RESEARCH

Location of Genomic Regions Contributing to *Phytophthora nicotianae* Resistance in Tobacco Cultivar Florida 301

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ABSTRACT

Black shank, caused by *Phytophthora nicotianae*, is typically the most important disease affecting tobacco (*Nicotiana tabacum* L.) production in the United States. Pedigree information suggests that most black shank resistance was derived from the cigar tobacco cultivar Florida 301. This resistance is thought to be polygenic in nature. The objectives of the current experiment were to (i) evaluate lines from a recombinant inbred line population derived from a cross between Florida 301 and the black shank-susceptible cultivar Hicks for partial resistance using replicated field and greenhouse testing, (ii) genotype the population and use quantitative trait loci (QTL) analyses to identify Florida 301 genomic regions associated with resistance, and (iii) compare results with those obtained from a previous QTL analysis of a population derived from a cross involving 'Beinhart 1000'. A total of 11 QTL affecting area under the disease progress curve were identified in both the field and greenhouse experiments. The QTL with the largest effect explained 16.9 and 18.6% of the phenotypic variation in the field and greenhouse experiments, respectively. This QTL was also found to have the largest effect on resistance in a Beinhart 1000 × Hicks doubled haploid mapping population. A major QTL found to affect resistance on linkage group 15 in the latter population, however, was not found to be important in the current population. Quantitative trait loci identification using greenhouse data was comparable to, if not superior to, use of field data.

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Abbreviations: AUDPC, area under the disease progress curve; BIC, Bayesian information criterion; h^2, narrow-sense heritability; LG, linkage group; LOD, logarithm of odds; PCR, polymerase chain reaction; PMI, Philip Morris International; QTL, quantitative trait loci/locus; RIL, recombinant inbred line.

The black shank disease of tobacco is caused by the soilborne oomycete *Phytophthora nicotianae* van Breda de Haan and is one of the most important diseases of cultivated tobacco worldwide. Systems to reduce economic loss from this disease incorporate the use of crop rotation, chemical control, and resistant cultivars. Genetic resistance is an economically attractive component of the overall strategy if it can be incorporated into genotypes that also contribute to high yields of cured leaf with acceptable quality.

The black shank pathogen was first reported in Indonesia (van Breda de Haan, 1896) and, since then, black shank has been observed in almost all tobacco growing countries (Erwin and Ribeiro, 1996). The disease was first reported in the United States in southern Georgia in 1915 and began to be recognized as an economically important problem in the 1930s. Tisdale (1931) described the development of black shank-resistant cigar tobacco cultivar Florida 301 from an initial cross between selections from 'Big Cuba' and 'Little Cuba'. Black shank resistance in Florida 301

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is partial in nature (the cultivar does not exhibit immunity) and is believed to be non-race specific.

Based on pedigree information, most U.S. flue-cured tobacco cultivars are believed to have derived the majority of their partial black shank resistance alleles from Florida 301 via early flue-cured tobacco varieties such as 'Oxford 1', 'Oxford 2', 'Oxford 1-181', 'Dixie Bright 101', and 'Dixie Bright 102' that were commercialized in the 1940s and 1950s. Immunity to race 0 of *P. nicotianae* can be provided by the *Php* and *Phl* genes introgressed into *N. tabacum* from *Nicotiana plumbaginifolia* Viv. and *Nicotiana longiflora* Cav., respectively (Valleau et al., 1960; Apple, 1962; Chaplin, 1962; Johnson et al., 2002). These genes provide no resistance to race 1, however. Recent wide-scale planting of cultivars possessing the *Php* gene has resulted in pathogen race shifts in many growing areas, and alternative races that are able to overcome this type of resistance now prevail (Sullivan et al., 2005). There is therefore now increased market demand for flue-cured and burley tobacco cultivars carrying greater levels of resistance to alternative races of *P. nicotianae*.

Studies to investigate the inheritance of the high level of partial resistance in Florida 301 have led to variable conclusions. Clayton (1958) suggested that resistance was simply inherited and controlled by recessive alleles. Moore and Powell (1959) reported resistance to be partially dominant and affected by modifying factors. Others have suggested Florida 301 resistance to be polygenic and additive in nature (Smith and Clayton, 1948; Crews et al., 1964; Chaplin, 1966). Incomplete penetrance and variable expressivity has probably complicated the interpretation of data generated to investigate the inheritance of resistance in this line.

Use of molecular markers might provide an increased understanding of the genetic control of black shank resistance in Florida 301 and could also result in development of selection methodologies that could increase the efficiency of developing new cultivars with high levels of black shank resistance. Using a limited number of microsatellite markers that were available at the time, Vontimitta and Lewis (2012a, 2012b) conducted research to identify quantitative trait loci (QTL) associated with a high level of black shank resistance in the cigar tobacco line Beinhart 1000. Genes affecting resistance in Beinhart 1000 and associated DNA markers may be of value to cultivar development efforts. A much greater number of microsatellite markers are now publicly available (Bindler et al., 2011; Tong et al., 2012), which makes characterization of black shank resistance in Florida 301 at the QTL level a reasonable and worthwhile objective.

