Characterization of *Phn15.1*, a Newly Identified *Phytophthora nicotianae* Resistance QTL in *Nicotiana tabacum*

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Abstract

Phytophthora nicotianae is an oomycete that causes black shank, one of the most economically important diseases affecting tobacco production worldwide. Identification and introgression of novel genetic variability affecting partial genetic resistance to this pathogen is important because of the increased durability of partial resistance over time as compared with genes conferring immunity. A previous mapping study identified a quantitative trait locus (QTL), hereafter designated as *Phn15.1*, with a major effect on *P. nicotianae* resistance in tobacco. In this research, we describe significantly improved resistance of nearly isogenic lines (NILs) of flue-cured tobacco carrying the introgressed *Phn15.1* region derived from highly resistant cigar tobacco cultivar Beinhart 1000. The *Phn15.1* region appeared to act in an additive or partially dominant manner to positively affect resistance. To more finely resolve the position of the gene or genes underlying the *Phn15.1* effect, the QTL was mapped with an increased

number of molecular markers (single-nucleotide polymorphisms) identified to reside within the region. Development and evaluation of subNILs containing varying amounts of Beinhart 1000-derived *Phn15.1*-associated genetic material permitted the localization of the QTL to a genetic interval of approximately 2.7 centimorgans. Importantly, we were able to disassociate the Beinhart 1000 *Phn15.1* resistance alleles from a functional *NtCPS2* allele(s) which contributes to the accumulation of a diterpene leaf surface exudate considered undesirable for flue-cured and burley tobacco. Information from this research should be of value for marker-assisted introgression of Beinhart 1000-derived partial black shank resistance into flue-cured and burley tobacco breeding programs.

Keywords: black shank, *Nicotiana*, partial resistance, *Phytophthora*, plant disease resistance, quantitative trait locus, tobacco

The black shank disease, caused by *Phytophthora nicotianae*, is the most economically important disease affecting tobacco (*Nicotiana tabacum* L.) production in many parts of the world. *P. nicotianae* is a soilborne oomycete that typically infects the roots and stems of tobacco plants, resulting in substantial crop loss in fields with sufficient inoculum density and environmental conditions conducive to disease progression. Multiple types of genetic resistance for reducing crop loss due to *P. nicotianae* have been described. The resistance genes *Php* and *Phl* were introgressed from *N. plumbaginifolia* and *N. longiflora*, respectively (Apple 1962; Chaplin 1962; Valleau et al. 1960), and provide what is believed to be immunity to race 0 isolates of *P. nicotianae*. An introgressed *N. rustica* chromosome segment designated as Wz also carries a gene or genes conferring a very high level of resistance to multiple races of *P. nicotianae* (Drake and Lewis 2013; Drake et al. 2015). Another system of *P. nicotianae* resistance is of the polygenic type. Polygenic resistance is controlled by genes at multiple loci with variable effects, is partial in nature, and is believed to be largely nonrace specific. The complexity of polygenic resistance makes a complete understanding difficult. Simply inherited monogenic *P. nicotianae* resistance mechanisms can be easily overcome by evolving pathogen populations when not combined with intermediate to high levels of polygenic partial resistance that are more durable over time. Therefore, successful breeding for enhanced long-term *P. nicotianae* resistance requires understanding of components of partial resistance and identification and introgression of new variability affecting the trait.

Past quantitative trait loci (QTL) mapping experiments have provided insight into the genetic control of partial *P. nicotianae* resistance in the highly black shank resistant cigar tobacco cultivar Florida 301

(Xiao et al. 2013) and highly resistant flue-cured tobacco cultivar K346 (Drake-Stowe et al. 2017). One major QTL, designated as *Phn7.1* (Ma et al. 2019), was found to be the largest contributor to resistance in these cultivars. The exceptionally high level of *P. nicotianae* resistance exhibited by cigar tobacco cultivar Beinhart 1000 has also been studied via QTL mapping experiments. Again, *Phn7.1* was found to be the largest contributor to resistance in this cultivar (Vontimitta and Lewis 2012). A second QTL with a large effect was identified on *N. tabacum* linkage group 15 that was not identified in the other QTL mapping studies and is hereafter referred to as *Phn15.1*. It is not likely that the favorable *Phn15.1* resistance allele found in Beinhart 1000 exists within current flue-cured or burley tobacco germplasm pools. Incorporation of this allelic variability into breeding programs could increase the level, range, and durability of genetic resistance to *P. nicotianae* in future cultivars of these market types. However, *Phn15.1* is very closely linked to *NtCPS2*, a gene involved in the biosynthesis of Z-abienol (Vontimitta et al. 2010). Z-abienol is a diterpene trichome exudate that contributes to flavor and aroma characteristics of Oriental and some cigar tobacco but is considered undesirable for flue-cured and burley tobacco. For successful utilization of *Phn15.1* in flue-cured and burley tobacco cultivar development, the favorable Beinhart 1000 *Phn15.1* alleles must be disassociated from the Beinhart 1000 *NtCPS2* allele. Previous mapping of *Phn15.1* was carried out using a relatively low density of microsatellite markers (Vontimitta and Lewis 2012). An increased density of DNA markers in the vicinity of the two loci could aid in marker-assisted transfer of *Phn15.1*-mediated partial black shank resistance to flue-cured and burley tobacco without undesired genetic linkages. For example, Brouwer and St. Clair (2004) more finely resolved multiple QTL for *Phytophthora* disease resistance in tomato using nearly isogenic lines (NILs) and subNILs with the goal of utilizing favorable allelic variability for resistance while selecting against undesirable genetic linkages. Fine mapping of *Phn15.1* coupled with the utilization of recently published genomic sequence information for *N. tabacum* (Edwards et al. 2017; Sierro et al. 2013, 2014) might also facilitate identification of causal DNA sequence variability underlying the desirable *Phn15.1* phenotypic effect.

