table 1). Amplified products were separated for 6 h at 3 V/cm constant voltage in 1.5% cold (4°C overnight) agarose gels (Sigma Chemical Co.) containing 0.15 µg of ethidium bromide per liter. Submerged gel electrophoresis was performed in 1× TBE buffer (0.09 M Tris borate, 0.002 M EDTA) containing 0.046 µg of ethidium bromide per liter. One lane on each gel contained a Lambda *Eco*RI-*Hind*III marker (Promega, Madison, WI) or a 100-bp DNA Ladder (Gibco BRL, Life Technologies, Grand Island, NY).

RESULTS

RAPD analyses of the K 326-BC₂F₁ and K 326-BC₂F₁ DH lines. A total of 60 easily scored and repeatable RAPD markers, 54 in coupling and 6 in repulsion phase, were identified linked to the *Ph* gene in the K 326-BC₂F₁ DH lines by BSA and RAPD analyses. A subset of 30 randomly chosen markers, 26 linked in coupling and 4 in repulsion phase (Table 1), was used for estimating linkage distance between the markers and *Ph*. Five marker recombination events were observed in the 149 K 326-BC₂F₁ DH lines analyzed with the 30 markers. Four events occurred in a single K 326-BC₂F₁ DH line. Two recombination events involved the RAPD

Table 1. Random amplified polymorphic DNA (RAPD) markers in coupling (n = 26) and repulsion phase (n = 4) linkage to the *Ph* gene from tobacco cultivar Coker 371-Gold, with their respective amplification programs^a

RAPD	Amplification program	RAPD	Amplification program
OPAD-3 ₇₁₀	2	UBC-248 ₄₃₀	1
OPAJ-15 ₅₃₅	2	UBC-265 ₁₀₁₀	2
OPAJ-12 ₅₂₅	2	UBC-30 ₄₉₀	1
OPAK-18 ₆₄₅	2	UBC-333 ₂₇₀	1
OPAN-2 ₇₆₀	2	UBC-422 ₄₂₀	2
OPE-10 ₄₃₀	1 ^b	UBC-427 ₅₇₅	2
OPH-18 ₄₃₀	2 ^c	UBC-443 ₃₆₀	1
OPM-16 ₂₅₀	1	UBC-443 ₅₆₀	1
OPN-8 ₄₄₀	3 ^d	UBC-469 ₃₈₅	2
OPR-1 ₁₁₆₀	1	UBC-564 ₃₄₅	2
OPW-3 ₄₁₀	2	UBC-613 ₆₄₀	2
OPZ-5 ₇₇₀	1	OPB-13 ₁₀₄₀ ^e	2
UBC-159 ₅₉₀	2	OPZ-7 ₃₇₀ ^e	1
UBC-169 ₆₈₀	1	UBC-365 ₅₆₅ ^e	2
UBC-247 ₅₇₀	2	UBC-671 ₁₂₃₀ ^e	2

^a RAPD band sizes have a standard deviation of 8 to 16 bp estimated using Seqaid Version 3.8 (Seqaid is available on-line from Indiana University, Bloomington, IN).

^b Program 1: 1 cycle 94°C/30 s; 3 cycles 94°C/1 min, 38°C/1 min, 72°C/2 min; 35 cycles 92°C/1 min, 40°C/1 min, 72°C/2 min + 1 s extension/cycle; 1 cycle 72°C/5 min.

^c Program 2: 1 cycle 94°C/30 s; 3 cycles 94°C/1 min, 38°C/1 min, 72°C/2 min; 35 cycles 92°C/10 s, 40°C/20 s, 72°C/2 min + 1 s extension/cycle; 1 cycle 72°C/5 min.

^d Program 3: 1 cycle 94°C/1 min; 15 cycles 92°C/30 s, 50°C/30 s – 0.5°C/cycle, 72°C/2 min; 20 cycles 92°C/10 s, 40°C/20 s, 72°C/2 min + 1 s extension/cycle; 1 cycle 72°C/5 min.

^e RAPD markers in repulsion-phase linkage to *Ph*.

OPAK-18₆₄₅. No recombinants were observed for RAPD markers linked in repulsion phase to *Ph*. Thus, determination of linkage distance between the 30 RAPD markers and *Ph* was not feasible.