The objectives of this research were to use a Florida 301 × Hicks recombinant inbred line (RIL) population to (i) gain increased understanding of the genetic control of black shank resistance in Florida 301, (ii) identify microsatellite markers associated with Florida 301 genomic regions contributing to this resistance, and (iii) compare genomic regions contributing to resistance in Florida 301 to those previously found to be associated with resistance in Beinhart 1000.

MATERIALS AND METHODS

Genetic Materials and Disease Evaluations

A RIL population consisting of 122 $F_{5:6}$ lines was derived from a cross between Florida 301 and the black shank-susceptible flue-cured tobacco cultivar Hicks using single seed descent. The RIL population along with the parental lines were evaluated for black shank incidence in three soil-borne disease nurseries during 2008 (Lower Coastal Plain Research Station, Kinston, NC; Upper Coastal Plain Research Station, Rocky Mount, NC; and Oxford Tobacco Research Station, Oxford, NC). The experimental design at each location was a randomized complete block design with three replications. Experimental units consisted of single 7 m-long plots of 12 plants each. Interrow spacing was 120 cm and within-row plant spacing was 56 cm. Starting at approximately 40 d after transplanting, the number of plants killed by black shank was recorded throughout the growing season at intervals of approximately 15 d. At the end of the season (approximately 120 d after transplanting), the area under the disease progress curve (AUDPC) was calculated for each plot using the trapezoidal method described by Madden et al. (2007).

The RIL population and parental lines were also evaluated for black shank resistance in a greenhouse experiment conducted at the Yunnan Academy of Tobacco Agricultural Sciences in Yuxi, China. The experimental design was a randomized complete block design with three replications. Experimental units consisted of eight plants contained within an eight-compartment segment of a Q32 nursery tray (Alibaba.com International, http://www.alibaba.com/). Seeds were germinated on growing medium (Jinye Fuda Industrial and Trading Company). Approximately 40 d after emergence, seedlings were transplanted to individual cells of the trays mentioned previously. Plants were inoculated 7 d after transplanting by first wounding the base of the plant stems with a knife. Approximately 5 g of *P. nicotianae* race 0 infested rice (*Oryza sativa* L.) grains were then added to soil near the wounded stem of each plant and lightly covered with soil. Infested rice grains were prepared by culturing sterilized rice grains at 28 to 30°C for 10 to 14 d with a selected *P. nicotianae* isolate of Chinese origin. After inoculation of plants, the temperature was maintained at approximately 32°C. Soil moisture was uniformly maintained by subirrigation. The number of dead plants for each genotype was recorded at 6 and 15 d after inoculation and used to calculate the AUDPC.

Genotyping

The cetyltrimethyl ammonium bromide (CTAB) method of Maguire et al. (1994) with minor modifications was used to isolate DNA from each RIL. The parental lines were first screened for polymorphisms at more than 5700 microsatellite marker loci. The entire RIL population was then genotyped with the polymorphic markers. Polymerase chain reactions (PCRs) for microsatellite marker genotyping were performed in 20 μL

volumes containing 20 to 40 ng of template DNA (dependent on primer combination), 1x PCR buffer (10 mM Tris-HCl, pH = 8.4, 50 mM KCl, and 1.5 mM $MgCl_2$), 10 to 15 pmol forward primer, 10 to 15 pmol reverse primer, 200 μM of each deoxyribonucleotide triphosphate (TaKaRa Biotechnology Co. Ltd.), and 0.75 U rTaq DNA polymerase (TaKaRa Biotechnology). Primer pair sequences have been published previously (Bindler et al., 2011; Tong et al., 2012). Polymerase chain reaction conditions consisted of a denaturation step at 95°C for 5 min and 30 cycles of 95°C for 30 sec, 57 to 62°C (dependent on primer combination) for 30 sec, and 72°C for 30 sec followed by a final extension step at 72°C for 7 min. Polymerase chain reaction products were separated on 6% nondenaturing polyacrylamide gels (220 V for 3.5 h). Amplification products were visualized by silver staining according to Bassam et al. (1991) with minor modifications. Amounts loaded varied from 1.5 to 3.0 μL, depending on the primer combination. Bands were scored as codominant markers and heterozygous genotypes were treated as missing data for further analyses.

Statistical Analysis, Map Construction, and Quantitative Trait Loci Identification

An analysis of variance was performed on the field and greenhouse AUDPC data separately using PROC GLM of SAS 9.1 (SAS Institute, 2004), and least squares means were produced for the RILs and the parental lines. Least squares means for the RILs were used for QTL identification (see below). Heritability on a plot and entry mean basis were calculated using univariate mixed model analysis as described by Holland et al. (2003).