The objectives of this research were to (i) remap *Phn15.1* in Beinhart 1000 with an increased density of single-nucleotide polymorphism

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(SNP) markers, (ii) utilize these markers to develop and study NILs and hybrids of flue-cured tobacco cultivars Hicks and K326 carrying the *Phn15.1* region derived from Beinhart 1000 in homozygous and heterozygous condition, and (iii) subsequently derive subNILs with varying amounts of *Phn15.1*-associated genetic material for the purpose of fine mapping the QTL. Evaluation of these materials allowed for investigation of the genetic effect of this QTL, enabled further resolution of the genomic region underlying the favorable effect of *Phn15.1*, and determined the potential for disassociating the Beinhart 1000 *Phn15.1* *P. nicotianae* resistance alleles from the functional Beinhart 1000 gene contributing to the accumulation of Z-abienol.

Materials and Methods

NIL development. NILs are useful in analyses of QTL because they isolate the alleles under study in a common background. Working toward the goal of mapping *Phn15.1* with increased marker density, we first constructed NILs by backcrossing the genomic region carrying *Phn15.1* from Beinhart 1000 into the genetic backgrounds of moderately resistant flue-cured tobacco cultivar K326 and highly susceptible flue-cured tobacco cultivar Hicks. At each backcross generation, we selected for an approximately 13.0-centimorgan (cM) region of the Beinhart 1000 genome previously reported to contain *Phn15.1* (Vontimitta and Lewis 2012) using *Phn15.1*-associated microsatellite markers PT30354 and PT61373, applying previously described microsatellite marker genotyping methodologies (Vontimitta and Lewis 2012). During the initial backcross generations, we selected against Beinhart 1000 microsatellite marker alleles associated with *Phn7.1*, another major effect *P. nicotianae* resistance QTL (Ma et al. 2019; Vontimitta and Lewis 2012). Backcrossing followed by self-pollination and selection for homozygosity at the relevant microsatellite loci was carried out to the BC₃F₃ generation

for the Hicks genetic background and to the BC₆F₃ generation for the K326 genetic background. Backcrossing was carried out in such a way that each family was independently derived from a unique BC₁F₁ individual. Five Hicks + *Phn15.1* BC₃F₃ NILs (designated as 375-8, 379-7, 377-5, 381-9, and 200-2) and four K326 + *Phn15.1* NILs (designated as 385-1, 386-6, 385-5, and 388-3) were selected and subjected to preliminary field testing to determine whether the favorable *P. nicotianae* resistance alleles from Beinhart 1000 had been transferred.

Phenotypic Evaluation of Initial NILs. The initial NILs described above were evaluated for field *P. nicotianae* resistance over a period of 3 years in mixed-race soilborne pathogen nurseries at Kinston, NC (Lower Coastal Plain Research Station), and Rocky Mount, NC (Upper Coastal Plain Research Station), along with the check cultivars Hicks, K326, K346 (a highly black shank-resistant flue-cured tobacco cultivar), and the NIL Hicks + *Phn7.1* (Ma et al. 2019). Two Hicks + *Phn15.1* NILs (379-7 and 375-8) were evaluated in comparison with Hicks in each environment during 2013, 2014, and 2015. Two K326 + *Phn15.1* NILs (385-1 and 386-6) were also evaluated in comparison with K326 in each of these environments. The Hicks + *Phn15.1* NIL 375-8 and the K326 + *Phn15.1* NIL 386-6 were also evaluated in comparison with their F₁ hybrids with Hicks and K326, respectively, during the 2016 and 2017 growing seasons in order to determine the level of dominance of the favorable *Phn15.1* allele.

