RESEARCH

# An Introgressed *Nicotiana rustica* Genomic Region Confers Resistance to *Phytophthora nicotianae* in Cultivated Tobacco

Katherine Drake and Ramsey S. Lewis*


## ABSTRACT

Black shank, caused by the pathogen *Phytophthora nicotianae*, is an important disease affecting tobacco (*Nicotiana tabacum* L.) production in many parts of the world. Host resistance offers an efficient means of reducing economic loss due to this pathogen. Previous observations suggested that a genomic region introgressed from *Nicotiana rustica* L. may affect resistance to black shank. The effect of this genomic region, designated as *Wz*, on resistance to multiple races of *P. nicotianae* had not previously been investigated in a systematic way, however. We used growth chamber and field experiments to evaluate resistance of a doubled haploid (DH) mapping population segregating for the *Wz* region. We also genotyped the population with amplified fragment length polymorphism (AFLP) markers found to be polymorphic between the parental lines. In addition, we determined resistance and marker genotypes for individuals of a larger $BC_1F_1$ population segregating for *Wz*. A set of 29 AFLP markers determined to be of *N. rustica* origin were found to cosegregate with each other and were associated with resistance to both race 0 and race 1 of *P. nicotianae* in the DH population. A selected subset of these markers was also found to cosegregate with resistance in the $BC_1F_1$ population. Consistent with other introgressed alien genomic regions in *N. tabacum*, no recombination was observed between these selected markers. The *N. rustica*-derived genetic variation and associated DNA markers will be of value for breeding for black shank resistance in tobacco.


Dep. of Crop Science, North Carolina State Univ., Raleigh, NC 27695. Received 24 Oct. 2012. *Corresponding author (ramsey_lewis@ncsu.edu).

**Abbreviations:** AFLP, amplified fragment length polymorphism; AUDPC, area under the disease progress curve; DAI, days after inoculation; DH, doubled haploid; EPS, end percent survival; *H*, entry mean heritability; $r_{Pearson}$, Pearson correlation coefficient; SCAR, sequence characterized amplified region.

Black shank, caused by the soil-borne pathogen *P. nicotianae* Breda de Haan, is one of the most destructive diseases of tobacco in many tobacco producing areas (Csinos et al., 1984; Shew and Lucas, 1991). *Phytophthora nicotianae* is a hemibiotrophic oomycete that can infect tobacco roots, stems, and leaves at any stage of plant development resulting in symptoms that include wilting, chlorosis, root and stem necrosis, stunting, and plant death (Csinos and Minton, 1983). Genetic resistance is an attractive means of combating black shank in tobacco and plays a major role in overall strategies to reduce the economic impact of this disease.

Both monogenic and multigenic types of resistance to *P. nicotianae* have been used to develop black shank-resistant tobacco cultivars. The first black shank-resistant cultivar, Florida 301, was developed by Tisdale (1931). This cultivar possesses resistance of the classic polygenic type, where resistance is partial, non-race specific, and controlled by multiple genes (Smith and Clayton, 1948; Crews et al., 1964; Chaplin, 1966; Xiao et al., 2012). Although this type of resistance has been used in numerous tobacco cultivars, it has been associated with lower yielding ability and reduced cured leaf quality (Chaplin and Ford, 1958). Monogenic resistance, controlled by single genes designated as *Php* and *Phl*, has been introgressed into *N. tabacum* from *Nicotiana plumbaginifolia* Viv. and *Nicotiana longiflora*

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Cav., respectively (Valleau et al., 1960; Apple, 1962a; Chaplin, 1962). Both genes confer immunity to race 0 of the black shank pathogen but confer no resistance to race 1 (Sullivan et al., 2005a). Wide-scale planting of varieties carrying these genes has led to race shifts and an increased prevalence of races other than race 0 (Litton et al., 1966; Sullivan et al., 2005b). Besides race 0, other described races of *P. nicotianae* include race 1 (Apple, 1962b, 1967), race 2 (van Jaarsveld et al., 2002), and race 3 (McIntyre and Taylor, 1978; Gallup and Shew, 2010). Race 0 and race 1 are currently of greatest economic importance, however.

Because complete resistance to all races is not known to exist within current tobacco cultivars, there is a desire to identify additional genetic factors that could extend the range and level of resistance to *P. nicotianae* in cultivated tobacco. Burk and Heggestad (1966) reported that *N. rustica* exhibited high levels of black shank resistance that might be of value if the responsible genes could be transferred to *N. tabacum*. A recent evaluation of 86 *N. rustica* accessions revealed that almost all of the accessions had very high resistance to *P. nicotianae* race 0 and that 20 accessions exhibited race 1 resistance that was superior to that of the highly resistant flue-cured tobacco variety 'K346' (Nifong et al., 2011).

Dominant resistance to *Pseudomonas syringae* pv. *tabaci* (the causal agent of the wildfire and angular leaf spot diseases) was transferred from *N. rustica* L. var. *brasilia* Schrank to *N. tabacum* by conventional interspecific hybridization and backcrossing (Woodend and Mudzengerere, 1992). A stable breeding line designated as "WZ" was developed from this effort. Several flue-cured tobacco hybrids with WZ in their pedigree have exhibited high levels of resistance to multiple races of *P. nicotianae* in field and laboratory experiments (Antonopoulos et al., 2010). These observed levels of resistance are somewhat unexpected based on the parentage of these cultivars. It may be possible that the introgressed *N. rustica* region (hereby designated as *Wz*) carries a gene or genes that also affect resistance to *P. nicotianae*. If so, this novel genetic variability could be of importance to future efforts to develop cultivars with increased levels of resistance to black shank. In addition, DNA markers associated with the introgressed alien genomic region could increase the efficiency of developing future tobacco cultivars carrying this resistance. The objectives of this study were to (i) generate doubled haploid and $BC_1F_1$ populations segregating for the introgressed *Wz* region, (ii) evaluate these populations for resistance to race 0 and race 1 of *P. nicotianae*, and (iii) determine if amplified fragment length polymorphism (AFLP) markers identified to reside within the *Wz* region were associated with black shank resistance.

# MATERIALS AND METHODS

## Genetic Material

As a preliminary experiment to determine if the introgressed *Wz* region was associated with black shank resistance, a doubled haploid (DH) population of limited size was developed from a cross between the breeding line WZ and the fertile version of flue-cured tobacco cultivar NC55, which has a low level of partial resistance. Resulting $F_1$ hybrid plants were crossed as females with *Nicotiana africana* Merxm. to produce haploid plants using the method of Burk et al. (1979). Haploid plants were chromosome doubled according to Kasperbauer and Collins (1972). A total of 71 DH plants were generated and self-pollinated to produce 71 DH lines.

A larger $BC_1F_1$ population that was expected to segregate 1:1 for the introgressed *N. rustica* region was also generated by hybridizing WZ with the highly black shank-susceptible flue-cured tobacco cultivar Hicks. A single $F_1$ individual was then backcrossed to Hicks to generate the $BC_1F_1$ population.