Linkage analysis was performed using the program JoinMap version 4.0 (Van Ooijen, 2006). The markers were roughly classified into 24 linkage groups using the grouping module and based on a logarithm of odds (LOD) score of 2.0 to 10.0. Marker order and genetic distances were calculated using the regression mapping algorithm with the parameters set as follows: Kosambi's mapping function (Kosambi, 1944), goodness-of-fit Jump threshold for removal loci = 5.0, number of added loci after which to perform a ripple = 1, recombination frequency ≤0.40, LOD score ≥1.0, and third round = Yes. Graphical representations of linkage groups and LOD score peaks were produced using the software MapChart (Voorrips, 2002).

Composite interval mapping (Zeng, 1994) and multiple interval mapping (Kao and Zeng, 1997; Kao et al., 1999) were performed on the field and greenhouse entry means separately using Windows QTL Cartographer version 2.5 (Wang et al., 2007). In composite interval mapping, log-likelihood values for statistically significant associations between genotype and AUDPC were generated by calculating LOD scores at 0.5 cM intervals along linkage groups. Logarithm of odds threshold significance levels were determined using 1000 permutations of the method of Churchill and Doerge (1994). Composite interval mapping was executed only to provide an initial model for further analysis using multiple interval mapping. Quantitative trait loci peaks from composite interval mapping with LOD threshold values greater than 3.2 and 3.4 for the field and greenhouse data, respectively, were used in initial models for multiple interval mapping. Models were created and tested in an iterative, stepwise fashion, searching for new QTL and testing the significance of the QTL after each search cycle. New models were accepted only when they had a decreasing effect on the value of the Bayesian information criterion (BIC) (Piepho and Gauch, 2001). After QTL were added according to the BIC, all possible interactions between QTL were tested for their significance and were retained in the model if they decreased the BIC. When developing models using multiple interval mapping, there is always a possibility of overfitting the model. To avoid overfitting, it was made sure that the proportion of the total phenotypic variation explained by the QTL did not exceed the trait entry mean heritability. The model with the minimum BIC was chosen, and the QTL effects were simultaneously estimated using the "summary" option of QTL Cartographer. Genetic variability explained by the identified QTL was calculated as the total phenotypic variation explained by QTL divided by the entry mean heritability of the trait. Confidence intervals for the identified QTL were calculated according to Lander and Botstein (1989) using 1.0 LOD score as the support interval.

RESULTS

Analyses of variance of field and greenhouse data sets indicated highly significant differences ($P < 0.0001$) for AUDPC among the RILs derived from the Florida 301 × Hicks cross. In both the field and greenhouse experiments, Florida 301 was at the extreme end of the distribution for low AUDPC while the AUDPC for Hicks was much higher (Fig. 1 and 2).

A total of 5729 microsatellite primer pairs were screened for their ability to reveal marker polymorphisms between Florida 301 and Hicks (Table 1). A low percentage of primer pairs (5.95%) were found to reveal polymorphisms between these parents. The vast majority of these primer pairs produced only single bands although a few (32 primer pairs) amplified two bands each. We attempted to place a total of 373 microsatellite markers on a linkage map by genotyping the entire population of 122 RILs using 341 primer pairs. The linkage map consisted of 339 markers distributed among 24 linkage groups (LGs) and covered a total length of 1176.54 cM (Supplemental Fig. S1). Thirty-four markers could not be assigned to any LG. The sizes of the LGs ranged from 22.21 to 120.59 cM, and the number of markers on each linkage group varied from 5 to 29. The distance between adjacent markers ranged from 0.07 to 29.71 cM with an average distance of 5.64 cM. To facilitate current and future discussion of QTL locations in *N. tabacum*, we have assigned LG numbers to correspond with those previously presented in the high-density microsatellite marker map generated by Philip Morris International (PMI) and published by Bindler et al. (2011). Assignment was based on comparison of groupings of the "PT" microsatellite markers in the current research with the PMI LGs outlined by Bindler et al. (2011). Groupings of "PT" markers in the current research were largely congruent with those of the high-density PMI map. There were some exceptions, however, that are indicated in Supplemental Fig. S1. Noncongruency between

