Origin of the Black Shank Resistance Gene, *Ph*, in Tobacco Cultivar Coker 371-Gold

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ABSTRACT

Johnson, E. S., Wolff, M. F., Wernsman, E. A., Atchley, W. R., and Shew, H. D. 2002. Origin of the black shank resistance gene, *Ph*, in tobacco cultivar Coker 371-Gold. *Plant Dis.* 86:1080-1084.

Flue-cured tobacco (*Nicotiana tabacum*) cultivar Coker 371-Gold (C 371-G) possesses a dominant gene, *Ph*, that confers high resistance to black shank disease, caused by race 0 of the soil-borne pathogen *Phytophthora parasitica* var. *nicotianae*. The origin of this gene is unknown. Breeding lines homozygous for the *Ph* gene were hybridized with NC 1071 and L8, flue-cured and burley genotypes known to possess qualitative resistance genes from *Nicotiana plumbaginifolia* and *N. longiflora*, respectively. The F_1 hybrids were out-crossed to susceptible testers and the progenies evaluated in field black shank nurseries and in greenhouse disease tests with *P. parasitica* var. *nicotianae* race 0. Results showed that *Ph* was allelic to *Php* from *N. plumbaginifolia* in NC 1071. Testcross populations of hybrids between burley lines homozygous for *Ph* and L8, possessing *Phl* from *N. longiflora*, showed that *Ph* and *Phl* integrated into the same tobacco chromosome during interspecific transfer. Nevertheless, the two loci were estimated to be 3 cM apart. Random amplified polymorphic DNA (RAPD) analyses of the testcross progenies confirmed that recombination between the two loci was occurring. Forty-eight RAPD markers linked to *Ph* in doubled haploid lines were used in cluster analyses with multiple accessions of *N. longiflora* and *N. plumbaginifolia*, breeding lines L8, NC 1071, and DH92-2770-40, and cultivars K 326, Hicks, and C 371-G. A cladogram or region tree confirmed the data obtained from field and greenhouse trials, that *Ph*, transferred from C 371-G to DH92-2770-40, and *Php* in NC 1071 were allelic and originated from *N. plumbaginifolia*.

Additional keywords: disease resistance genes, gene phylogeny

Black shank disease of tobacco is caused by the hemibiotrophic oomycete *Phytophthora parasitica* var. *nicotianae* (Breda de Haan) Tucker, which attacks tobacco roots, stems, and leaves at any stage of development. Disease development is rapid under conditions of warm temperatures and high humidity (16). Annually, 1 to 2% of the flue-cured tobacco crop is lost to black shank in North Carolina, and this results in millions of dollars in lost revenue.

Three physiological races of *P. parasitica* var. *nicotianae* are present in the United States. The most common strain, race 0, is defined as being nonpathogenic on *Nicotiana plumbaginifolia* Viv. (1). Race 1 is pathogenic on *N. plumbaginifolia*, but is less fit than race 0 and occurs under intense selection pressure resulting from continuous cultivation of race 0-resistant cultivars (2). Races 2 and 3 and

isolate 63 of *P. parasitica* var. *nicotianae* (18,20,24) have been reported in South Africa, Connecticut, and the Hunsur tract in Karnataka, India, respectively.

Both oligogenic and monogenic resistance sources to *P. parasitica* var. *nicotianae* are known in tobacco. In 1931, Tisdale transferred oligogenic black shank resistance to cigar cultivar Florida 301 (Fla 301). This resistance source confers partial resistance to both race 0 and race 1 of *P. parasitica* var. *nicotianae* (27). Initially, Fla 301 resistance was the only source available to tobacco breeders, and it was used to develop all black shank-resistant flue-cured and burley cultivars in the United States up to 1964. As such, 71% of all tobacco cultivars released since 1964 also have Coker 139 and Hicks in their pedigrees (5). Severe losses in tobacco cultivars with a high level of Fla 301 resistance have been documented in plants under drought stress (26). This prompted the search for more reliable sources of black shank resistance. Consequently, dominant, monogenic resistance from *N. plumbaginifolia* (1,7) and *Nicotiana longiflora* Cav. (26) were introgressed into tobacco breeding lines.