## Field Evaluations

As an initial investigation, the DH population, the parental lines (NC55 and WZ), and the check breeding line 'NC1071' were evaluated for field black shank resistance. NC1071 has immunity to race 0 of *P. nicotianae* due to its *Php/Php* genotype but is highly susceptible to race 1 isolates. This line is often used as a race indicator in black shank field experiments. Field experiments were performed in disease nurseries naturally infested with *P. nicotianae* at three North Carolina locations (Cunningham Research Station, Kinston, NC, Upper Coastal Plain Research Station, Rocky Mount, NC, and Oxford Tobacco Research Station, Oxford, NC) during 2010 and 2011. There is a known prevalence of *P. nicotianae* race 1 in all of these disease nurseries. The experimental design was a randomized complete block with three replications in each environment. Experimental units consisted of single 12-plant rows at both the Cunningham Research Station and the Upper Coastal Plain Research Station and 22-plant rows at the Oxford Tobacco Research Station. Plots were transplanted and managed in accordance with common cultural practices for flue-cured tobacco production in North Carolina (North Carolina Cooperative Extension, 2011).

Approximately 35 d after transplanting, the number of plants killed by black shank in each plot was recorded. Disease counts were repeated approximately every 21 d in 2010 and every 28 d in 2011. At the end of the season (approximately 110 d after transplanting), end percent survival (EPS) and the area under the disease progress curve (AUDPC) were calculated for each plot. Area under the disease progress curve was calculated using the trapezoidal method described by Das et al. (1992).

## Growth Chamber Evaluations

Artificial inoculations were used in growth chamber experiments to determine the level of race specific black shank resistance of lines of the DH population and to more closely investigate associations between black shank resistance and specific AFLP markers (see below) using the $BC_1F_1$ population. Cultures of *P. nicotianae* were initiated on carrot agar 1 mo before transplanting plants. Carrot agar medium was prepared by mixing 50 mL of organic carrot juice (Bolthouse Juice Products LLC) and 20 g of agar (Sigma Chemical Co.) in 950 mL distilled water. Autoclaved medium was poured into 100 by 20 mm petri dishes. An agar plug containing either a race 0 or race 1 isolate of *P. nicotianae* (provided by Dr. David Shew, North Carolina State University Plant Pathology Department) was placed on the medium and allowed to grow

until hyphae were spread across most of the plate (after about 7 d at room temperature without light). Sterilized oat (*Avena sativa* L.) grains were then spread over the plate in a single layer. Cultures were considered ready to use when hyphae could be seen covering the majority of the oat grains (after approximately 15 d at room temperature without light).

The DH population, the parental lines (NC55 and WZ), and the control line NC1071 were evaluated for race-specific resistance in a growth chamber at the North Carolina State University Phytotron. The experimental design was a split-plot design with three replications performed over time. The mainplot factor was pathogen race (race 0 or race 1) and the subplot factor was line or cultivar (71 DH lines plus NC55, WZ, and NC1071). Lines or cultivars were randomized within mainplots. Subplots consisted of six plants contained in a six-compartment segment of a "3601" Com-Pack plastic tray insert (T.O. Plastics Inc.). Seeds were germinated on Metro Mix 200 growing medium (Sun Gro Horticulture) under plastic domes at 25°C with a 16:8 h light:dark photoperiod. Approximately 2 wk after emergence, seedlings were transplanted to individual cells of the plastic Com-Pack inserts containing a 2:1 peat:sand mixture. Two weeks after transplanting, each plant was inoculated by placing three *P. nicotianae*-infested oat grains into the soil in the corners of each cell of the plastic insert. The growth chamber was maintained at a temperature of 30°C for 16 h of daylight and decreased to 25°C at night. Uniform soil moisture was maintained via subirrigation. Disease incidence was recorded 7 d postinoculation and every 7 d thereafter for a total duration of 35 d. End percent survival and AUDPC were calculated for each subplot.

A larger population of $BC_1F_1$ individuals was also evaluated for black shank resistance using a race 0 isolate in a growth chamber experiment to better study the potential for recombination between markers found to be tentatively associated with resistance using the DH population. One-hundred forty $BC_1F_1$ plants, along with nine plants of each of the parental lines, were transplanted into individual cells (8 by 8 by 5 cm) of 18-cell plastic trays (Landmark Plastic Corporation). The parental lines were randomly interspersed among the 140 $BC_1F_1$ plants. Seedlings were germinated as described above. Two weeks after transplanting, each plant was inoculated by inserting five oat grains into the soil of each pot at a depth of approximately 1 cm. The growth chamber was maintained at a temperature of 30°C for 16 h of daylight and decreased to 25°C at night. Soil moisture was maintained through subirrigation. Plants were monitored every day for 31 d postinoculation, and the number of days of survival was recorded for each individual.

## Identification of Amplified Fragment Length Polymorphism Markers Associated with *Wz*-Mediated Resistance

Work was performed to first identify markers tentatively associated with the introgressed *N. rustica* region and to then determine if the identified markers were significantly associated with black shank resistance. Leaf tissue samples were collected from each of the doubled haploid lines, each of the 140 $BC_1F_1$ individuals, the parental lines, and *N. rustica* accession TW 117 approximately 3 wk after seeding. Deoxyribonucleic acid was isolated using a modified cetyltrimethylammonium bromide procedure (Afandor et al., 1993; Johnson et al., 1995), with the exception that a BIO 101 FastPrep machine (BIO 101) was used to grind leaf samples. Deoxyribonucleic acid was quantified using a Hoefer fluorometer (Hoefer Scientific Instruments). After quantification, DNA was diluted to 25 ng $\mu L^{-1}$ and stored at −20°C.

The two parents of the DH population (WZ and NC55) and *N. rustica* were initially genotyped using a total of 224 AFLP primer combinations. The objective was to identify AFLP markers polymorphic between WZ and NC55 that were likely derived from *N. rustica*. Amplified fragment length polymorphism reactions were performed according to the protocol of Milla et al. (2005), which is based on the original protocol described by Vos et al. (1995). Labeled *Eco*RI plus three primers were purchased from LI-COR Inc. All other AFLP primers and adapters were purchased from Sigma Genosys. After amplification, AFLP fragments were denatured at 94°C and separated by polyacrylamide gel electrophoresis on a LI-COR 4300 DNA sequencer using 8% v/v denaturing polyacrylamide gels. Amplified fragment length polymorphism bands were scored as binary data (band presence indicated by 1 and absence indicated by 0) using the software package AFLP-Quantar 1.0 (Geerlings et al., 1999). To determine the size of specific fragments, IRDye 700- or 800-labeled molecular weight standards (50–700 bp) also were loaded on each gel.

Candidate coupling phase markers (those that were amplified for WZ and *N. rustica* but not for NC55) were then used to genotype the entire population of 71 DH lines. Candidate repulsion phase markers (those that were amplified for NC55 but not WZ or *N. rustica*) were also identified and used to genotype members of the DH population. A selected subset of seven AFLP markers (selected based on band intensity) found to be associated with resistance in the DH population were subsequently used to genotype the 140 $BC_1F_1$ individuals that were evaluated for black shank resistance in the growth chamber experiment.