Experiments in each environment during the years 2013, 2014, and 2015 were organized as randomized complete block designs with three replications. During 2016 and 2017, experiments were organized as randomized complete block designs with eight replications. During 2013, experimental units consisted of single 22-plant rows whereas, in 2014, 2015, 2016, and 2017, experimental units

Fig. 1. Comparison of **A**, Hicks + *Phn15.1* and **B**, K326 + *Phn15.1* nearly isogenic lines (NILs) for area under the disease progress curve (AUDPC) relative to control genotypes. Hicks + *Phn15.1* NILs were of the BC₃F₃ generation. K326 + *Phn15.1* NILs were of the BC₆F₃ generation. Means are averaged over six 2013, 2014, and 2015 field environments. Means with the same letters are not significantly different at the $P < 0.05$ level.

Fig. 2. Comparison of **A**, Hicks + *Phn15.1* and **B**, K326 + *Phn15.1* homozygous and heterozygous nearly isogenic lines (NILs) for area under the disease progress curve (AUDPC) relative to control genotypes. Hicks + *Phn15.1* NILs were of the BC₃F₃ generation. K326 + *Phn15.1* NILs were of the BC₆F₃ generation. Means are averaged over four 2016 and 2017 field environments. Means with the same letters are not significantly different at the $P < 0.05$ level.

consisted of single 12-plant rows. Interrow and within-row plant spacing at each environment were 120 and 56 cm, respectively.

Beginning approximately 40 days posttransplanting, the number of plants killed by *P. nicotianae* was recorded at intervals of approximately 15 days. Data were collected over shorter intervals (approximately every 7 days) during periods of rapid disease progression later in the growing season. Area under the disease progress curve (AUDPC) was calculated for each plot according to Madden et al. (2007). PROC MIXED of SAS (version 9.4; SAS Institute, Cary, NC, U.S.A.) was then used to analyze plot AUDPC data over environments, with a Kenward-Roger degrees of freedom adjustment (Kenward and Roger 1997; Schaalje et al. 2001). Mean separations were performed using Fisher's protected least significant difference at $\alpha = 0.05$.

Development of subNILs for mapping of *Phn15.1*. Having found Hicks + *Phn15.1* and K326 + *Phn15.1* NILs to exhibit field *P. nicotianae* resistance significantly superior to that of their recurrent parents and, thus, presumably carrying the desirable Beinhart 1000 *Phn15.1* genomic region, our next objective was to more finely resolve the *Phn15.1* QTL through evaluation of subNILs with varying amounts of Beinhart 1000-derived genetic material associated with the *Phn15.1* region. A custom 30K Infinium iSelect HD Bead-Chip SNP chip (Illumina Inc., San Diego, CA, U.S.A.) was first used to identify a set of 4,112 SNP markers that differentiated K326 and Beinhart 1000, and a second set of 1,898 SNP markers that differentiated Hicks and Beinhart 1000. Haldane's mapping function within JoinMap (Van Ooijen 2006) was then employed to map the latter set of SNP markers, along with a set of previously reported microsatellite markers (Vontimitta and Lewis 2012), using a previously described doubled-haploid mapping population derived from a cross between Beinhart 1000 and Hicks (Vontimitta and Lewis 2012). Composite interval mapping within R package R/qtl (Broman and Sen 2009) was used to identify QTL affecting black shank resistance in this original mapping population using the AUDPC data of Vontimitta and Lewis (2012). Conditional genotypic probabilities were calculated using an error probability rate of 0.001, and minimum logarithm of odds values were calculated using a window size of 20 cM and three covariate markers. QTL effects of the final model were determined using the "fitqtl" and "refineqtl" functions. In addition, the five established Hicks + *Phn15.1* BC₅F₃ NILs and four established K326 + *Phn15.1* BC₆F₃ NILs were genotyped with polymorphic SNP markers to assess their genetic composition and estimate the amount of Beinhart 1000 germplasm that was retained genomewide after the backcrossing process.

The next objective was to develop subNILs with new recombination breakpoints within the Beinhart 1000 *Phn15.1* region initially introgressed into Hicks + *Phn15.1* BC₅F₃ NIL 375-8 and K326 + *Phn15.1* BC₆F₃ NIL 386-6. These NILs were selected based upon superior *P. nicotianae* resistance exhibited in field experiments. To generate genotypes with new breakpoints in the vicinity of *Phn15.1*, we developed Hicks + *Phn15.1* BC₇F₁ and K326 + *Phn15.1* BC₈F₁ families derived from the aforementioned NILs that were expected to segregate 1:1 for the presence or absence of Beinhart 1000 *Phn15.1*-associated SNP markers. A subset of 12 well-spaced, readily genotyped, *Phn15.1*-associated polymorphic SNP