Segregation analyses on the root response of *N. tabacum* lines that contain

dominant monogenic interspecific sources of black shank resistance have been ongoing, and conflicting results have been reported. In 1962, transfer of two types of black shank resistance sources from *N. plumbaginifolia* was reported. Type 0 resistance was conferred by a single dominant factor and was only effective against race 0 (1). Type 0,1 resistance was effective against both race 0 and race 1 of *P. parasitica* var. *nicotianae* (1) and could have been a Fla 301 type seed contaminant. In 1970, all accessions of *N. longiflora* tested were reported to be resistant to both race 0 and race 1 of *P. parasitica* var. *nicotianae*, whereas *N. plumbaginifolia* had high resistance to race 0 only (15). In 1971, no segregation for black shank resistance in F_2 populations of *N. longiflora* $\times$ *N. plumbaginifolia* was observed, and the authors concluded that the two sources of black shank resistance were homologous (8).

The flue-cured tobacco cultivar Coker 371-Gold (C 371-G), released in 1986, possessed the highest level of black shank resistance observed among U.S. flue-cured cultivars at that time. The origin of the resistance genes in this cultivar was unknown. Black shank resistance in C 371-G was reported to be similar to that of NC 1071, a breeding line with black shank resistance derived from *N. plumbaginifolia* (9). Black shank resistance in C 371-G is derived from a single dominant gene, designated *Ph*, pyramided upon the Fla 301 quantitative genetic resistance (6).

Breeding lines NC 1071 and L8 have dominant monogenic black shank resistance derived from *N. plumbaginifolia* and *N. longiflora*, respectively (12,26). Testcross populations from F_1 hybrids between breeding lines homozygous for *Ph* and lines with known sources of dominant monogenic black shank resistance from *N. plumbaginifolia* and *N. longiflora* are suitable to study the allelic relationships among these black shank resistance sources. The objective of this study was to determine if *Ph* from C 371-Gold was allelic to the dominant resistance genes from *N. plumbaginifolia* and/or *N. longiflora*.

MATERIALS AND METHODS

Plant materials. The *Ph* gene was transferred from flue-cured cultivar C 371-G to a burley tobacco population through

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Accepted for publication 21 May 2002.

the production of a three-way hybrid involving two black shank-susceptible burley breeding lines, doubled haploid (DH) 408 and DH 410. DH 408 was hybridized with C 371-G, and the F₁ hybrid was crossed to DH 410. Twenty-six plants possessing the burley phenotype were identified from the segregating three-way cross and intercrossed at random to produce 13 full-sib families. Equal numbers of seeds from each cross were combined to form a random-mated burley population segregating for *Ph*. Following three cycles of mass selection for black shank resistance in a field disease nursery, resistant inbred lines were produced (E. A. Wernsman, unpublished data). The S₄ line, TG 593-13, known to be homozygous for *Ph*, was used in this study.

To determine if *Ph* and the dominant monogenic black shank resistance gene in *N. longiflora* (designated *Phl* in this paper) are allelic, TG 593-13 was hybridized with L8. Testcross progenies were produced by crossing the F₁ hybrid (*Ph/Phl*) to the black shank-susceptible burley cultivar KY 14.

The K 326-derived BC₁F₁ line DH92-2770-40, possessing *Ph*, was hybridized with NC 1071 to determine if *Ph* and the dominant black shank resistance gene in NC 1071 derived from *N. plumbaginifolia* (designated *Php* in this paper) are allelic. The F₁ hybrid *Ph/Php* was crossed as the female parent with black shank-susceptible tester DH94-1577-1 to produce the test-cross progeny.

Field evaluation of the *Ph/Phl* and *Ph/Php* testcross progenies in black shank nurseries. A sample of 440 *Ph/Phl* testcross progenies from the cross [TG 593-13 × L8] × KY 14 and 444 *Ph/Php* testcross progenies from [DH92-2770-40 × NC1071] × DH94-1577-1 were evaluated in randomized complete block (RCB) design experiments in black shank nurseries at the Upper Coastal Plain Research Station at Rocky Mount, NC, in 1997. Each RCB consisted of 22-plant plots containing parental lines TG 593-13, L8, NC 1071, and DH92-2770-40 as resistant checks, testers KY 14 and DH94-1577-1 as susceptible checks replicated twice, and the test-cross progenies.