## Statistical Analysis

Analyses of variance for EPS and AUDPC were performed on the replicated field and growth chamber evaluations of the DH population using PROC GLM of SAS 9.1 (SAS Institute, 2004) and means were calculated for each line or cultivar for each disease measure. Entry mean heritabilities were calculated according to Holland et al. (2003). PROC CORR was used to calculate Pearson correlation coefficients between EPS and AUDPC. Correlations were also calculated between resistance data from field and growth chamber environments. PROC GLM was also used to perform simple *F*-tests to determine if specific AFLP markers were significantly associated with black shank resistance in the field experiments and also with resistance to both race 0 and race 1 of *P. nicotianae* in the growth chamber experiment. For the $BC_1F_1$ population, simple *t* tests were performed using PROC TTEST of SAS to determine the significance in the number of days of survival after inoculation between the two marker groups (with and without marker present) for each of the seven markers in the selected subset.

## Investigating Potential Linkage between *Wz* and *Php*

To determine if the black shank resistance gene *Php* (introgressed from *N. plumbaginifolia*) and *Wz* might have been

introgressed on the same *N. tabacum* chromosome, WZ was first hybridized with NCTG-61, a North Carolina State University breeding line homozygous for *Php*. The $F_1$ was then crossed with black shank-susceptible cultivar Hicks, which possesses neither *Wz* or *Php*. Thirty-six progeny from the (WZ × NC 61) × Hicks cross were genotyped with the selected subset of seven AFLP markers identified to be associated with the introgressed *Wz* region and also with an unpublished *Php* sequence characterized amplified region (SCAR) marker (forward primer: 5′-TCCCATGCTGTCCCAATCCCAC-3′; reverse primer: 5′-TCCCATGCTGCGATGTTGGA-3′) derived from random amplified polymorphic DNA marker SOPZ-5 published by Johnson et al. (2002). A chi-square test for independence using a 2 × 2 contingency table was performed on the genotypic data to test for independent segregation between *Wz* and *Php*.

# RESULTS

## Replicated Field and Growth Chamber Evaluations

Analyses of variance indicated highly significant differences among entries in the replicated field evaluations ($P < 0.0001$). For EPS in the field, the DH population exhibited wide variation that ranged from 7.5 to 77.4% (Fig. 1a). Area under the disease progress curve ranged from 5252.6 to 9064.7 (higher numbers indicates greater survival rates) for the field evaluation (Fig. 1b). End percent survival and AUDPC were highly correlated with each other, with the Pearson correlation coefficient ($r_{Pearson}$) = 0.96 ($P < 0.0001$). The WZ parental line exhibited significantly greater black shank resistance than NC55 ($P < 0.05$) according to either disease measure. The means for EPS and AUDPC for WZ were 68.91% and 8788.4, respectively, while the corresponding means for NC55 were 37.1% and 7218.1. The EPS and AUDPC means for NC1071 (the indicator line for race 1 presence) were not significantly different than those for NC55. Entry mean heritabilities (*H*s) for AUDPC and EPS in the field study were $H = 0.83$ and $H = 0.90$, respectively.

The DH lines also exhibited wide and significant ($P < 0.0001$) variation after inoculation with either race 0 or race 1 in the growth chamber. End percent survival and AUDPC were highly correlated with each other, with $r_{Pearson} = 0.94$ ($P < 0.0001$) for the race 0 inoculations and $r_{Pearson} = 0.98$ ($P < 0.0001$) for race 1 inoculations. The correlations for EPS and AUDPC between race 0 and race 1 inoculations were $r_{Pearson} = 0.47$ ($P < 0.0001$) and $r_{Pearson} = 0.58$ ($P < 0.0001$), respectively. For race 0 inoculations, EPS ranged from 0 to 100% (Fig. 2a) and AUDPC ranged from 506 to 3500 (Fig. 2b). For race 1 inoculation, EPS also ranged from 0 to 100% (Fig. 3a) while AUDPC ranged from 1206 to 3500 (Fig. 3b). The WZ parent was significantly superior ($P < 0.01$) to the partially resistant parent, NC55, after inoculation with race 0. WZ was numerically superior to NC55 after the race 1 inoculation, but the difference was not significant. NC1071 exhibited 100% EPS after inoculation with race 0 and an EPS of 22.2% after inoculation with race 1. Significant

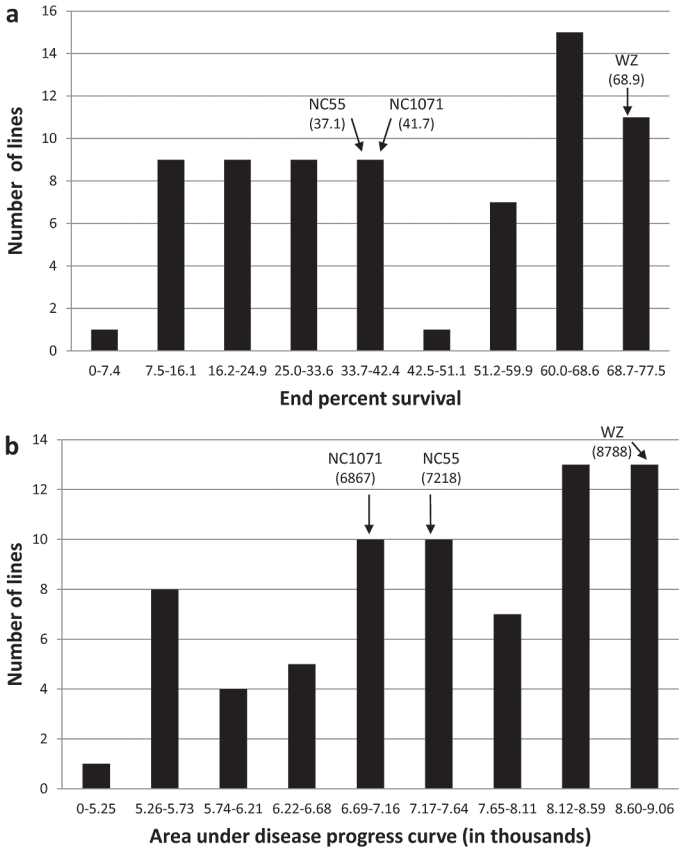


Figure 1. Frequency histogram for field black shank resistance of the WZ × NC55 doubled haploid population as measured by (a) end percent survival and (b) area under disease progress curve. Means are averaged over six North Carolina environments. For area under disease progress curve, higher numbers indicate greater levels of resistance. Means for parental lines (NC55 and WZ) and NC1071 (race 1 indicator line) are indicated in parenthesis and by arrows.

differences ($P < 0.0001$) between WZ and NC1071 were observed for the two disease measures after inoculation with race 1. These two lines were not significantly different after inoculation with race 0. The race 0 and race 1 isolates used in the experiment differed in their aggressiveness (degree of pathogenic fitness) as significant differences were observed between main plots (races) for both EPS and AUDPC. Entry mean heritabilities for AUDPC and EPS after race 0 inoculation were both $H = 0.94$. Entry mean heritabilities for AUDPC and EPS after race 1 inoculation were $H = 0.87$ and $H = 0.90$, respectively. The correlations between field resistance and resistance to race 0 inoculation in the growth chamber were $r_{Pearson} = 0.88$ ($P < 0.0001$) and $r_{Pearson} = 0.92$ ($P < 0.0001$) for AUDPC and EPS, respectively. The correlations between field resistance and resistance to race 1 inoculation were $r_{Pearson} = 0.76$ ($P < 0.0001$) and $r_{Pearson} = 0.72$ ($P < 0.0001$) for AUDPC and EPS, respectively.