Black shank disease evaluation of K 326-BC₂F₁ DH lines in the field. Black shank severity was similar at both locations, except for one of the experiments at Kinston. This accounted for most of the variability in disease pressure observed between locations (Table 2). If this experiment with only 35% BSDI was eliminated, only a 2% difference in %BSDI was observed between locations.

Black shank incidence reached 100% in the susceptible checks within 66 ± 6 days after transplanting, indicating high levels of *P. parasitica* var. *nicotianae* at both locations. Mean DIF was 83 ± 7 days, and BSDI was 54 ± 23% for the check K 326, which possesses a low level of partial Fla 301 black shank resistance.

K 326-BC₂F₁ DH lines with *Ph* and their testcross hybrids survived the entire growing season (98 days) at both locations (Table 2). Conversely, K 326-BC₂F₁ DH lines, their testcross hybrids, and susceptible checks without *Ph* succumbed to black shank within 75 days of transplanting.

The 30 RAPD markers linked to the *Ph* locus in the K 326-BC₁F₁ bulks accurately predicted the genotypes of the 149 K 326-BC₂F₁ DH lines classified in the haploid state. Mean DIF and BSDI for the K 326-BC₂F₁ DH lines with coupling markers were not significantly different from each other, their testcross hybrids, or the parental lines with *Ph* (Table 2). Mean DIF and BSDI for the K 326-BC₂F₁ DH lines lacking the coupling markers were significantly different from those of lines with the markers and from the susceptible checks K 326 and DH94-1577-1 (Table 2).

Genotype identification and disease evaluation of advanced K 326-BC lines. Disease nursery data for the K 326-BC₂F₁ DH lines confirmed that *Ph* and the RAPD markers linked to this gene were inherited as a block. Markers OPZ-5₇₇₀ and OPZ-7₃₇₀ linked in coupling and repulsion phase, respectively, were used in MAS to produce advanced generations of K 326-BC populations. Selection of plants with

OPZ-5₇₇₀ identified *Ph/ph* heterozygous plants. Selection for OPZ-5₇₇₀ and against OPZ-7₃₇₀ in the segregating K 326-BC₄S₁ population identified *Ph/Ph* homozygous plants (Fig. 2).

Weather conditions were conducive to disease development in the black shank nursery at UCPRS in Rocky Mount, NC, in 1998. Mean DIF was 79 days and BSDI was 48% for the susceptible check K 326. The mean DIF and BSDI values for K 326 in 1998 were well within one standard deviation of the mean values obtained for this line at this location in 1997. The black shank disease response in the 11 K 326-BC₄S₁ lines selected by MAS with OPZ-5₇₇₀ and OPZ-7₃₇₀ was in complete agreement with genotypes predicted by the markers. *Ph/Ph* and *Ph/ph* genotypes with RAPD marker OPZ-5₇₇₀ survived the entire growing season in the black shank nursery. The *ph/ph* K 326-BC₄S₁ lines lacking OPZ-5₇₇₀ had a disease response similar to that of the susceptible check K 326.

Mean DIF and BSDI for *Ph/Ph* and *Ph/ph* genotypes were not significantly different, while mean DIF and BSDI for the testcross hybrids of the two genotypic classes were significantly different. Disease responses of the *Ph/Ph* and *Ph/ph* genotypes were not significantly different from that of the DH check possessing *Ph*. However, disease response of the *Ph/Ph* and *Ph/ph* genotypes was significantly different from that of the susceptible check K 326 lacking *Ph*, but possessing low, partial Fla 301 resistance (Table 3).

All 30 RAPD markers identified in the K 326-BC₁F₁ BSA were present in the five K 326-BC₅ plants screened. This indicated that *Ph* was segregating as expected and that no recombination within the chromosomal block had occurred. Three plants were heterozygous for *Ph* and two were homozygous for the susceptible allele (Fig. 2). Banding patterns were similar between the early and advanced K 326-BC generations.