markers was selected for fine mapping experiments. An SNP marker within *NtCPS2* (Sallaud et al. 2012) found to reside within the QTL region (Vontimitta et al. 2010) was also included. Kompetitive allele-specific PCR (KASP) markers were designed (LGC Genomics, Beverly, MA, U.S.A.) for convenient genotyping at each SNP locus (SNP flanking sequences available at https://solgenomics.net/cview/map.pl?map_version_id=178). Performance of some KASP markers was optimized by introduction of a "pre-KASP" amplification step and carrying out the KASP cycle as a nested reaction. Pre-KASP primers were designed using GSP, a web tool for genome specific primer design in polyploid species (Wang et al. 2016). Homologous regions for the GSP web tool were identified via BLAST searches against the published *N. tabacum* genome sequence of Edwards et al. (2017). In total, 480 individuals from the Hicks + *Phn15.1* BC₇F₁ and 480 individuals from the K326 + *Phn15.1* BC₈F₁ families were genotyped using markers on opposite sides of the introgressed genomic region from Beinhart 1000: SNP markers *Nt1AA7078* and *NtCPS2*, representing an approximately 30-cM region, or markers *NtAD4594* and *PT30354*, representing a slightly larger (36.8 cM) region to identify recombination events with the genomic region carrying the *P. nicotianae* resistance QTL. Recombinant individuals were subsequently genotyped using the remaining SNP markers selected for characterization of the *Phn15.1*-associated region.

Field evaluation of recombinant subNILs for fine-mapping of *Phn15.1*. After eliminating a number of recombinant BC₇F₁ or BC₈F₁ individuals based on genetic redundancy, 25 Hicks + *Phn15.1* BC₇F₁ plants and 21 K326 + *Phn15.1* BC₈F₁ plants were self-pollinated to produce corresponding BC₇F₂ or BC₈F₂ recombinant families that were evaluated for field *P. nicotianae* resistance during 2016 at the previously mentioned Kinston and Rocky Mount, NC, locations. These families were still segregating for an expected 1:2:1 ratio at the *Phn15.1*-associated SNP marker loci. The Hicks + *Phn15.1* BC₇F₂ and K326 + *Phn15.1* BC₈F₂ families were evaluated in separate experiments. In addition to the recombinant families, the recurrent parental lines and original BC₅F₃ or BC₆F₃ NILs were

Table 2. Percentage of polymorphic non-linkage group 15 single-nucleotide polymorphism (SNP) markers derived from the recurrent parent in Hicks + *Phn15.1* (BC₅F₃ generation) and K326 + *Phn15.1* (BC₆F₃ generation) nearly isogenic lines (NILs)

NILs	Recurrent parent SNP alleles (%)
Hicks + <i>Phn15.1</i> BC ₅ F ₃ NIL 375-8 ^a	98.9
Hicks + <i>Phn15.1</i> BC ₅ F ₃ NIL 379-7 ^a	99.1
Hicks + <i>Phn15.1</i> BC ₅ F ₃ NIL 377-5	98.8
Hicks + <i>Phn15.1</i> BC ₅ F ₃ NIL 381-9	99.1
Hicks + <i>Phn15.1</i> BC ₅ F ₃ NIL 200-2	98.8
K326 + <i>Phn15.1</i> BC ₆ F ₃ NIL 385-1 ^a	99.6
K326 + <i>Phn15.1</i> BC ₆ F ₃ NIL 386-6 ^a	98.7
K326 + <i>Phn15.1</i> BC ₆ F ₃ NIL 385-5	99.5
K326 + <i>Phn15.1</i> BC ₆ F ₃ NIL 388-3	85.9

^a Indicates that this NIL was tested for *Phytophthora nicotianae* resistance for 3 years (2013, 2014, and 2015).

Table 1. Estimates of quantitative trait loci (QTL) positions, effects, and percentage of phenotypic variance explained for area under the disease progress curve from composite interval mapping in the Beinhart 1000 × Hicks doubled-haploid population

QTL	LG ^a	Marker		Position (cM) ^b	LOD ^c	Effect	Variance (%) ^d
		Left	Right				
<i>Phn7.1</i>	7	<i>Nt1AF4104</i>	<i>PT61512</i>	76.4	15.96	77.2	35.3
<i>Phn15.1</i>	15	<i>Nt1AD4594</i>	<i>PT5116</i>	108.9	12.43	66.1	25.5
<i>Phn17.1</i>	17	<i>Nt1AA2233</i>	<i>Nt1AG0010</i>	88.0	4.86	35.5	8.5

^a *Nicotiana tabacum* linkage group.

^b Position in centimorgans (cM).

^c LOD = logarithm of the odds.