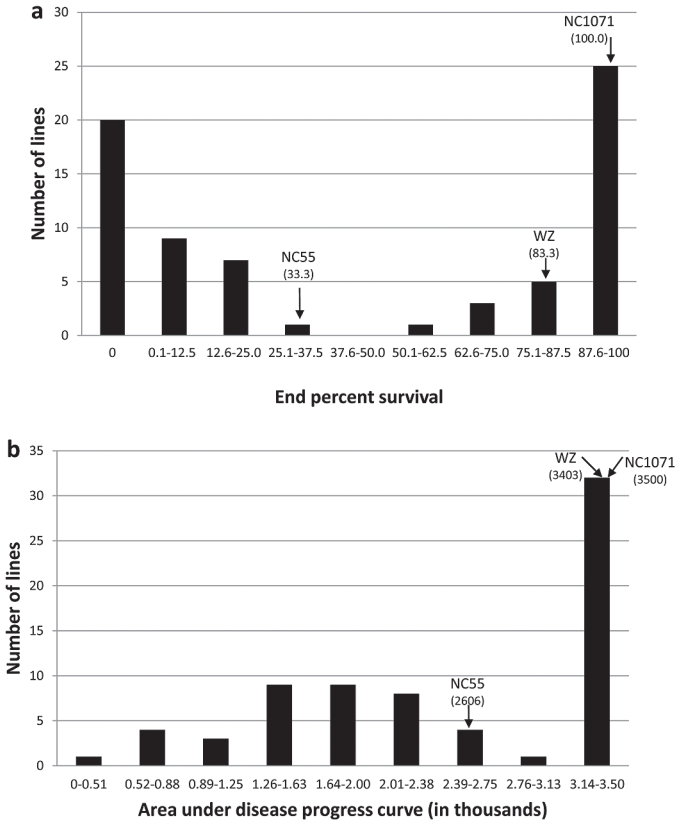


Figure 2. Frequency histogram for *Phytophthora nicotianae* race 0 resistance of WZ × NC55 doubled haploid population in growth chamber inoculations as measured by (a) end percent survival and (b) area under disease progress curve. For area under disease progress curve, higher numbers indicate greater levels of resistance. Means for parental lines (NC55 and WZ) and NC1071 (race 1 indicator line) are indicated in parenthesis and by arrows.

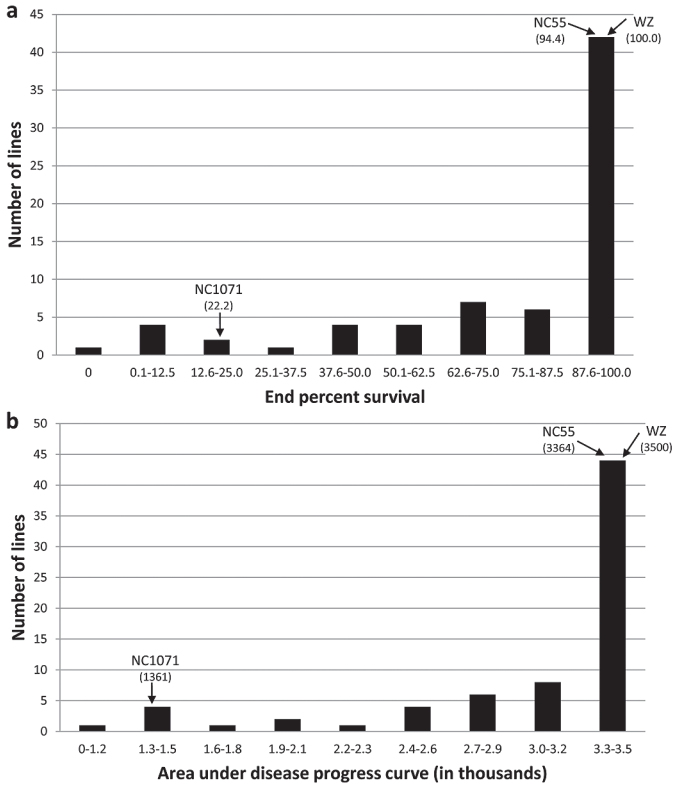


Figure 3. Frequency histogram for *Phytophthora nicotianae* race 1 resistance of WZ × NC55 doubled haploid population in growth chamber inoculations as measured by (a) end percent survival and (b) area under disease progress curve. For area under disease progress curve, higher numbers indicate greater levels of disease resistance. Means for parental lines (NC55 and WZ) and NC1071 (race 1 indicator line) are indicated in parenthesis and by arrows.

## Associations between Amplified Fragment Length Polymorphism Markers and Black Shank Resistance

A total of 224 AFLP primer combinations were screened for their ability to reveal polymorphisms between WZ and NC55. A band's presence or absence in *N. rustica* accession TW117 suggested its origin being from *N. rustica* or from *N. tabacum*, respectively. A total of 46 bands (Table 1) were initially identified that were polymorphic between WZ and NC55. Twenty-nine of these bands were putative coupling phase markers as they were present for WZ and *N. rustica* but absent for NC55. Seventeen bands were considered as putative repulsion phase markers as they were present for NC55 but absent for WZ and *N. rustica*. As an initial investigation into potential associations between these markers and black shank resistance, the complete set of 46 AFLP markers was used to genotype the population of 71 WZ × NC55 DH lines. Statistically significant ($P < 0.05$) segregation distortion was not observed for any of the markers in this population. Using both field resistance data and race-specific growth chamber inoculation data, *F*-tests revealed significant differences ($P < 0.0001$) between the means of the two marker groups (with the marker versus not having the marker) for each of the 46 markers tested for both EPS and AUDPC (Tables 1 and 2). For putative coupling phase markers derived from *N. rustica*, the genotypic group possessing the marker had significantly higher levels of black shank resistance than the group that did not. For putative repulsion phase markers derived from *N. tabacum*, the genotypic group possessing the marker exhibited significantly lower levels of resistance than the group lacking the marker. Notably, these associations were observed for growth chamber inoculations with either the race 0 or race 1 *P. nicotianae* isolates (Table 2).