The experiments were bordered by the black shank susceptible cultivar Hicks to monitor and maintain the activity of *P. parasitica* var. *nicotianae* in the tests. Black shank was assessed biweekly with individual plants rated as resistant if there was no sign of disease at the final disease reading, 98 days after transplanting as previously described (6). Transplant and cultural practices were those used in conventional tobacco production. Allelism was determined by χ^2 analyses.

Greenhouse tests to confirm field results. In the field, black shank symptoms are caused primarily by race 0 of *P. parasitica* var. *nicotianae*, but occasionally race

1 can also cause similar disease symptoms. Therefore, greenhouse root inoculations of 200 *Ph/Phl* testcross plants and four plants each of TG 593-13, L8, the F₁ hybrid (TG 593-13 × L8), and KY 14 were performed to confirm that plants lost in the field to black shank were infected with *P. parasitica* var. *nicotianae* race 0. Plants were transplanted into MetroMix 200 sterile medium in square 8.5-cm plastic pots and grown under an 18-h photoperiod for 3 weeks at 26°C in a growth room. The plants were moved to a greenhouse 1 week prior to inoculation.

Inoculum preparation and disease evaluation. Pure cultures of *P. parasitica* var. *nicotianae* race 0 were grown on 5% oatmeal agar in 100 × 20 mm petri plates for 2 weeks in the dark at room temperature. The mycelia and agar medium of 15 plates were added to 1 liter of deionized water, homogenized in a Waring Blender for 30 s, and kept on ice until needed. Prior to inoculation, plants were fertilized (20-20-20) and irrigated to saturation. Two holes, 1 cm in diameter and 4 to 5 cm deep, were made on each side of the plant in a pot, and 10 ml of the *P. parasitica* var. *nicotianae* race 0 inoculum was poured into each hole. Holes were filled with surrounding soil to prevent dehydration of the inoculum. Disease development was monitored daily for 2 weeks, beginning 3 days following inoculation. Plants were rated resistant if they showed no symptoms and susceptible if death occurred from black shank.

Cladistic analyses for *Ph* from Coker 371-G. DNA was extracted and standard-

ized by fluorometry to 5 ng μl^{-1} (14) from immature leaves of *N. plumbaginifolia* accessions TW 106 and TW 108, *N. longiflora* (*brevifolia*) accessions TW 79 and TW 80, *N. longiflora* (*grandifolia*) accessions TW 78 and TW 81, tobacco lines DH92-2770-40, NC 1071, and cultivars L8, Coker 371-Gold, K 326, Coker 139, and Hicks. Random amplified polymorphic DNA (RAPD) analyses were performed using 48 decamer primers from Operon Technologies (Alameda, CA) and the University of British Columbia (Vancouver, Canada). These primers generated 43 RAPD markers in coupling- and 5 in repulsion-phase linkage to *Ph* (Table 1).

RAPD amplifications were performed using each of the 48 primers in 15- μl RAPD reactions (28). Each reaction contained 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 pmol of a decamer primer. For a primer control, template DNA was replaced with sterile water (Sigma). Each reaction was overlaid with 50 μl of sterile mineral oil (Sigma).

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

Table 1. Operon (OP) and University of British Columbia (UBC) decamer primers generating random amplified polymorphic DNA (RAPD) markers linked to the *Ph* gene from tobacco cultivar Coker 371-G^a