^d Percent phenotypic variance explained.

included as controls. The experimental design in each environment was a randomized complete block design with eight replications. Experimental units were single, 12-plant rows with previously described plant spacing. Data were collected as previously described but with shorter intervals (approximately 8 days) between observations. AUDPC was calculated for each plot and PROC MIXED

was used to analyze data over environments. Linear contrasts using the CONTRAST function of SAS were performed to determine the statistical significance of differences in field resistance between genotypic classes (segregating for Beinhart 1000 allele versus fixed for Hicks or K326 allele) at each SNP marker locus. Significance tests were reported as $-\log_{10}(P)$ values (also known as LogWorth)

Fig. 3. Genetic composition of Hicks + *Phn15.1* BC₇F₂ sub-nearly isogenic lines (subNILs) evaluated for field *Phytophthora nicotianae* resistance in two different North Carolina environments during 2016. Area under the disease progress curve (AUDPC) means averaged over both environments are located on the second left-hand column. Genotypes are ranked in descending order of resistance. The linkage map is shown at the top of the figure, along with map distances (centimorgans) and corresponding LogWorth values indicated in parentheses. LogWorth values were used to report the level of statistical significance of linear contrasts that were performed between genotypic classes (segregating for Beinhart 1000 allele versus fixed for Hicks allele at each single-nucleotide polymorphism marker locus). LSD = least significant difference.

to more easily make comparisons at extreme levels of significance. The $-\log_{10}(P)$ values of 1.3, 2, and 3 correspond to traditional P -value significance thresholds of 0.05, 0.01, and 0.001, respectively.

During the 2016 growing season, plants from each of the selected BC₇F₂ or BC₈F₂ families were genotyped to identify individuals homozygous for Beinhart 1000 SNP alleles at the *Phn15.1*-associated loci and self-pollinated to produce genetically stable BC₇F₃ or BC₈F₃ families for field testing at the Kinston and Rocky Mount

locations during the 2017 growing season. Some recombinant genotypes were not tested during the 2017 season due to genetic redundancy, and some recombinant BC₇F₂ or BC₈F₂ families were represented by multiple BC₇F₃ or BC₈F₃ lines to strengthen statistical comparisons of interest. In total, 26 Hicks + *Phn15.1* BC₇F₃ subNILs and 19 K326 + *Phn15.1* BC₈F₃ subNILs were evaluated during the 2017 growing season. Methodology and analyses of the 2017 experiments were identical to those used during 2016, except that tests

Fig. 4. Genetic composition of K326 + *Phn15.1* BC₈F₂ sub-nearly isogenic lines (subNILs) evaluated for field *Phytophthora nicotianae* resistance in two different North Carolina environments during 2016. Area under the disease progress curve (AUDPC) means averaged over both environments are located on the second left-hand column. Genotypes are ranked in descending order of resistance. The linkage map is shown at the top of the figure, along with map distances (centimorgans) and corresponding LogWorth values indicated in parentheses. LogWorth values were used to report the level of statistical significance of linear contrasts that were performed between genotypic classes (segregating for Beinhart 1000 allele versus fixed for K326 allele at each single-nucleotide polymorphism marker locus). LSD = least significant difference.

for significant differences in AUDPC between genotypic classes at each SNP marker locus were between individuals fixed for the recurrent parent allele and those fixed for the Beinhart 1000 allele.

Results

Evaluation of Initial *Phn7.1* NILs. The Hicks + *Phn15.1* BC₅F₃ NILs 375-8 and 379-9 were compared with Hicks, a Hicks + *Phn7.1* NIL (Ma et al. 2019), and K326 in a total of six environments. Both Hicks + *Phn15.1* NILs exhibited significantly ($P < 0.05$) lower AUDPC as compared with Hicks but significantly greater ($P < 0.05$) AUDPC as compared with the Hicks + *Phn7.1* NIL and K326 (Fig. 1). Similarly, the K326 + *Phn15.1* NILs 385-1 and 386-1 NILs exhibited significantly lower AUDPC values as compared with

K326 per se (Fig. 1). AUDPC for these NILs was greater than but not significantly different from the AUDPC observed for highly black-shank-resistant cultivar K346. *Phn15.1* appeared to act in an additive or partially dominant manner in both the Hicks and K326 genetic backgrounds because nearly isogenic hybrids that were heterozygous for the Beinhart 1000 *Phn15.1* allele were intermediate in value for AUDPC between their parental inbred lines (Fig. 2).

Remapping of *P. nicotianae* resistance with increased marker density. The 1,898 SNP markers plus 276 previously described microsatellite markers identified to be polymorphic between Hicks and Beinhart 1000 were used to remap *P. nicotianae* resistance QTL in the original doubled-haploid population derived from a cross

Fig. 5. Genetic composition of K326 + *Phn15.1* BC₅F₃ sub-nearly isogenic lines (subNILs) evaluated for field *Phytophthora nicotianae* resistance in two different North Carolina environments during 2016. Area under the disease progress curve (AUDPC) means averaged over both environments are located on the second left-hand column. Genotypes are ranked in descending order of resistance. The linkage map is shown at the top of the figure, along with map distances (centimorgans) and corresponding LogWorth values indicated in parentheses. LogWorth values were used to report the level of statistical significance of linear contrasts that were performed between genotypic classes (segregating for Beinhart 1000 allele versus fixed for K326 allele at each single-nucleotide polymorphism marker locus). LSD = least significant difference.

between these two lines (Vontimitta and Lewis 2012). *Phn7.1* and *Phn15.1* were found to be the QTL with the largest and second-largest effects, respectively, on *P. nicotianae* resistance in this population (Table 1). The *Phn15.1* QTL explained 25.5% of the observed variation for AUDPC.