A selected subset of seven coupling phase AFLP markers (Table 3) was used to genotype a larger (WZ × Hicks) × Hicks $BC_1F_1$ population of 140 plants that was also evaluated for days survival after inoculation with race 0 of *P. nicotianae*. The number of days survived postinoculation (days after inoculation [DAI]) for this population ranged from 5 d to still living at day 31 when the experiment was terminated. The distribution was bimodal with 86 plants dying within 9 d of inoculation and 43 plants surviving the entire observation period (Fig. 4). WZ was highly superior to Hicks as all nine WZ plants survived the

**Table 1. Amplified fragment length polymorphism (AFLP) markers associated with *Wz* and corresponding *F*-test values, $R^2$ values, and additive effects for field evaluation of doubled haploid population.**

| | EPS† | | | AUDPC‡ | | |
|---|---|---|---|---|---|---|
| AFLP marker§ | *F*-value | $R^2$ | Additive effect | *F*-value | $R^2$ | Additive effect |
| E-AGA/M-CCG-150¶ | 265.7# | 0.80 | 20.0 | 123.8# | 0.65 | 903.3 |
| E-AGG/M-CCG-675¶ | 265.7# | 0.80 | 20.0 | 123.8# | 0.65 | 903.3 |
| E-AGG/M-CCG-344¶ | 252.5# | 0.80 | 19.6 | 120.3# | 0.65 | 869.5 |
| E-AAA/M-CGC-96¶ | 263.1# | 0.80 | 20.1 | 128.4# | 0.66 | 916.5 |
| E-ATC/M-CCC-232¶ | 265.7# | 0.80 | 20.0 | 123.8# | 0.65 | 903.3 |
| E-ATG/M-CCG-359¶ | 250.1# | 0.80 | 20.2 | 111.7# | 0.64 | 908.8 |
| E-ATG/M-CCG-361¶ | 250.1# | 0.80 | 20.2 | 111.7# | 0.64 | 908.8 |
| E-ATT/M-CCA-482¶ | 180.1# | 0.74 | 19.2 | 94.6# | 0.60 | 872.1 |
| E-ATT/M-CCA-302¶ | 231.4# | 0.78 | 19.6 | 105.8# | 0.62 | 885.9 |
| E-ATC/M-CCC-323¶ | 221.9# | 0.78 | 19.6 | 102.4# | 0.62 | 890.0 |
| E-ATC/M-CCC-175¶ | 221.9# | 0.78 | 19.6 | 102.4# | 0.62 | 890.0 |
| E-ATG/M-CCG-256¶ | 264.1# | 0.80 | 20.4 | 120# | 0.65 | 913.1 |
| E-ATG/M-CCG-88¶ | 264.1# | 0.80 | 20.4 | 120# | 0.65 | 913.1 |
| E-AGC/M-CGG-299¶ | 211.3# | 0.76 | 19.7 | 111.8# | 0.63 | 897.2 |
| E-ATG/M-CGA-157¶ | 240.7# | 0.79 | 19.8 | 111.2# | 0.63 | 896.2 |
| E-AGC/M-CGA-113¶ | 173.7# | 0.72 | 19.3 | 87.3# | 0.57 | 857.9 |
| E-ATT/M-CGG-167¶ | 213.0# | 0.76 | 19.6 | 115.2# | 0.63 | 894.9 |
| E-AGC/M-CGT-657¶ | 265.7# | 0.80 | 20.0 | 123.8# | 0.65 | 903.3 |
| E-AGC/M-CGT-289¶ | 265.7# | 0.80 | 20.0 | 123.8# | 0.65 | 903.3 |
| E-AAT/C-CGA-332¶ | 265.7# | 0.80 | 20.0 | 123.8# | 0.65 | 903.3 |
| E-ATT/M-CCT-542¶ | 255.9# | 0.79 | 19.9 | 118.8# | 0.64 | 898.0 |
| E-ATT/M-CCT-413¶ | 255.9# | 0.79 | 19.9 | 118.8# | 0.64 | 898.0 |
| E-ATT/M-CCT-223¶ | 255.9# | 0.79 | 19.9 | 118.8# | 0.64 | 898.0 |
| E-ATT/M-CCT-206¶ | 255.9# | 0.79 | 19.9 | 118.8# | 0.64 | 898.0 |
| E-ATT/M-CGT-544¶ | 265.7# | 0.80 | 20.0 | 123.8# | 0.65 | 903.3 |
| E-ATT/M-CGT-413¶ | 265.7# | 0.80 | 20.0 | 123.8# | 0.65 | 903.3 |
| E-ATT/M-CGT-223¶ | 265.7# | 0.80 | 20.0 | 123.8# | 0.65 | 903.3 |
| E-ATT/M-CGT-206¶ | 265.7# | 0.80 | 20.0 | 123.8# | 0.65 | 903.3 |
| E-AGA/M-CTT-288¶ | 199.2# | 0.75 | 19.5 | 101.6# | 0.60 | 873.5 |
| E-ATT/M-CCA-324†† | 207.4# | 0.77 | –19.6 | 104.4# | 0.62 | –895.9 |
| E-AGC/M-CGG-102†† | 206.7# | 0.76 | –19.6 | 109.6# | 0.62 | –888.5 |
| E-ATG/M-CGA-577†† | 219.9# | 0.77 | –19.7 | 106.4# | 0.62 | –893.8 |
| E-ATG/M-CGA-412†† | 219.9# | 0.77 | –19.7 | 106.4# | 0.62 | –893.8 |
| E-ATT/M-CGG-107†† | 199.0# | 0.75 | –19.6 | 111.1# | 0.62 | –893.0 |
| E-AAT/C-CGA-162†† | 244.0# | 0.78 | –19.9 | 118.8# | 0.64 | –900.0 |
| E-AGA/M-CCG-169†† | 244.0# | 0.78 | –19.9 | 118.8# | 0.64 | –900.0 |
| E-ATT/M-CCT-587†† | 255.9# | 0.79 | –19.9 | 118.8# | 0.64 | –898.0 |
| E-ATT/M-CCT-407†† | 255.9# | 0.79 | –19.9 | 118.8# | 0.64 | –898.0 |
| E-AGG/M-CCG-295†† | 232.3# | 0.78 | –19.5 | 115.8# | 0.64 | –865.9 |
| E-AAA/M-CGC-679†† | 241.2# | 0.78 | –20.0 | 122.8# | 0.65 | –912.5 |
| E-AAA/M-CGC-487†† | 241.2# | 0.78 | –20.0 | 122.8# | 0.65 | –912.5 |
| E-AAA/M-CGC-208†† | 241.2# | 0.78 | –20.0 | 122.8# | 0.65 | –912.5 |
| E-ATT/M-CGT-590†† | 155.9# | 0.70 | –18.7 | 83.6# | 0.55 | –835.0 |
| E-ATT/M-CGT-408†† | 244.0# | 0.78 | –19.9 | 118.8# | 0.64 | –900.0 |
| E-AGA/M-CTT-247†† | 244.0# | 0.78 | –19.9 | 118.8# | 0.64 | –900.0 |
| E-ATC/M-CCC-223†† | 257.0# | 0.79 | –19.9 | 118.6# | 0.64 | –895.2 |

†EPS, end percent survival.

