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ARTICLE

Analysis of a *Nicotiana tabacum* L. Genomic Region Controlling Two Leaf Surface Chemistry Traits

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cis-Abienol and sucrose esters are *Nicotiana tabacum* leaf surface components that likely influence plant resistance to pests. Their breakdown products also contribute to flavor and aroma characteristics of certain tobacco types. Mapping of genes involved in the biosynthesis of these compounds could permit development of molecular-based tools for generating tobacco types with novel cured leaf chemistry profiles. A doubled haploid mapping population segregating for major genes (*Abl* and *BMVSE*) affecting the ability to accumulate *cis*-abienol and sucrose esters was generated and genotyped with a large set of microsatellite markers. The two genes were found to reside on chromosome A of the *N. tabacum* genome with a distance of 8.2 cM (centimorgans) between them. Seventeen microsatellite markers were also placed on this linkage group, several of which exhibited complete cosegregation with *Abl* and *BMVSE*. Results should aid breeding efforts focused on modification of this aspect of tobacco cured leaf chemistry.

KEYWORDS: Tobacco; leaf surface chemistry; *cis*-abienol; sucrose esters; gene mapping; molecular markers

INTRODUCTION

The sucrose esters (6-*O*-acetyl-2,3,4-tri-*O*-acyl-α-D-glucopyranosyl-β-D-fructofuranosides) and the labdanoid diterpene *cis*-abienol (**Figure 1**) are among the natural products that can be found on the leaf surfaces of tobacco (*Nicotiana tabacum* L.). These compounds are exuded by trichomes and may play important roles in plant defense against insects, plant pathogens, and other microbes (*1*−*3*). Their breakdown products also contribute in a substantial way to flavor and aroma characteristics of tobacco products (*1*, *4*).

Agronomic factors, environmental conditions, and stage of plant development can influence levels of sucrose esters and *cis*-abienol on leaf surfaces. Plant genetics also plays a very important role, however, and genes controlling trichome morphology, activity, and density likely contribute to quantitative variation for these chemistries (*1*, *5*−*8*). In addition, allelic variation at loci involved in the direct biosynthesis of these exudates can affect their levels in qualitative and possibly quantitative manners (*7*, *9*).

cis-Abienol synthesis occurs in trichome gland cells and is controlled by a single dominant gene designated as *Abl* (*10*) that may encode for *cis*-abienol synthase (*3*). Tobacco sucrose esters are also produced in trichome glands (*11*), but their synthesis is more complex. The sucrose esters of *N. tabacum* consist of an array of isomers in which each isomer contains an acetyl group at the C-6 position of the glucosyl moiety and three C_3 to C_8 straight or branched aliphatic acids at the C-2, C-3, and C-4 positions (*3*). Numerous possible combinations can result and the sucrose esters for *N. tabacum* have been divided into six groups (group I through group VI), with each group differing in molecular weight by a single methylene unit (*1*). Groups III through VI all contain at least one 3-methylvaleryl group esterified at the 2-, 3-, or 4-position. Gwynn et al. (*7*) reported that a single gene designated as *BMVSE* controlled the biosynthesis of sucrose esters classified into these groups.

Substantial variability is present among cultivars of different market classes for their ability to accumulate sucrose esters and *cis*-abienol (*1*, *12*). In general, flue-cured, burley, and Maryland tobaccos do not produce *cis*-abienol. These tobacco types also produce low levels of groups I and II sucrose esters, and only trace levels of groups III through VI. High quantities of *cis*-abienol and sucrose ester groups III through VI are produced by most Oriental and many cigar tobacco cultivars, however. These molecules and their volatile breakdown products are believed to be the major contributors to the aromatic and flavor properties provided by cured leaf of these market classes (*4*).

Cultivar development programs for any tobacco type must take into consideration genes that affect cured leaf chemistry, including leaf surface exudates, so that characteristics of new cultivars meet present market requirements. In addition, opportunities may also exist for development of tobacco cultivars with novel leaf chemistry combinations that do not correspond to current market classes. There is also interest in using genetic modification to increase deposition of certain leaf surface phytochemicals for the purpose of enhancing natural pest resistance or for boosting possibilities of large-scale isolation of compounds of potential commercial value (ingredients of organic pesticides,