The set of polymorphic SNP markers was also used to characterize the genome-wide genetic composition of the five initial Hicks +

Phn15.1 BC₅F₃ and four initial K326 + *Phn15.1* BC₆F₃ NILs that were established by DNA marker-assisted backcrossing of the Beinhart 1000 *Phn15.1* genomic region into the genetic backgrounds of Hicks and K326. For eight of the nine genotyped NILs, at least 98.7% of the non-*Phn15.1*-associated SNP marker alleles were derived from either the Hicks or K326 recurrent parental lines (Table 2), indicating a very high degree of effectiveness of five or

Fig. 6. Genetic composition of Hicks + *Phn15.1* BC₇F₃ sub-nearly isogenic lines (subNILs) evaluated for field *Phytophthora nicotianae* resistance in two different North Carolina environments during 2016. Area under the disease progress curve (AUDPC) means averaged over both environments are located on the second left-hand column. Genotypes are ranked in descending order of resistance. The linkage map is shown at the top of the figure, along with map distances (centimorgans) and corresponding LogWorth values indicated in parentheses. LogWorth values were used to report the level of statistical significance of linear contrasts that were performed between genotypic classes (segregating for Beinhart 1000 allele versus fixed for Hicks allele at each single-nucleotide polymorphism marker locus). LSD = least significant difference.

six backcrosses for restoring the recurrent parent genome. No Beinhart 1000 *Phn7.1*-associated makers were detected in the newly created NILs. Based upon the new linkage map created for the Beinhart 1000 × Hicks mapping population, the estimated sizes of the introgressed Beinhart 1000 *Phn15.1* chromosome segment in Hicks + *Phn15.1* BC₅F₃ NILs 375-8 and 379-7 (each tested for 3 years) were 57.3 and 52.7 cM, respectively. The estimated sizes of the introgressed Beinhart 1000 *Phn15.1* chromosome segment in K326 + *Phn15.1* BC₅F₃ NILs 385-1 and 386-6 (each also tested for 3 years) were 68.3 and 71.2 cM, respectively.

Generation and testing of Hicks + *Phn7.1* subNILs. Hicks + *Phn15.1* subNILs carrying varying amounts of the initial introgressed Beinhart 1000 *Phn15.1*-associated chromosome segment within the genetic backgrounds of Hicks and K326 were developed by identifying recombination events within the region of interest. In total, 25 Hicks + *Phn15.1* BC₇F₂ and 21 K326 + *Phn15.1* BC₈F₂ families were evaluated for field *P. nicotianae* resistance in separate experiments in two environments during the 2016 growing season. The Hicks-derived materials exhibited greater overall loss due to disease as compared with the K326-derived materials but results of significance tests between genotypic classes were very similar. Linear contrasts for each genotypic class (homozygous for recurrent parent allele versus other) identified SNP markers *NtCPS2*, *NtIAD4594*, *NtIAA5923*, and *NtIAD1096* within a 5.2-cM window to be most highly associated with AUDPC (Figs. 3 and 4). Families with Beinhart 1000 alleles at these loci exhibited, on average, significantly lower levels of disease development.

During 2017, 19 K326 + *Phn15.1* BC₈F₃ subNILs were evaluated for resistance to *P. nicotianae*. Disease incidence during 2017 was lower than that for 2016 but results were again similar for both sets of materials. K326 subNILs carrying Beinhart 1000 alleles at SNP marker loci *NtCPS2*, *NtIAD4594*, *NtIAA5923*, and *NtIAD1096* exhibited the most significantly reduced AUDPC (Fig. 5). On the other side of the studied introgressed region (represented by markers *NtIAA7078*, *NtIAB3612*, *NtIAF5707*, and *NtIAC9276*), subNILs with Beinhart 1000 alleles exhibited significantly greater mean values for AUDPC (Fig. 5).

During 2017, 26 Hicks + *Phn15.1* BC₇F₃ subNILs were evaluated for resistance. Again, subNILs carrying Beinhart 1000 alleles at SNP marker loci *NtIAA5923* and *NtIAD1096* (spanning a 2.7-cM region) exhibited the most significant reduction in AUDPC (Fig. 6). Also, again, markers on the other side of the introgressed genomic region were also very significantly associated with AUDPC but Beinhart 1000 alleles were associated with greater levels of disease incidence. Importantly, Hicks + *Phn15.1* BC₇F₃ subNILs resulting from a double-crossover event leading to the replacement of the Beinhart 1000 allele at *NtCPS2*, on average, exhibited a significantly lower mean AUDPC value as compared with Hicks per se (Fig. 6), indicating that it is possible to separate the favorable Beinhart 1000 alleles associated with *Phn15.1* from the wild-type *NtCPS2* Beinhart 1000 allele encoding for a functional *NtCPS2* enzyme.