‡AUDPC, area under the disease progress curve.

§AFLP markers are designated by primer pair combination followed by estimated band size.

¶Coupling phase AFLP marker.

#Significant at the 0.0001 probability level.

††Repulsion phase AFLP marker.

entire observation period while all nine Hicks plants died between 6 and 7 d after inoculation. After genotyping this population with the seven selected AFLP markers, 55 were found to be positive for each of the seven markers while 85 were found to be negative for all of the markers. A chi-square test indicated significant segregation distortion in this population ($P = 0.01$), with the *Wz* region being transmitted to female gametes at a lower-than-expected frequency. No recombination was observed between the seven AFLP markers.

Significant differences ($P < 0.0001$) were found for DAI between plants with each of the seven markers versus those without (Table 3). The group of plants with the seven associated markers had a mean DAI of 28.5 while the group without the markers had a mean DAI of 7.5. The majority of plants with the *Wz* markers had a very high DAI and those without had a low DAI. Eight plants with a low DAI tested positive for the seven coupling markers, and one plant with a DAI of 31 tested negative for the *Wz* markers (Fig. 4).

## Investigation of Independent Segregation between *Wz* and *Php*

To investigate the possibility that *Wz* and *Php* may have been introgressed on the same *N. tabacum* chromosome, we genotyped 36 individuals from the three-way cross, (WZ × NC 61) × Hicks, with three of the seven AFLP markers mentioned above and a SCAR marker that indicates the presence of *Php*. Seventeen individuals tested positive for the SOPZ-5 SCAR marker linked to *Php* and 23 tested positive for the three AFLP markers associated with *Wz*. Thirteen plants tested positive for both *Php* and the three *Wz*-associated markers. Four plants possessed only the *Php* marker, 10 possessed only the *Wz* markers, and nine plants had neither. A chi-square test for independence failed to reject the null hypothesis that *Wz* and *Php* segregate independently of each other ($P = 0.14$).

# DISCUSSION

Woodend and Mudzengerere (1992) described the transfer of wildfire and angular leaf spot resistance from *N. rustica* to *N. tabacum*. Data presented in this paper convincingly demonstrate that one or more closely linked genes affecting resistance to *P. nicotianae* were also inadvertently transferred to *N. tabacum* in their efforts. Prior research found nearly all studied *N. rustica* accessions to exhibit very high levels of resistance *P. nicotianae* race 0 and some accessions to also exhibit high levels of resistance to race 1 (Nifong et al., 2011). The number of genes controlling black shank resistance in these materials is unknown. Data presented in this paper indicate that tobacco lines possessing the introgressed *Wz* region from *N. rustica* carry a gene or genes that confer a very high level of resistance to race 0 and that the *Wz* region also affects resistance to race 1. End percent survival for the breeding line WZ after inoculation

**Table 2. Amplified fragment length polymorphism (AFLP) markers associated with *Wz* and corresponding *F*-test values, $R^2$ values, and additive effects for race 0 and race 1 growth chamber inoculations of the doubled haploid population.**