DISCUSSION

The dominant nature of the *Ph* gene was confirmed in black shank nursery evaluations of the 149 K 326-BC₂F₁ DH lines, in

that disease responses of the *Ph/Ph* homozygous DH lines were indistinguishable from those of their testcross hybrids and parental checks containing the gene. The ANOVA of the disease response showed that the variation in response of *Ph/Ph* K 326-BC₂F₁ DH lines was due to environmental differences between experiments and not genetic differences among the *Ph/Ph* DH lines.

Disease severity in lines lacking the coupling phase markers was variable and ranged from high to low black shank resistance. This varying level of resistance was assumed to be mediated by genes from Fla 301. The evaluation of a testcross hybrid with its parental line negated the need for multiple trials over locations and years in black shank nurseries to differentiate between genotypes possessing high Fla 301 resistance and those having the *Ph* gene. Testcross hybrids of lines with high Fla 301 resistance expressed a lower level of black shank resistance than those of *Ph/Ph* DH parental lines.

Twenty-six RAPD markers in coupling and four in repulsion phase linkage to *Ph* were identified in a K 326-BC₁F₁ DH population by BSA and RAPD analyses. Banding patterns produced by 28 of the RAPD primers were highly reproducible in all generations tested and in both haploid and diploid states. However, the intensity of RAPD bands for primers UBC-443 and UBC-613 was repeatedly very weak in the diploid BC₅ generation compared with the haploid BC₂F₁ generation. It is very unlikely that these observations were due to artifacts of RAPD amplification (9,13), as no major changes in DNA template:primer ratio were made. A possible cause may be a dilution effect or incomplete dominance as a result of competition for RAPD reaction components between alleles at this locus and other loci. If the match between the primer and the binding site at the alternative locus is better than that in the block containing *Ph*, then competitive amplification by two copies at the alternative locus in the diploid state could have partially or completely blocked the formation of the expected products from UBC-613 and UBC-443. It was shown that loss of a band in RAPD analyses with a

Table 2. Mean squares and significance levels for days of survival in the field (DIF) and black shank disease incidence (BSDI) in 149 K326-BC₂F₁ tobacco doubled haploid (DH) lines and their testcross hybrids in black shank nurseries at Rocky Mount and Kinston, NC, in 1997

Source	Mean square (lines with coupling markers)			Mean square (lines lacking coupling markers)		
	df	DIF	BSDI	df	DIF	BSDI
Experiment (Expt)	14	6.47* ^a	0.03***	14	1,502.1***	1.57
Replication (Expt)	15	2.44	0.01	15	156.6***	0.19**
Line vs. testcross hybrid	1	0.53	0.00	1	422.5***	0.20
Line within cross	136	3.45	0.01	163	109.1***	0.14**
Error	151	3.68	0.01	202	50.6	0.07
Lines with vs. lines without coupling markers	1	20.26***	1.02***			
Lines vs. DH 94-1577-1	1	31.66***	1.40***	1	11.5***	0.38**
Lines vs. K 326	1	14.51***	0.80***	1	5.7*	0.22

^a *, **, *** = significance at the 0.05, 0.01, and 0.005 probability levels, respectively.

single primer for eight *Xanthomonas campestris* strains, with the potential to amplify two bands at unrelated loci, was due to preferred synthesis of one band over the other product (26).

The RAPD markers all appear to be tightly linked in the block containing *Ph* and were useful in MAS of this gene into K 326. Thus, the 30 RAPD markers used in this study were just as informative as all 60 identified. We used OPZ-5₇₇₀ in coupling and OPZ-7₃₇₀ in repulsion phase

linkage to produce advanced K 326 backcross generations possessing *Ph*. The success of MAS was confirmed by disease evaluation of the K 326-BC₄S₁ generation in a black shank nursery. Three backcross generations per year using MAS in greenhouse plantings were possible, compared with one generation per year with DH production and field testing to identify *Ph/Ph* homozygous lines.

The RAPD markers in coupling phase linkage to *Ph* were not present in lines with

high Fla 301 resistance. This implies that *Ph* and the genes for the Fla 301 black shank resistance are not in the same linkage group or are more than 50 cM apart.