Discussion

Partial disease resistance in plants is generally considered to be non-race-specific and more durable over time. Such genetic systems consequently play important roles in overall strategies to reduce economic loss from plant pathogens. However, partial resistance is also generally under polygenic control and, therefore, it is often necessary to combine favorable alleles at multiple loci with varying effects in order to produce breeding lines with high levels of resistance. Thus, increasing the maximum level of possible genetic resistance in breeding programs is dependent upon the introduction of novel resistance alleles. DNA markers can aid in the identification and transfer of such alleles from exotic source materials into elite germplasm.

Phn15.1 was previously reported to be a large-effect *P. nicotianae* resistance QTL in tobacco (Vontimitta and Lewis 2012), and a favorable allele at this QTL was identified in cigar tobacco cultivar Beinhart 1000 (Vontimitta and Lewis 2012) that is not believed to be present in current flue-cured or burley tobacco germplasm pools (Drake-Stowe et al. 2017; Xiao et al. 2013). Thus, this allele could be of great value for increasing the range and level of resistance to

P. nicotianae in these market classes. In research described here, we used marker-assisted selection to transfer the Beinhart 1000 genomic region carrying *Phn15.1* to black-shank-susceptible old-line, flue-cured tobacco cultivar Hicks and to moderately resistant flue-cured tobacco cultivar K326. Nearly isogenic versions of these cultivars were significantly more resistant than the parental cultivars in multienvironment testing, thus strongly validating the previously reported effect for this QTL. Indeed, K326 NILs were nearly as resistant as K346, an industry standard for high partial soilborne pathogen resistance in flue-cured tobacco. Field evaluation of F₁ hybrids heterozygous for the introgressed region suggested that the Beinhart 1000 *Phn15.1* allele acts in an additive or partially dominant manner to positively affect *P. nicotianae* resistance. Therefore, to maximize resistance, both parents of new experimental F₁ hybrid cultivars would have to carry this allele, although even heterozygous F₁ hybrids could be significantly improved for resistance.

Deleterious linkage drag effects that can negatively affect yield or quality are often observed when chromosome regions are introgressed from exotic source material into elite germplasm. Indeed, the Beinhart 1000 *Phn15.1* allele is closely linked to an allele of *NtCPS2* encoding for a functional enzyme that contributes to the accumulation of the diterpene Z-abienol on the tobacco leaf surface. This trichome exudate is considered an undesirable flavor and aroma component for flue-cured and burley tobacco. Because Z-abienol and related diterpenes have been reported to have activity against oomycetes (Steede et al. 2017), it was reasonable to speculate that Z-abienol itself might actually contribute to *P. nicotianae* resistance. We failed to observe a change in field *P. nicotianae* resistance in transgenic versions of Beinhart 1000 in which genes involved in the Z-abienol biosynthetic pathway were silenced, however (Steede et al. 2017). Therefore, for successful use of *Phn15.1*-mediated black shank resistance in flue-cured and burley tobacco, it is necessary to disassociate the Beinhart 1000 alleles contributing to partial *P. nicotianae* resistance from the Beinhart 1000 *NtCPS2* allele. We consequently desired to more finely resolve the genomic positions of the gene or genes underlying *Phn15.1*-based partial resistance to *P. nicotianae*, particularly with respect to *NtCPS2*. Development and testing of subNILs with varying amounts of introgressed DNA from Beinhart 1000 permitted the identification of an approximately 2.7-cM region where the responsible gene or genes reside. The identified closely linked DNA markers should allow tobacco researchers to utilize this allelic variability to improve black shank resistance in breeding programs.