| | Race 0 inoculation | | | | | | Race 1 inoculation | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | EPS[†] | | | AUDPC[‡] | | | EPS | | | AUDPC | | |
| AFLP marker[§] | *F*-value | $R^2$ | Additive effect | *F*-value | $R^2$ | Additive effect | *F*-value | $R^2$ | Additive effect | *F*-value | $R^2$ | Additive effect |
| E-AGA/M-CCG-150[¶] | 573.5[#] | 0.89 | 41.3 | 186.6[#] | 0.73 | 837.2 | 40.6[#] | 0.37 | 17.8 | 33.6[#] | 0.33 | 380.6 |
| E-AGG/M-CCG-675[¶] | 573.5[#] | 0.89 | 41.3 | 186.6[#] | 0.73 | 837.2 | 40.6[#] | 0.37 | 17.8 | 33.6[#] | 0.33 | 380.6 |
| E-AGG/M-CCG-344[¶] | 529.8[#] | 0.89 | 41.1 | 186.7[#] | 0.74 | 818.0 | 41.5[#] | 0.39 | 16.6 | 33.8[#] | 0.34 | 352.6 |
| E-AAA/M-CGC-96[¶] | 591.3[#] | 0.90 | 41.6 | 193.5[#] | 0.74 | 847.7 | 41.2[#] | 0.38 | 18.1 | 33.9[#] | 0.34 | 385.9 |
| E-ATC/M-CCC-232[¶] | 573.5[#] | 0.89 | 41.3 | 186.6[#] | 0.73 | 837.2 | 40.6[#] | 0.37 | 17.8 | 33.6[#] | 0.33 | 380.6 |
| E-ATG/M-CCG-359[¶] | 509.4[#] | 0.89 | 41.2 | 161.4[#] | 0.72 | 834.3 | 34.9[#] | 0.35 | 17.7 | 29.1[#] | 0.31 | 379.7 |
| E-ATG/M-CCG-361[¶] | 509.4[#] | 0.89 | 41.2 | 161.4[#] | 0.72 | 834.3 | 34.9[#] | 0.35 | 17.7 | 29.1[#] | 0.31 | 379.7 |
| E-ATT/M-CCA-482[¶] | 287.0[#] | 0.82 | 39.9 | 122.1[#] | 0.66 | 806.3 | 30.1[#] | 0.32 | 17.2 | 25.4[#] | 0.29 | 369.4 |
| E-ATT/M-CCA-302[¶] | 494.9[#] | 0.89 | 40.9 | 160.2[#] | 0.71 | 831.0 | 34.9[#] | 0.35 | 17.7 | 29.1[#] | 0.31 | 379.7 |
| E-ATC/M-CCC-323[¶] | 475.1[#] | 0.88 | 40.8 | 153.5[#] | 0.71 | 829.0 | 33.5[#] | 0.35 | 17.7 | 27.9[#] | 0.31 | 379.4 |
| E-ATC/M-CCC-175[¶] | 475.1[#] | 0.88 | 40.8 | 153.5[#] | 0.71 | 829.0 | 33.5[#] | 0.35 | 17.7 | 27.9[#] | 0.31 | 379.4 |
| E-ATG/M-CCG-256[¶] | 578.7[#] | 0.90 | 41.9 | 179.8[#] | 0.73 | 845.8 | 37.7[#] | 0.36 | 17.8 | 31.3[#] | 0.32 | 380.2 |
| E-ATG/M-CCG-88[¶] | 578.7[#] | 0.90 | 41.9 | 179.8[#] | 0.73 | 845.8 | 37.7[#] | 0.36 | 17.8 | 31.3[#] | 0.32 | 380.2 |
| E-AGC/M-CGG-299[¶] | 389.2[#] | 0.85 | 40.4 | 154.4[#] | 0.70 | 821.2 | 40.3[#] | 0.38 | 17.9 | 33.8[#] | 0.34 | 385.6 |
| E-ATG/M-CGA-157[¶] | 514.7[#] | 0.89 | 41.1 | 166.8[#] | 0.72 | 832.9 | 36.3[#] | 0.36 | 17.7 | 30.2[#] | 0.32 | 380.0 |
| E-AGC/M-CGA-113[¶] | 388.2[#] | 0.85 | 40.5 | 140.7[#] | 0.68 | 807.8 | 28.4[#] | 0.30 | 16.1 | 24.7[#] | 0.27 | 348.7 |
| E-ATT/M-CGG-167[¶] | 342.0[#] | 0.83 | 40.0 | 149.2[#] | 0.69 | 813.6 | 36.8[#] | 0.35 | 17.3 | 30.7[#] | 0.31 | 370.7 |
| E-AGC/M-CGT-657[¶] | 573.5[#] | 0.89 | 41.3 | 186.6[#] | 0.73 | 837.2 | 40.6[#] | 0.37 | 17.8 | 33.6[#] | 0.33 | 380.6 |
| E-AGC/M-CGT-289[¶] | 573.5[#] | 0.89 | 41.3 | 186.6[#] | 0.73 | 837.2 | 40.6[#] | 0.37 | 17.8 | 33.6[#] | 0.33 | 380.6 |
| E-AAT/C-CGA-332[¶] | 573.5[#] | 0.89 | 41.3 | 186.6[#] | 0.73 | 837.2 | 40.6[#] | 0.37 | 17.8 | 33.6[#] | 0.33 | 380.6 |
| E-ATT/M-CCT-542[¶] | 554.0[#] | 0.89 | 41.3 | 180.1[#] | 0.73 | 836.0 | 39.2[#] | 0.37 | 17.8 | 32.5[#] | 0.33 | 380.4 |
| E-ATT/M-CCT-413[¶] | 554.0[#] | 0.89 | 41.3 | 180.1[#] | 0.73 | 836.0 | 39.2[#] | 0.37 | 17.8 | 32.5[#] | 0.33 | 380.4 |
| E-ATT/M-CCT-223[¶] | 554.0[#] | 0.89 | 41.3 | 180.1[#] | 0.73 | 836.0 | 39.2[#] | 0.37 | 17.8 | 32.5[#] | 0.33 | 380.4 |
| E-ATT/M-CCT-206[¶] | 554.0[#] | 0.89 | 41.3 | 180.1[#] | 0.73 | 836.0 | 39.2[#] | 0.37 | 17.8 | 32.5[#] | 0.33 | 380.4 |
| E-ATT/M-CGT-544[¶] | 573.5[#] | 0.89 | 41.3 | 186.6[#] | 0.73 | 837.2 | 40.6[#] | 0.37 | 17.8 | 33.6[#] | 0.33 | 380.6 |
| E-ATT/M-CGT-413[¶] | 573.5[#] | 0.89 | 41.3 | 186.6[#] | 0.73 | 837.2 | 40.6[#] | 0.37 | 17.8 | 33.6[#] | 0.33 | 380.6 |
| E-ATT/M-CGT-223[¶] | 573.5[#] | 0.89 | 41.3 | 186.6[#] | 0.73 | 837.2 | 40.6[#] | 0.37 | 17.8 | 33.6[#] | 0.33 | 380.6 |
| E-ATT/M-CGT-206[¶] | 573.5[#] | 0.89 | 41.3 | 186.6[#] | 0.73 | 837.2 | 40.6[#] | 0.37 | 17.8 | 33.6[#] | 0.33 | 380.6 |
| E-AGA/M-CTT-288[¶] | 544.7[#] | 0.89 | 41.3 | 186.6[#] | 0.73 | 838.5 | 36.8[#] | 0.35 | 17.3 | 30.7[#] | 0.31 | 370.7 |
| E-ATT/M-CCA-324[††] | 352.0[#] | 0.85 | −40.3 | 130.9[#] | 0.68 | −815.5 | 31.4[#] | 0.33 | −17.4 | 26.7[#] | 0.30 | −374.9 |
| E-AGC/M-CGG-102[††] | 327.4[#] | 0.83 | −39.6 | 135.7[#] | 0.67 | −799.9 | 32.1[#] | 0.32 | −16.6 | 26.2[#] | 0.28 | −350.7 |
| E-ATG/M-CGA-577[††] | 377.4[#] | 0.85 | −40.5 | 139.0[#] | 0.68 | −815.0 | 32.7[#] | 0.33 | −17.2 | 27.5[#] | 0.30 | −370.0 |
| E-ATG/M-CGA-412[††] | 377.4[#] | 0.85 | −40.5 | 139.0[#] | 0.68 | −815.0 | 32.7[#] | 0.33 | −17.2 | 27.5[#] | 0.30 | −370.0 |
| E-ATT/M-CGG-107[††] | 278.3[#] | 0.80 | −39.5 | 127.7[#] | 0.65 | −796.6 | 33.4[#] | 0.33 | −16.8 | 28.2[#] | 0.29 | −361.3 |
| E-AAT/C-CGA-162[††] | 422.5[#] | 0.86 | −40.7 | 156.1[#] | 0.70 | −819.3 | 36.8[#] | 0.35 | −17.3 | 30.7[#] | 0.31 | −370.7 |
| E-AGA/M-CCG-169[††] | 422.5[#] | 0.86 | −40.7 | 156.1[#] | 0.70 | −819.3 | 36.8[#] | 0.35 | −17.3 | 30.7[#] | 0.31 | −370.7 |
| E-ATT/M-CCT-587[††] | 554.0[#] | 0.89 | −41.3 | 180.1[#] | 0.73 | −836.0 | 39.2[#] | 0.37 | −17.8 | 32.5[#] | 0.33 | −380.4 |
| E-ATT/M-CCT-407[††] | 554.0[#] | 0.89 | −41.3 | 180.1[#] | 0.73 | −836.0 | 39.2[#] | 0.37 | −17.8 | 32.5[#] | 0.33 | −380.4 |
| E-AGG/M-CCG-295[††] | 390.2[#] | 0.86 | −40.4 | 154.4[#] | 0.70 | −799.0 | 37.4[#] | 0.36 | −16.2 | 30.8[#] | 0.32 | −342.7 |
| E-AAA/M-CGC-679[††] | 429.0[#] | 0.86 | −41.0 | 160.5[#] | 0.71 | −829.0 | 37.4[#] | 0.36 | −17.6 | 31.0[#] | 0.32 | −375.7 |
| E-AAA/M-CGC-487[††] | 429.0[#] | 0.86 | −41.0 | 160.5[#] | 0.71 | −829.0 | 37.4[#] | 0.36 | −17.6 | 31.0[#] | 0.32 | −375.7 |
| E-AAA/M-CGC-208[††] | 429.0[#] | 0.86 | −41.0 | 160.5[#] | 0.71 | −829.0 | 37.4[#] | 0.36 | −17.6 | 31.0[#] | 0.32 | −375.7 |
| E-ATT/M-CGT-590[††] | 270.6[#] | 0.80 | −39.1 | 105.0[#] | 0.61 | −761.8 | 25.6[#] | 0.27 | −15.2 | 19.8[#] | 0.23 | −314.2 |
| E-ATT/M-CGT-408[††] | 422.5[#] | 0.86 | −40.7 | 156.1[#] | 0.70 | −819.3 | 36.8[#] | 0.35 | −17.3 | 30.7[#] | 0.31 | −370.7 |
| E-AGA/M-CTT-247[††] | 422.5[#] | 0.86 | −40.7 | 156.1[#] | 0.70 | −819.3 | 36.8[#] | 0.35 | −17.3 | 30.7[#] | 0.31 | −370.7 |
| E-ATC/M-CCC-223[††] | 553.7[#] | 0.89 | −41.2 | 180.0[#] | 0.73 | −835.7 | 39.2[#] | 0.37 | −17.8 | 32.5[#] | 0.33 | −380.4 |

[†]EPS, end percent survival.