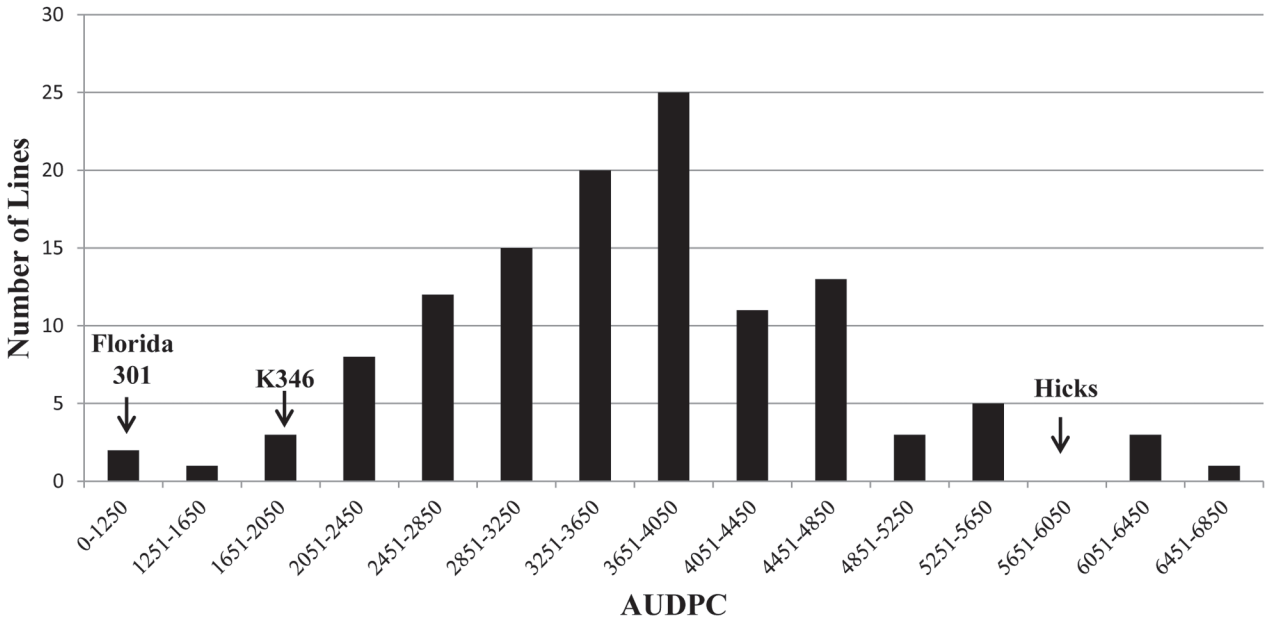

Figure 1. Distribution of field black shank area under the disease progress curve (AUDPC) for recombinant inbred lines derived from the Florida 301 × Hicks cross.

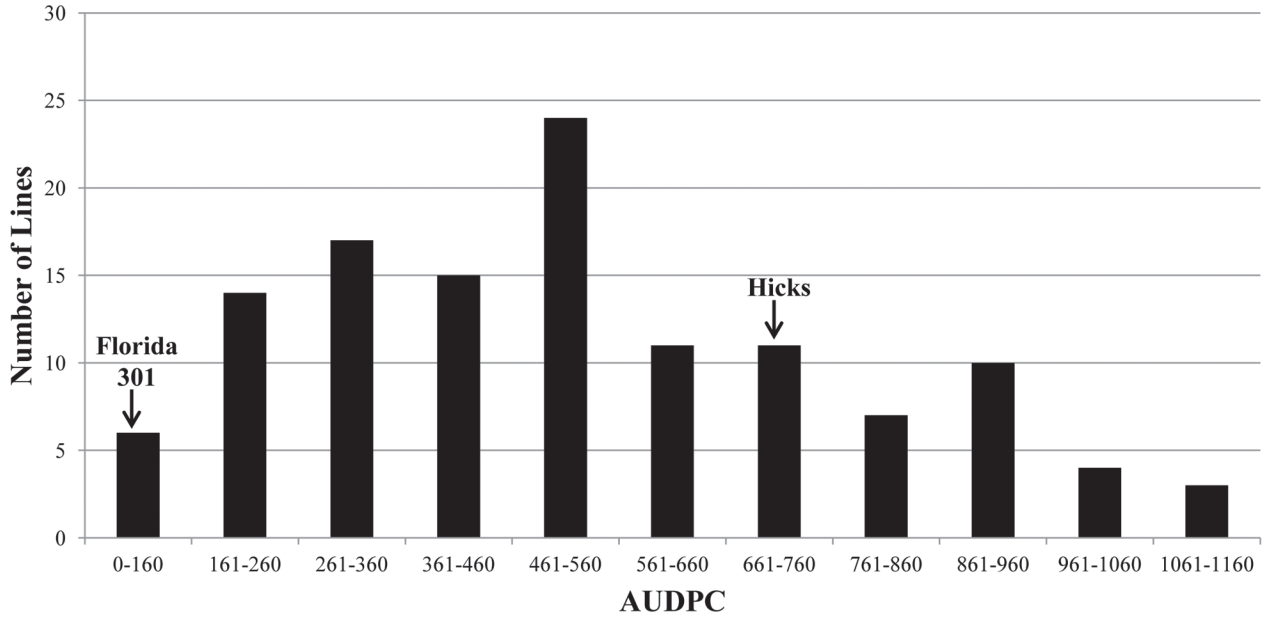

Figure 2. Distribution of greenhouse black shank area under the disease progress curve (AUDPC) for recombinant inbred lines derived from the Florida 301 × Hicks cross.