Marker-assisted selection for *Ph* can be used in conjunction with greenhouse evaluations with race 1 of *P. parasitica* var. *nicotianae* to pyramid black shank resistance to both races of the pathogen (32). Selection for resistance to *P. parasitica* var. *nicotianae* race 1 in populations heterogeneous for Fla 301 resistance but homozy-

Fig. 2. Amplification products produced by primers OPZ-5 and OPZ-7 in tobacco haploid and diploid K 326-derived backcross generations on a 1.5% agarose gel. Random amplified polymorphic DNA (RAPD) markers **A**, OPZ-5₇₇₀ and **B**, OPZ-7₃₇₀, framed by stippling, were identified linked in coupling and repulsion phase, respectively, to the *Ph* gene from Coker 371-Gold in the K 326-BC₁F₁ generation. The RAPD markers accurately identified all *Ph* genotypes in all generations tested in both haploid and diploid plants. The markers were used to select *Ph* heterozygous or homozygous plants for further backcross to K 326. "R" represents the dominant resistant *Ph* allele and "r" the susceptible *ph* allele. Lane PC is a primer control in which the DNA template was replaced with sterile distilled water. Lane M is either a 100 bp or Lambda *EcoRI-HindIII* marker.

Table 3. Mean squares and significance levels for days of survival in the field (DIF) and black shank disease incidence (BSDI) in 11 K 326-BC₄S₁ tobacco families and their testcross hybrids tested in a black shank nursery at Rocky Mount, NC, in 1998

Source	df	Mean square	
		DIF	BSDI
Replication	1	4.37	0.01
Genotype	24	83.92* a	0.15*
Error	24	16.10	0.03
<i>Ph/Ph</i> vs. <i>Ph/ph</i> genotypes with OPZ-5 ₇₇₀	1	1.20	0.11
<i>Ph/Ph</i> testcrosses vs. <i>Ph/ph</i> testcrosses	1	12.91***	0.51***
<i>Ph/ph</i> with OPZ-5 ₇₇₀ vs. checks with <i>Ph</i>	1	2.32	0.18
<i>Ph/ph</i> with OPZ-5 ₇₇₀ vs. K 326 with OPZ-7 ₃₇₀	1	11.28**	0.59***
<i>Ph/Ph</i> with OPZ-5 ₇₇₀ vs. checks with <i>Ph</i>	1	1.12	0.08
<i>Ph/Ph</i> with OPZ-5 ₇₇₀ vs. K 326 with OPZ-7 ₃₇₀	1	12.48***	0.69***

a *, **, *** = significance at the 0.05, 0.01, and 0.005 probability levels, respectively.

gous for *Ph* should permit the development of genotypes with very high levels of resistance to both races. Further, screening DH lines or haploid plants with OPZ-5₇₇₀ prior to disease evaluation in the black shank nursery would eliminate all genotypes in any generation without *Ph*. Thus, space in a disease nursery could be used more efficiently.

Although tight linkage is a prerequisite for successful MAS, the presence of all 26 coupling RAPD markers in the K 326-BC₂F₁ *Ph/Ph* DH lines and K 326-BC₅ *Ph/ph* heterozygous plants indicated reduced recombination between the introgressed chromosomal block containing *Ph* and its tobacco heterologous segment. Recombination between the markers and *Ph* was highly suppressed in the introgressed block, as no recombinants were observed in the 149 K 326-BC₂F₁ DH or 11 BC₄S₁ lines. The single K 326-BC₂F₁ DH line lacking four of the RAPD markers in coupling phase linkage to the *Ph* gene most likely lost a segment of the introgressed block. Thus, the markers may or may not be physically close to the gene.

In cytological studies of substitution lines, a substituted *N. plumbaginifolia* chromosome failed to associate with its *N. tabacum* counterpart during meiosis (10). It was speculated that a similar situation may exist for RAPD markers identified in *N. tabacum* linked to a black root rot resistance gene from *N. debneyi* (4). Low recombination in introgressed regions around interspecific resistance genes has also been reported in tomato and wheat (8,24,35).

Suppressed recombination in regions around an introgressed interspecific resistance gene makes reducing the effect of linkage drag difficult, when it is significant. However, suppressed recombination also facilitates the use of markers to select for multiple disease resistance. In tobacco, marker-assisted selection may be useful in developing cultivars with resistance to

black shank, black root rot (4), tobacco wildfire (33), root-knot nematodes (34), blue mold (19), TMV (30), and *Potato virus Y* (21).

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