[‡]AUDPC, area under the disease progress curve.

[§]AFLP markers are designated by primer pair combination followed by estimated band size.

[¶]Coupling phase AFLP marker.

[#]Significant at the 0.0001 probability level.

[††]Repulsion phase AFLP marker

with race 1 was actually higher than EPS for the same line after inoculation with race 0. The race 1 isolate used in our study was less aggressive than the race 0 isolate that was used, however. The level of apparent partial resistance can be dependent on isolates used in inoculations and also on environmental conditions. It could therefore be possible

**Table 3. Student *t* test values for amplified fragment length polymorphism (AFLP) markers used to genotype $BC_1F_1$ population segregating for *Wz* and inoculated with *Phytophthora nicotianae* race 0.**

| AFLP marker† | Days survival after inoculation *t*-value |
|---|---|
| E-AGA/M-CCG-150‡ | 21.0§ |
| E-AGG/M-CCG-675‡ | 21.3§ |
| E-AGG/M-CCG-344‡ | 21.3§ |
| E-AAA/M-CGC-96‡ | 21.0§ |
| E-ATC/M-CCC-232‡ | 16.5§ |
| E-ATG/M-CCG-359‡ | 19.9§ |
| E-ATG/M-CCG-361‡ | 19.9§ |

†AFLP markers are designated by primer pair combination followed by estimated band size.

‡Coupling phase AFLP marker.

§Significant at the 0.0001 probability level.

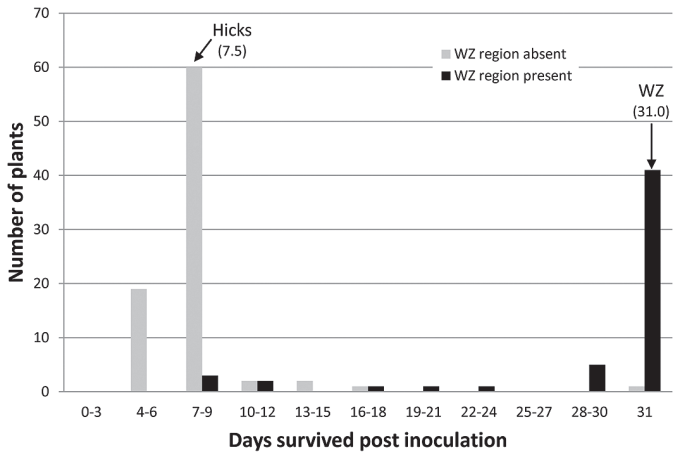


Figure 4. Frequency histogram for days survived post inoculation for (WZ × Hicks) × Hicks $BC_1F_1$ population inoculated with race 0 of *Phytophthora nicotianae*. Days survived is shown for individuals with and without the introgressed *Nicotiana rustica Wz* region as determined using seven *Wz* associated amplified fragment length polymorphism markers. Means for parental lines (Hicks and WZ) are indicated in parenthesis and by arrows.

that the resistance to race 1 isolates, on average, may actually be less than resistance to race 0 isolates. One argument needs to be pointed out, however. If the *Wz* region contains only a single gene affecting black shank resistance, it may be a gene with a large partial effect and not one that provides complete immunity. This is evidenced by the fact that some plants carrying *Wz* were killed with the race 0 isolate in controlled growth chamber experiments. No plants of NC1071 (homozygous for *Php*) were killed in these experiments. It seems unlikely that a single gene with a large partial effect would act in a race-specific manner although this possibility cannot be ruled out. It is possible that *Wz* has differential interactions with different *P. nicotianae* isolates due to observations that cultivars known to possess this genomic region perform differently for resistance in black shank nurseries in different growing regions of the United States (Brian Smeeton, personal communication, 2012). Further research is underway to investigate questions related to partial versus complete resistance and interactions with diverse *P. nicotianae* isolates.

Amplified fragment length polymorphism markers found to be associated with *Wz* will be useful for selecting for the *N. rustica*-derived variation affecting black shank resistance in segregating tobacco populations. All AFLP markers initially identified as putative coupling and repulsion phase markers for *Wz* were ultimately demonstrated to be associated with the alien region. This should not be surprising as the level of AFLP polymorphism among lines of *N. tabacum* is generally low (Ren and Timko, 2001), and introgression of alien *Nicotiana* chromatin generally introduces substantial DNA marker polymorphism into cultivated tobacco (Lewis, 2011). No segregation was observed between the AFLP markers associated with *Wz*. Such repressed recombination has also been observed between markers associated with other disease resistance genes transferred from wild *Nicotiana* relatives (Johnson et al., 2002).

The *Wz* region may have value in developing future tobacco cultivars with enhanced black shank resistance. This is especially the case since *Wz* apparently has a large effect against at least some isolates of race 1. Race 1 of *P. nicotianae* is currently of greatest concern because of race shifts caused by the widespread deployment of cultivars carrying *Php* or *Phl*. We have shown here that, because *Wz* and *Php* segregate independently, the two genomic regions can easily be combined into single inbred lines. Some have speculated that the black shank resistance gene in WZ acts in a manner similar to *Php* and *Phl*, in that it confers immunity to race 0 but little resistance to race 1 (Bukuta, 2002). The current results suggest otherwise. First, there was a very significant observed effect of *Wz* on resistance against a race 1 isolate in our controlled growth chamber experiments. Second, WZ as a line, per se, exhibited high levels of resistance in field experiments where a substantial level of race 1 was present (as evidenced by large scale death of NC1071). This would not be expected based on the pedigree, as WZ was developed by backcrossing the *N. rustica*-derived wildfire and angular leaf spot resistance into a *N. tabacum* genetic background that confers a low level of resistance to black shank (Brian Smeeton, personal communication, 2010). Third, some individuals possessing *Wz* succumbed to disease after inoculation with race 0, indicating that the resistance may not be of the immunity type displayed by *Php* and *Phl*. NC1071 exhibited 100% EPS after inoculation with this isolate. Finally, it cannot be ruled out that more than a single gene within the *Wz* region may be affecting black shank resistance in the *N. tabacum* genetic background.

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