Table 1. Microsatellite marker polymorphisms between Florida 301 and Hicks.

Source	Marker prefix	Number of primer pairs screened	Number of primer pairs producing polymorphisms	Percent polymorphic	Number of markers genotyped on population
Derived from Tobacco Genome Initiative	TM	2953	226	7.65	234
Derived from NCBI and TIGR†	TME	1576	39	2.47	40
Bindler et al. (2011)	PT	1218	94	7.83	99
Total		5729	341	5.95	373

†NCBI, National Center for Biotechnology Information; TIGR, The Institute for Genomic Research.

Table 2. Summary of multiple interval mapping results for field and greenhouse evaluation of the Florida × Hicks recombinant inbred line population for resistance to *Phytophthora nicotianae*.

QTL†	Linkage group‡	Left marker	Right marker	Position (cM)	Effect (AUDPC)§		LOD¶ value		Percent phenotypic variance explained	
					Field	Greenhouse	Field	Greenhouse	Field	Greenhouse
1	7	PT30174	PT30165	62.02	–45.09	–49.69	8.79	11.7	16.9	18.6
2	24	TME0466	PT60308	9.53	–32.09	–44.93	3.52	7.04	9.2	15.3
3	14	TM10552	TM10983	4.94	–28.05	–47.39	3.17	8.17	6	10.7
4	3	TM10173	TM10175	23.44	–	–28.71	–	4.06	–	5.6
5	6	TM10470	TM10922	67.38	–28.24	–21.03	4.19	2.60	7.1	5.3
6	4a	TM10821	TM10914	83.75	–24.96	–35.95	2.79	5.02	4.2	6.9
7	11a	PT30137	TM11157	10.56	41.27	31.84	5.96	4.90	6.6	1.2
8	19	TM11026	TME0065	1.01	21.20	31.84	2.14	4.77	3.4	6.3
9	24	PT51264	PT61153	37.29	–26.29	–	2.60	–	6.1	–
10	22	TM10368	TM11161	33.62	–22.69	–27.36	2.49	3.15	3.7	5.2
11	9/18	TM10606	PT30110	13.86	–	30.96	–	3.46	–	4.4
12	20	TME0324a	TM10386	15.11	–25.30	–	3.21	–	4.3	–
13	8/18	TM10584	TME0137	26.94	–26.29	–	3.92	–	4.2	–
14	8/18	TM10358	PT30351	78.25	–	–20.93	–	2.88	–	1.7
1 × 10					–7.71	–	0.33	–	0.4	–
1 × 12					–12.46	–	0.77	–	–0.9	–
1 × 15					–	–27.13	–	4.10	–	5.0
2 × 10					–27.94	–	3.59	–	4.6	–
4 × 6					–	14.00	–	0.95	–	0.5
7 × 8					–29.93	–	3.29	–	5.0	–
8 × 15					–	–25.33	–	3.13	–	1.3
12 × 13					23.59	–	2.69	–	2.0	–

†QTL, quantitative trait loci.

‡Linkage groups correspond to those published by Bindler et al. (2011).

§AUDPC, area under the disease progress curve. Negative effect indicates that the favorable allele affecting AUDPC was derived from Florida 301. A positive effect indicates that the favorable allele was derived from Hicks.

¶LOD, logarithm of odds.

the two linkage maps could be due to differences in chromosome structure in the genomes of different tobacco types, amplification of alternative PCR products under different amplification conditions, differences in areas with suppressed or enhanced recombination, or sampling or genotyping errors.