RAPD	Amplification program ^b	RAPD	Amplification program	RAPD	Amplification program
OPB-13 ₁₀₄₀ ^c	2	OPAJ-12 ₅₃₅	2	UBC-265 ₁₀₁₀	2
OPZ-7 ₇₇₀ ^c	1	OPAJ-15 ₅₃₅	2	UBC-296 ₃₀₀	1
UPC-365 ₅₆₅ ^c	2	OPAK-18 ₆₄₅	2	UBC-297 ₄₀₀	1
UBC-484 ₆₀₀ ^c	2	OPAL-14 ₃₀₀	1	UBC-333 ₂₇₀	1
UBC-671 ₁₂₃₀ ^c	2	OPAN-2 ₇₆₀	2	UBC-387 ₁₀₀₀	1
OPE-10 ₄₃₀	1	OPAN-9 ₆₀₀	2	UBC-412 ₆₀₀	2
OPH-18 ₄₃₀	2	UBC-30 ₄₉₀	2	UBC-422 ₄₂₀	2
OPM-16 ₂₅₀	1	UBC159 ₅₉₀	2	UBC-427 ₅₇₅	2
OPN-8 ₄₄₀	3	UBC-162 ₄₀₀	2	UBC-443 ₃₆₀	2
OPR-1 ₁₁₆₀	1	UBC-171 ₄₀₀	1	UBC-443 ₅₆₀	2
OPR-7 ₆₀₀	2	UBC-183 ₉₀₀	1	UBC447 ₅₆₄	2
OPU-9 ₈₀₀	2	UBC-184 ₈₀₀	1	UBC-469 ₃₈₅	2
OPW-3 ₄₁₀	2	UBC-227 ₉₅₀	1	UBC-564 ₃₄₅	2
OPZ-5 ₇₇₀	1	UBC-247 ₅₇₀	2	UBC-571 ₈₀₀	2
OPAD-3 ₇₁₀	2	UBC-248 ₄₃₀	1	UBC-613 ₆₄₀	2
OPAE-5 ₂₁₀₀	1	UBC-259 ₆₀₀	1	UBC-664 ₈₃₁	2

^a Standard errors of ± 12 bp for RAPD fragment size were estimated using the "shareware" package Seqaid (22).

^b Amplification programs used to generate the RAPD markers: 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. 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. Program 3: 1 cycle 94°C/1 min; 15 cycles 92°C/30 s, 50°C/30 s minus 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.

^c RAPD markers in repulsion-phase linkage with the *Ph* gene. All other RAPD markers are in coupling-phase linkage with *Ph*.

gels (Sigma) containing 0.15 μg of ethidium bromide per liter. Submerged gel electrophoresis was performed in 1 $\times$ TBE buffer (0.09 M Tris borate, 0.002 M EDTA) (23) containing 0.046 μg of ethidium bromide per liter. One lane on each gel contained a 100-bp DNA Ladder (Gibco BRL, Life Technologies, Grand Island, NY).

RAPD marker distribution was used to study the relationships among the three *Nicotiana* species. Parsimony analyses were performed using the program PAUP (10) to produce a region or gene tree for *Ph.*

Determination of genetic recombination in *Ph/Phl* hybrids by RAPD analyses. Six RAPD markers in coupling-phase linkage to *Ph*, OPN-8₄₄₀, UBC-30₄₉₀, UBC-159₅₉₀, UBC-248₄₃₀, UBC-427₅₇₅, and UBC-469₃₈₅, were present only in C 371-G, DH92-2770-40, NC 1071, and the two *N. plumbaginifolia* accessions. Subsequent to root inoculations, DNA was extracted from disease-free plants of 16 TG 593-13 × L8 (*Ph/Phl*) testcross progenies, TG 593-13, the TG 593-13 × L8 hybrid, and L8, and standardized to 5 ng µl⁻¹ by fluorometry. RAPD amplifications using

each of the six decamer primers separately and product separation were conducted as previously described.

RESULTS

Field and greenhouse black shank evaluations of testcross progenies. Seven plants were lost to black shank from 440 *Phl/Phl* testcross progenies evaluated in the field disease nursery. Inoculum level in the black shank nursery was high, as the susceptible check and tester KY 14 only survived an average of 48.5 days (100% disease incidence [DI]) after transplanting. All plants of the parental lines TG 593-13 and L8 survived the full season (98 days, 0% DI). One plant of the hybrid TG 593-13 × L8 was lost to disease.