Literature Cited

- Apple, J. L. 1962. Transfer of resistance to black shank (*Phytophthora parasitica* var *nicotianae*) from *Nicotiana glauca* to *N. tabacum*. (Abstr.) Phytopathology 52:1.
- Broman, K. W., and Sen, S. 2009. A Guide to QTL Mapping with R/qtl in Statistics for Biology and Health. Springer, New York, NY, U.S.A.
- Brouwer, D. J., and St. Clair, D. A. 2004. Fine mapping of three quantitative trait loci for late blight resistance in tomato using near isogenic lines (NILs) and sub-NILs. Theor. Appl. Genet. 108:628-638.
- Chaplin, J. F. 1962. Transfer of black shank resistance from *Nicotiana glauca* to flue-cured *N. tabacum*. Tob. Sci. 6:184-189.
- Drake, K. E., and Lewis, R. S. 2013. An introgressed *Nicotiana rustica* genomic region confers resistance to *Phytophthora nicotianae* in cultivated tobacco. Crop Sci. 53:1366-1374.
- Drake, K. E., Moore, J. M., Bertrand, P., Fortnum, B., Peterson, P., and Lewis, R. S. 2015. Black shank resistance and agronomic performance of flue-cured tobacco lines and hybrids carrying the introgressed *Nicotiana rustica* region. Wz. Crop Sci. 55:1-8.
- Drake-Stowe, K., Bakaher, N., Goepfert, S., Philippon, B., Mark, R., Peterson, P., and Lewis, R. S. 2017. Multiple disease resistance loci affect soilborne disease resistance in tobacco (*Nicotiana tabacum*). Phytopathology 107:1055-1061.
- Edwards, K., Fernandez-Pozo, N., Drake-Stowe, K., Humphry, M., Evans, A. D., Bombarely, A., Allen, F., Hurst, R., White, B., Kernodle, S. P., Bromley, J. R., Sanchez-Tamburino, J. P., Lewis, R. S., and Mueller, L. A. 2017. A reference genome for *Nicotiana tabacum* enables map-based cloning of homeologous loci implicated in nitrogen utilization efficiency. BMC Genomics 18:448.
- Kenward, M. G., and Roger, J. H. 1997. Small sample inference for fixed effects from restricted maximum likelihood. Biometrics 53:983-997.
- Ma, J. M., Heim, C., Humphry, M., Nifong, J. M., and Lewis, R. S. 2019. Genetic analysis of *Phn7.1*, a major QTL conferring partial resistance to *Phytophthora nicotianae* in *Nicotiana tabacum*. Mol. Breed. 39:11.

- Madden, L. V., Hughes, G., and van der Bosch, F. 2007. The Study of Plant Disease Epidemics. American Phytopathological Society, St. Paul, MN, U.S.A.
- Sallaud, C., Giacalone, C., Töpfer, R., Goepfert, S., Bakaher, N., Rösti, S., and Tissier, A. 2012. Characterization of two genes for the biosynthesis of the labdane diterpene Z-abienol in tobacco (*Nicotiana tabacum*) glandular trichomes. *Plant J.* 72:1-7.
- Schaalje, G. B., McBride, J. B., and Fellingham, G. W. 2001. Approximations to distributions of test statistics in complex mixed linear models using SAS Proc MIXED. Pap. 26.262 in: SAS Users Group Int. (SUGI) 26 Proc.
- Sierro, N., Battey, J. N. D., Ouadi, S., Bakaher, N., Bovet, L., Willig, A., Goepfert, S., Peitsch, M. C., and Ivanov, N. V. 2014. The tobacco genome sequence and its comparison with those of tomato and potato. *Nat. Commun.* 5:3833.
- Sierro, N., van Oeveren, J., van Eijk, M. J. T., Martin, F., Stormo, K. E., Peitsch, M. C., and Ivanov, N. V. 2013. Whole genome profiling physical map and ancestral annotation of tobacco Hicks Broadleaf. *Plant J.* 75:880-889.
- Steede, W. T., Ma, J. M., Eickholt, D. P., Drake-Stowe, K. E., Kernodle, S. P., Shew, H. D., Daneshmandi, D. A., and Lewis, R. S. 2017. The tobacco trichome exudate Z-abienol and its relationship with plant resistance to *Phytophthora nicotianae*. *Plant Dis.* 101:1214-1221.
- Valleau, W. E., Stokes, G. W., and Johnson, E. M. 1960. Nine years experience with the *Nicotiana longiflora* factor for resistance to *Phytophthora parasitica* var. *nicotianae* in control of black shank. *Tob. Sci.* 4:92-94.
- Van Ooijen, J. 2006. JoinMap 4, Software for the Calculation of Genetic Linkage Maps in Experimental Populations. Kyazma B.V., Wageningen, The Netherlands.
- Vontimitta, V., Daneshmandi, D. A., Steede, T., and Lewis, R. S. 2010. Analysis of a *Nicotiana tabacum* genomic region controlling two leaf surface chemistry traits. *J. Agric. Food Chem.* 58:294-300.
- Vontimitta, V., and Lewis, R. S. 2012. Mapping of quantitative trait loci affecting resistance to *Phytophthora nicotianae* in tobacco (*Nicotiana tabacum* L.) line Beinhart-1000. *Mol. Breed.* 29:89-98.
- Wang, Y., Tiwari, V. K., Rawat, N., Gill, B. S., Huo, N., You, F. M., Coleman-Derr, D., and Gu, Y. Q. 2016. GSP: A web-based platform for designing genome-specific primers in polyploids. *Bioinformatics* 32:2382-2383.
- Xiao, B., Drake, K., Vontimitta, V., Tong, Z., Zhang, X., Li, M., Leng, X., Li, Y., and Lewis, R. S. 2013. Location of genomic regions contributing to *Phytophthora nicotianae* resistance in tobacco cultivar Florida 301. *Crop Sci.* 53:473-481.