Using composite interval mapping, three genomic regions on PMI LGs 7, 14, and 24 were found to be significantly associated with the AUDPC (LOD scores > 3.2) in the field study, and a single genomic region on LG 7 was found to be significantly associated with the AUDPC in the greenhouse experiment (LOD score > 3.4). These QTL were included in the initial models for multiple interval mapping, and eight additional QTL were added using the BIC for the field data and 10 additional QTL were added for the greenhouse experiment. For the field AUDPC data, the final multiple interval mapping model included 11 QTL located on LGs 4a, 6, 7, 8/18, 11a, 14, 19, 20, 22, and 24 along with five epistatic interactions (Table 2; Fig. 3). The final model for the greenhouse experiment contained 11 QTL main effects and three epistatic interactions (Table 2; Fig. 3). The favorable alleles at nine of the 11 QTL positions identified using the field data were from the Florida 301 parent while the favorable alleles at eight of the 11 QTL positions for the greenhouse experiment were from the Florida 301 parent. Eight of the QTL identified using the field data were also identified using the greenhouse data. Logarithm of odds scores were generally higher for these QTL using the greenhouse data as compared to the field data. High LOD scores of 8.79 and 11.70 were observed for the QTL identified on LG 7 using the field and greenhouse data, respectively. This QTL explained 16.9 and 18.6% of the phenotypic variation in these data sets, respectively. Quantitative trait loci identified on LGs 14 and 24 also had relatively high LOD scores (greater than 7.0 for the greenhouse data) and each explained greater than 10% of the phenotypic variation in the greenhouse experiment. Most of the remaining QTL were minor, accounting for much lower percentages of the observed phenotypic variation (<7.0%). The final QTL model for field AUDPC explained 82.4 and 90.0% of the phenotypic and genotypic variation, respectively. For the greenhouse data, the final model explained 88.0 and 98.9% of the phenotypic and genotypic variation. Entry mean heritabilities (H) for the field and greenhouse experiments were $H = 0.92$ and $H = 0.89$, respectively.