One plant was lost to black shank from the 444 *Ph/Php* testcross. The susceptible check and tester DH94-1577-1 survived an average of 84 days in the black shank nursery. No plants were lost from the parental lines NC 1071 and DH92-2770-40 or their F₁ hybrid. Map distance between *Ph* and *Phl* was estimated by linkage and χ^2 analyses at 3.18 cM ($P < 0.005$). Thus, *Ph* and *Php* were determined to be allelic (Table 2).

Root inoculations performed in the greenhouse with race 0 of *P. parasitica* var. *nicotianae* corroborated field observations for the *Ph/Phl* testcross progeny of TG 593-13 $\times$ L8. Typical black shank symptoms were observed on KY 14, the susceptible check, 3 days postinoculation. All inoculated KY 14 plants were dead from black shank within 5 days after root inoculation. Three of 200 testcross progenies were lost to black shank 5 to 10 days after inoculation (Table 2). No plants were lost from TG 593-13, TG 593-13 $\times$ L8, and L8 from root black shank infections.

Genetic recombination in *Ph/Phl* hybrids visualized by RAPD analyses. RAPD analyses on the 16 testcross plants using the six RAPD markers, OPN-8₄₄₀, UBC-30₄₉₀, UBC-159₅₉₀, UBC-248₄₃₀, UBC-427₅₇₅, and UBC-469₃₈₅, in coupling-phase linkage to *Ph* further support the hypothesis that the three plants lost to black shank could have resulted from genetic recombination events. Crossing over occurred within and around the region containing the six RAPDs, resulting in partial to complete loss of the markers while resistance was maintained (Table 3). The three black shank-susceptible testcross plants were not available for RAPD analyses; however, at least eight recombination events occurred between the *Ph* and *Phl* segments present in the 16 testcross progenies analyzed (Table 3). Thus *Ph* and *Phl* are not allelic, and recombination can occur between the loci to generate genotypes susceptible to black shank.

Cladistic analyses. The 48 RAPD markers, 43 in coupling- and five in repulsion-phase linkage to the *Ph* locus, proved to be cladistically informative. Polymorphisms generated by the RAPD markers (Fig. 1) could be separated into 11 unique banding types in the three *Nicotiana* species and were used in cluster analyses to

Table 2. Linkage relationships between *Ph* from Coker 371-Gold, *Phl* from *Nicotiana longiflora*, and *Php* from *Nicotiana plumbaginifolia*^a

Black shank R gene	Observed frequencies	χ^2	<i>P</i>	Estimated linkage distance (cM)
	R : S ^b			
<i>Ph</i> and <i>Phl</i> ^c	433 : 7	128.58	0.00	3.18
<i>Ph</i> and <i>Php</i>	443 : 1	145.35	0.00	0.45
<i>Ph</i> and <i>Phl</i> ^d	197 : 3	58.91	0.00	3.00

^a χ^2 values for goodness-of-fit to expected ratios were calculated for field data from the Upper Coastal Plain Research Station, Rocky Mount, NC, in 1997 and for greenhouse root inoculation trials in 1998.

b A 3 resistant:1 susceptible ratio is expected if the two loci are segregating independently. Expected genotypic frequencies are $\frac{1}{4}$ *PhPhl/phphl*: $\frac{1}{4}$ *Phphl/phphl*: $\frac{1}{4}$ *phPhl/phphl*: $\frac{1}{4}$ *phphl/phphl*.

^c Testcross progenies from TG 593-13 × L8 evaluated in the field.

^d Testcross progeny of TG 593-13 evaluated in the greenhouse.

Table 3. Recombination of six random amplified polymorphic DNA (RAPD) markers in black shank root resistant *Ph/Phl* testcross progenies^a