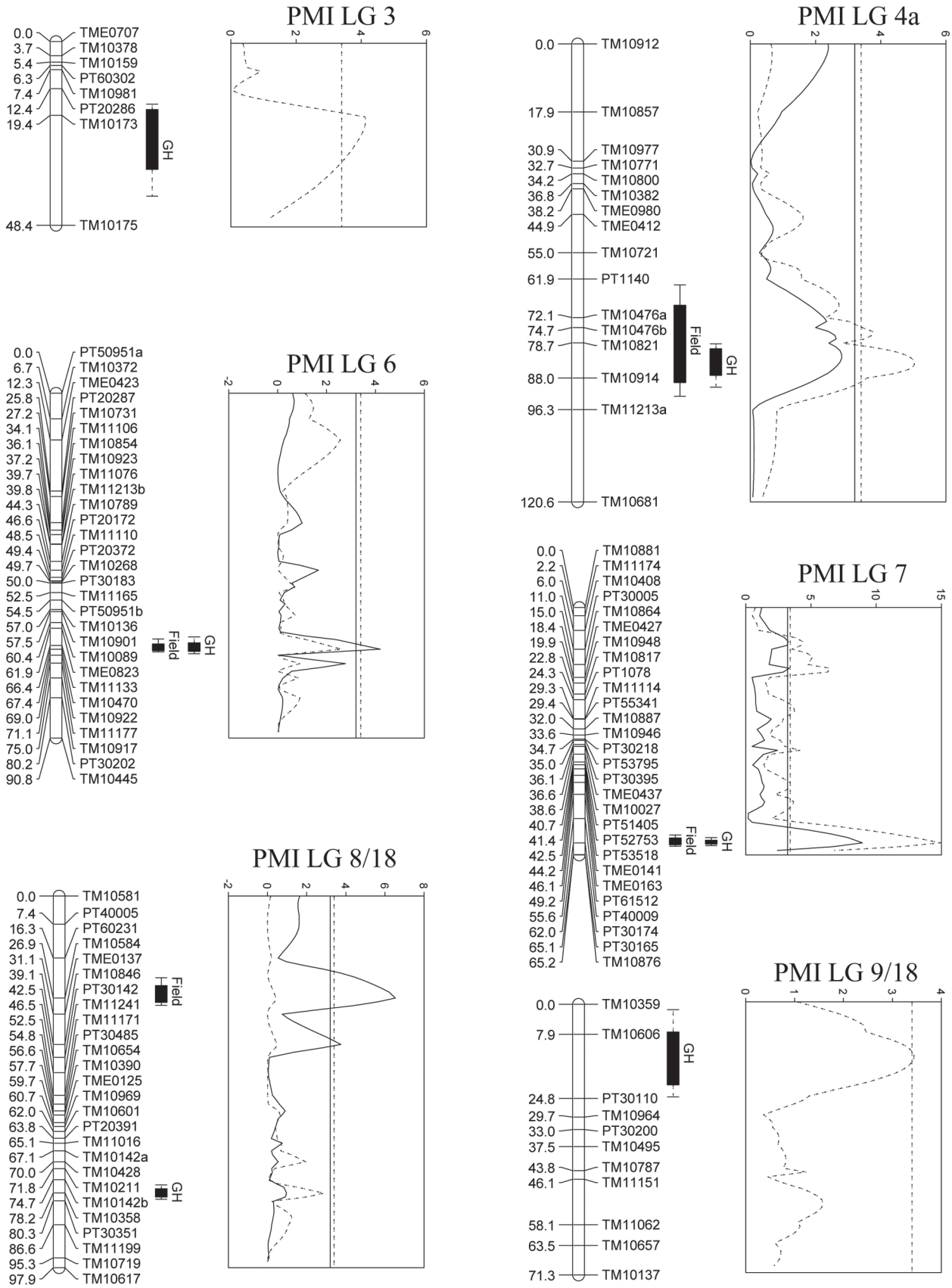

Figure 3. Logarithm of odds (LOD) score peaks for linkage groups found to be significantly associated with *Phytophthora nicotianae* resistance in soil-borne disease nurseries or through greenhouse inoculations. Solid and dashed lines relate to data for field and greenhouse (GH) area under the disease progress curve, respectively. Vertical bars represent 1 LOD and 2 LOD confidence intervals. PMI, Philip Morris International; LG, linkage group; GH, greenhouse.

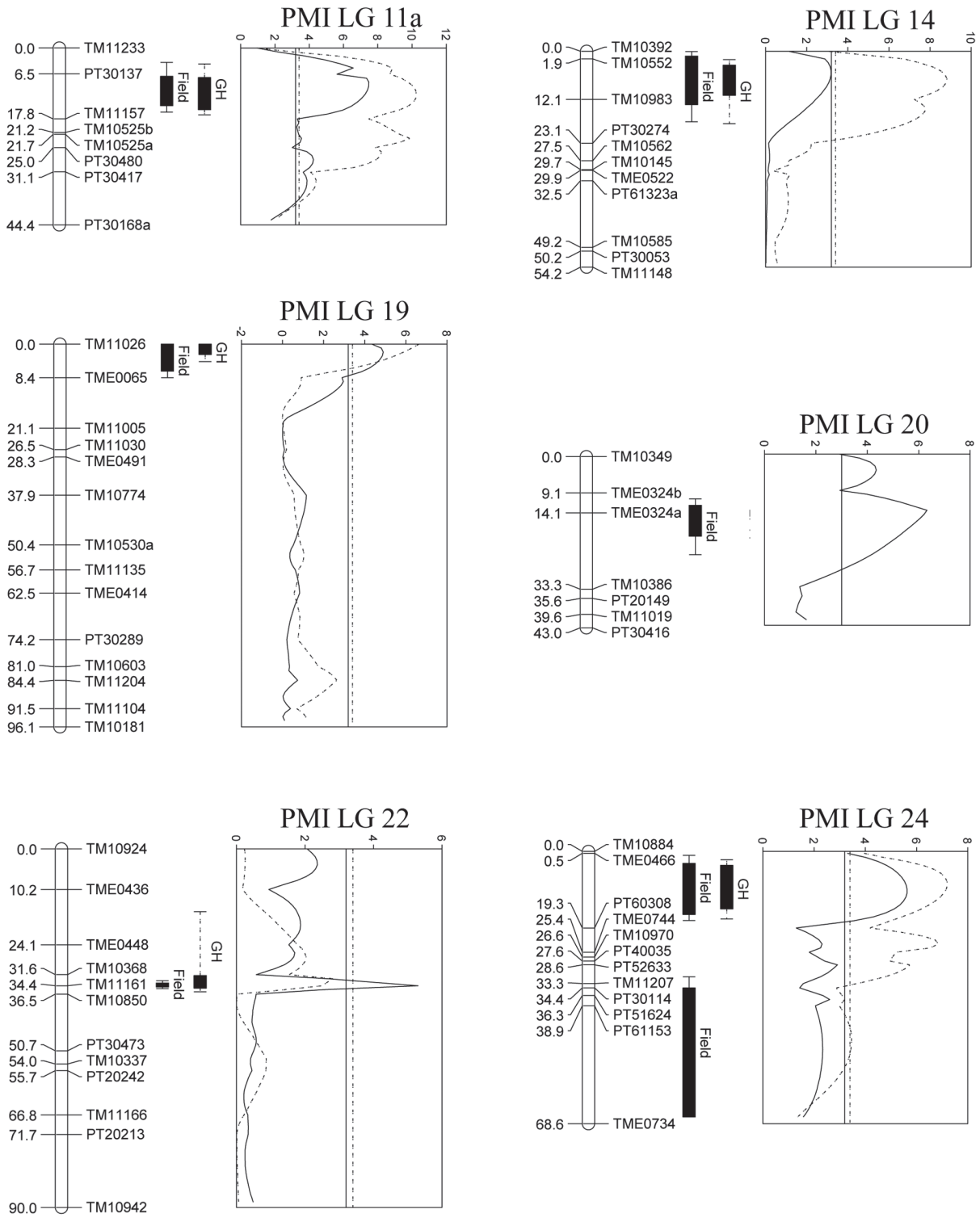

Figure 3. Continued.

DISCUSSION

Previous conclusions on the genetic control of the high level of partial resistance to *P. nicotianae* exhibited by Florida 301 have been inconsistent (Smith and Clayton, 1948; Clayton, 1958; Moore and Powell, 1959; Crews et al., 1964; Chaplin, 1966). Results from the current QTL study suggest that the Florida 301 type of resistance is of the classic polygenic type and is controlled by a combination of genetic factors with large, medium, and small effects. The favorable allele was contributed by Florida 301 at nine of the 11 QTL included in the final model generated to describe AUDPC in the field. For greenhouse AUDPC, the favorable allele was contributed by Florida 301 at eight of the 11 QTL included in the final model. It is possible that the black shank-susceptible cultivar Hicks may contribute black shank resistance QTL with small

effects. No Florida 301 × Hicks RIL exhibited a level of black shank resistance that was significantly greater than that of Florida 301, however.

Vontimitta and Lewis (2012a, 2012b) previously reported on QTL influencing black shank resistance in a doubled haploid mapping population derived from a cross involving Beinhart 1000, a cigar tobacco cultivar that consistently exhibits greater levels of resistance than does Florida 301. In our study of the Florida 301 × Hicks RIL population, the QTL identified to have the greatest effect and to control the largest percentage of the phenotypic variation in both the field and greenhouse experiments was located on LG 7. This QTL explained 16.9 and 18.6% of the observed variation in the field and greenhouse experiments, respectively. This QTL was also found to have the largest effect and to control the largest percentage of the variation (25.4%) in field evaluation of the Beinhart 1000 × Hicks doubled haploid population (Vontimitta and Lewis, 2012b). In each population, the corresponding confidence interval for this QTL was positioned over microsatellite marker locus PT30174. The identification of this particular QTL in a second population further validates the position and contribution of allelic variation at this locus to black shank resistance in *N. tabacum*.

The QTL on LG 4a that had an intermediate effect in the Florida 301 × Hicks RIL population may also have had a small effect in the Beinhart 1000 × Hicks doubled haploid population. This is stated with a limited degree of confidence, however, because only a single marker locus on this linkage group was shared between the two studies. The remaining QTL identified in the current study were not found to be significant in the Beinhart 1000 × Hicks doubled haploid population. The fact that a majority of the markers were not shared between the two studies makes a rigorous comparison difficult, however. The results of the current study and those of Vontimitta and Lewis (2012a, 2012b) suggest that Beinhart 1000 and Florida 301 share a major gene affecting black shank resistance but probably differ in allelic variability at a fair number of additional loci with smaller effects. The QTL on LG 15 that exhibited the second largest effect in the Beinhart 1000 × Hicks population (explaining 20.4% of the phenotypic variance for field resistance) was not identified in the study of the Florida 301 × Hicks RIL population despite a fairly high number of shared marker loci between the two populations in this genomic region. The favorable allele affecting black shank resistance at this QTL in Beinhart 1000 is linked to two genes influencing the accumulation of *cis*-abienol and sucrose esters on the tobacco leaf surface (Vontimitta et al., 2010). Florida 301 does not accumulate this chemistry to appreciable levels on its leaf surface (Germplasm Resources Information Network, 2012). Beinhart 1000 consistently exhibits greater levels of field resistance to black shank than does Florida 301. It may be possible that the QTL on LG 15 explains a large portion of the difference in resistance between the two lines. If so, it is highly unlikely that this genetic variation currently exists in U.S. flue-cured or burley tobacco germplasm. Markers associated with this region could be useful for precisely transferring this variation to desired genetic backgrounds.

In the present study, data from both field and greenhouse evaluations of the RIL population were subjected to QTL analyses. Analysis of field and greenhouse data produced confidence intervals that overlapped for eight QTL. Similar results were observed in a comparison between QTL analyses using field and growth chamber data (Vontimitta and Lewis, 2012b). In the current study, LOD scores were higher for identified QTL using the greenhouse data. Confidence intervals tended to be of a similar size or were smaller for analyses using the greenhouse data as compared to analyses using the field data. These comparisons suggest that the same genetic factors acting to provide resistance in a field situation also affect resistance in artificial inoculation settings in greenhouses or growth chambers. Given this expectation and the assumption that better control of nongenetic variation would be achieved in greenhouse or growth chamber situations, it might be preferred to conduct future black shank QTL studies using artificial inoculations. Such experiments require less space and less time and can be performed any time of the year.

In conclusion, data from this investigation suggests that genetic control of black shank resistance in Florida 301 is of the classic polygenic type and that it is controlled by a combination of a few genes with large effects and a greater number of genes with small to intermediate effects. Microsatellite markers linked to QTL found to affect black shank resistance point to genomic regions that might be fine mapped with additional markers to more finely resolve regions that might be subjected to marker-assisted backcrossing or used in other forms of marker-assisted selection.

Supplemental Information Available

Supplemental material is included with this manuscript.

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