Genotype	Disease reaction	OPN-8 ₄₄₀ ^b	UBC-159 ₅₉₀	UBC-427 ₅₇₅	UBC-469 ₃₈₅	UBC-248 ₄₃₀	UBC-30 ₄₉₀
L8	R	0 ^c	0	0	0	0	0
TG 593-13	R	1	1	1	1	1	1
TG 593-13 × L8	R	1	1	1	1	1	1
[TG 593-13 × L8] × KY 14	R	1	1	1	1	1	1
[TG 593-13 × L8] × KY 14	R	1	1	1	1	1	1
[TG 593-13 × L8] × KY 14	R	1	1	1	1	1	1
[TG 593-13 × L8] × KY 14	R	1	1	1	1	1	1
[TG 593-13 × L8] × KY 14	R	1	1	1	1	1	1
[TG 593-13 × L81 × KY 14	R	1	1	1	1	1	1
[TG 593-13 × L8] × KY 14	R	1	1	1	1	1	1
[TG 593-13 × L8] × KY 14	R	0	0	0	0	0	0
[TG 593-13 × L81 × KY 14	R	0	0	0	0	0	0
[TG 593-13 × L8] × KY 14	R	0	0	0	0	0	0
[TG 593-13 × L81 × KY 14	R	0	0	0	0	0	0
[TG 593-13 × L8] × KY 14	R	0	0	0	0	0	0
[TG 593-13 × L8] × KY 14	R	0	0	0	0	0	0
[TG 593-13 × L8] × KY 14	R	1	1	1	0	0	1
[TG 593-13 × L8] × KY 14	R	1	1	1	1	1	1
[TG 593-13 × L8] × KY 14	R	1	1	1	1	0	1

^a Crossing over between the *Ph* and *Phl* segments is apparent as some progenies have lost some or all of the RAPD markers.

^b RAPD fragment, size 440 bp, generated by Operon primer N-8. Primers from the University of British Columbia are abbreviated UBC.

^c Indicates presence (1) or absence (0) of RAPD fragment.

generate a region or gene tree for *Ph* (Fig. 2). This is one of the best three that fit the RAPD data, trees varying only in the ranking of genotypes within a branch. The data from RAPD and cladistic analyses showed that the region containing *Ph* from C 371-Gold is more closely related to *N. plumbaginifolia* and NC 1071 than L8, any of the *N. longiflora* accessions or the ancestral *N. tabacum* lines tested (Fig. 2).

DISCUSSION

The homology of the dominant black shank resistance genes *Php* and *Phl* from *N. plumbaginifolia* and *N. longiflora*, respectively, has long been debated (1,8,15). Here we present field, greenhouse, and RAPD data which show that *Ph* from C 371-G is allelic to *Php* from *N. plumbaginifolia* in NC 1071. Conversely, *Php* and *Ph* are not allelic, but homologous in function to *Phl* in breeding line L8 developed from the closely related species *N. longiflora*.

Testcross populations from *Ph/Phl* hybrids were designed so that confounding effects of Fla 301 black shank resistance would be negligible. Black shank resistance in the burley breeding line TG 593-13 is mediated by the *Ph* gene from C 371-G. Testcross, rather than selfed populations from the hybrid TG 593-13 × L8, were used to maximize the probability of finding susceptible, double recessive genotypes if the *Ph* and *Phl* genes were not allelic. Susceptible recombinants were observed at low frequencies in both greenhouse and field disease trials.

F₁ hybrids of DH92-2770-40 × NC 1071 did not produce gametes lacking a dominant allele for black shank resistance. We conclude, therefore, that the *Ph* gene in DH92-2770-40, from C 371-G, is allelic to *Php* from NC 1071. Field trials with the *Ph/Phl* testcross progenies showed that susceptible genotypes were produced at a frequency of approximately 1.5%. This frequency would represent one-half of the recombination events between the two loci. Hence, *Ph* and *Phl* appear to be linked approximately 3 cM apart.

These data also explain the results of a study of the relationship between the black shank resistance genes in *N. plumbaginifolia* and *N. longiflora* conducted in 1971 (8). These authors observed one susceptible recombinant in an F₂ population of 4,810 individuals from a cross between two segmental substitution lines containing black shank resistance from *N. plumbaginifolia* and *N. longiflora*, respectively. If the *Ph* and *Phl* loci are 3 cM apart, as estimated in our study, then the probability of obtaining a double recessive recombinant in an F₂ population would be 2.25×10^{-4} , or 1 in 5,000. As this is such a rare occurrence, the authors dismissed their findings and declared the two black shank resistance loci homologous (8).

Black shank susceptible recombinant gametes could arise in the *Ph/Phl* testcross

progenies by either recombination or loss of the chromosomal segments carrying the resistance genes. Proof that the double recessive gamete arose from recombination events could be ascertained if other recombination events were observed between the *Ph* and *Phl* segments in the tobacco genome. In tobacco, recombination is greatly suppressed between hybrids containing an interspecific chromosomal segment and its heterolog in the tobacco genome (3,11). Six RAPD markers, which distinguish the *Ph* and *Phl* introgressed regions in the three *Nicotiana* species, recombined with at least 8 crossover events occurring in 16 plants tested from *Ph/Phl* testcross progenies. Since the RAPD markers are recombining in resistant testcross progenies, we conclude that double recessive genotypes most likely arose by recombination events.

Data from field and greenhouse disease trials showed that *Ph* and *Phl* are different genes with homologous function. Thus, cladistic analyses could be used to further study the relationship between the introgressed regions containing these genes. A cladogram or gene tree for *Ph* generated from 48 RAPD markers linked to the region containing the gene supported field and greenhouse data. The cladogram generated by parsimony analyses, an algorithm using the least number of character

changes to explain the data (10,19), depicted shared similarities between the three *Nicotiana* species for the region containing the *Ph* gene from C 371-G. Genotypes in close proximity are more closely related to each other, with respect to *Ph*, than those further apart on the tree. By definition, cladograms do not reflect ancestral relationships (10). This analysis confirmed that the region containing *Ph* in C 371-G is more closely related to that of *Phl* in NC 1071, from *N. plumbaginifolia*, and different from the *Phl* region derived from *N. longiflora*.

Since *Ph* and *Phl* integrated in close proximity in the *N. tabacum* genome, they exhibit clustering characteristic of other resistance loci. Extensive research into the structure, function, and origin of cloned plant disease resistance genes is currently under way. Dominant resistance genes, such as *Ph* or *Phl*, effective against hemibiotrophs such as *P. parasitica* var. *nicotianae* and involved in signal transduction and activation of the HR, have been found to occur in clusters of either allelic or closely related genes (4,21). Four arrangements of resistance loci, two of which may be applicable to *Ph* and *Phl*, have been described (13). They may be members of a complex resistance locus comprised of tandem arrays of closely linked resistance gene homologues with differing

Fig. 1. Banding patterns of the cladistically informative random amplified polymorphic DNA (RAPD) markers UBC-159₆₀₀ linked in coupling-phase, and UBC-484₅₉₀ linked in repulsion-phase to *Ph*. Banding patterns of the RAPD markers in the three *Nicotiana* species were analyzed to determine the origin of *Ph* from Coker 371-Gold. Amplification products (15 µl) were separated on 1.5% agarose gels. RAPD marker size has a standard deviation of ±9 bp.

Fig. 2. One of three most parsimonious cladograms based on random amplified polymorphic DNA (RAPD) polymorphisms between the predecessors of Coker 371-Gold and the three *Nicotiana* species for the region containing the *Ph* gene.

specificities. Alternatively, *Ph* and *Phl* may be members of a major resistance complex such as the region on chromosome 6 in tomato which contains resistance genes *Cf-2/-5* to *Cladosporium fulvum*, *Mi* to *Meloidogyne* spp., and *Meu* to *Macrosiphum euphorbiae* (17). Linkage between bacterial wilt and black shank resistance has been reported (25). Cloning of *Ph* and *Phl* genes will provide the information needed to classify these resistance loci.

ACKNOWLEDGMENTS

We gratefully acknowledge Kurt Wollenberg, Department of Genetics, NCSU, for performing parsimony analyses. We also thank John Tysinger, Todd Campbell, Lori Linger, Ramsey Lewis, and Susana Milla for assisting with fieldwork and data collection. The authors wish to thank Verne Sisson, Oxford Tobacco Research Station, Oxford, NC, for kindly providing seed of *N. plumbaginifolia* and *N. longiflora* accessions. This work was funded by Philip Morris USA and the North Carolina Tobacco Research Commission.

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