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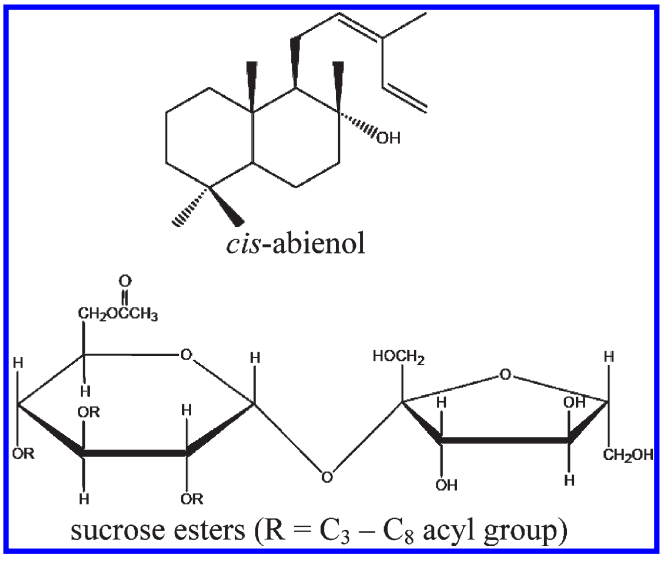

Figure 1. Chemical structures for tobacco leaf surface components *cis*-abienol and sucrose esters.

chemical intermediates for industrial and pharmaceutical synthesis, etc.).

Abl and *BMVSE* are both thought to have been contributed to the amphidiploid species *N. tabacum* by a diploid donor species closely related to modern-day *N. tomentosiformis* (*1*). Although both genes have been localized to chromosome A of the tobacco genome by monosomic analyses (*9*, *13*), information on the relative genomic positions of the *Abl* and *BMSVE* loci is not currently available. Such information would be important so as to know the expected frequency of coinheritance of the two genetic factors in segregating populations in cultivar development programs. In addition, the availability of closely linked DNA markers could facilitate selection for different combinations of alleles at these two loci during initial stages of plant growth in a comprehensive plant breeding program designed to develop new cultivars with desired leaf chemistry profiles in combination with important agronomic traits. DNA marker information might also contribute to future research to isolate genes involved in the biosynthesis of these two classes of secondary metabolites.

Microsatellite markers have been used to demonstrate sufficient polymorphism among tobacco varieties and have permitted development of the first linkage map for this species (*14*). The first objective of this research was to generate a doubled haploid mapping population derived from a cross between the cigar tobacco cultivar 'Beinhart-1000' (a *cis*-abienol and groups III through VI sucrose ester accumulator) and the flue-cured tobacco cultivar 'Hicks' (a variety that does not produce *cis*-abienol or groups III through VI sucrose esters). The second goal was to evaluate this population for accumulation of *cis*-abienol and sucrose esters on leaf surfaces, and determine the recombination frequency between the two genes (*Abl* and *BMVSE*) responsible for their synthesis. The final objective was to identify and map a series of microsatellite markers linked to these genes. Results could have practical utility for future cultivar development efforts, or for investigations aimed at understanding the synthesis of leaf surface compounds and their role in plant−pest interactions.

MATERIALS AND METHODS

Genetic Materials. Cigar tobacco cultivar Beinhart-1000 was hybridized with flue-cured tobacco cultivar Hicks. The former accumulates *cis*-abienol and sucrose esters, while the latter does not. An array of F_1-derived haploid plants was generated from this cross using the method of Burk et al. (*15*). A population of 117 doubled haploid lines was subsequently produced using the chromosome doubling procedure of Kasperbauer and Collins (*16*). Seed for field evaluation was produced by self-pollinating doubled haploid individuals.

Leaf Surface Chemistry Evaluations. The 117 doubled haploid lines and the two parental cultivars were grown in the field during 2008 at the Central Crops Research Station, Clayton, NC. The experimental design was a randomized complete block design with two replications. Plots consisted of five plants. Inter-row spacing and within-row spacing were 1.14 and 0.56 m, respectively.

A sharpened 1.6 cm stainless steel punch was used to remove two leaf disks from each of the five plants from each plot (10 total disks per plot) 62 days after transplanting. Leaf disks were collected from the uppermost leaves (10−15 cm in length) approximately 2 to 3 cm from the midrib. Disks from each plot were immediately transferred to 20 mL scintillation vials and stored on ice for transport to the laboratory.

Sample preparation was a modification of the procedure described by Severson et al. (*12*). In the laboratory, leaf disks were washed twice with 7.5 mL of HPLC grade CH_2Cl_2 (Fisher Scientific, Pittsburgh, PA) by vortexing each 7.5 mL aliquot for 30 s. The combined washes contain over 99% of the total leaf surface components (*17*). Approximately 0.5 g of anhydrous Na_2SO_4 was added to each scintillation vial, and vials were allowed to stand overnight. Extracts were then filtered through 10−20 μm porosity fritted glass funnels into 25 mL screw cap test tubes, and the vials were rinsed twice with 1 mL of CH_2Cl_2. 1.00 mL of an internal standard solution containing 50 μg/mL of heptadecane (C-17 alkane) and heptadecanol (C-17 alcohol) in toluene was added. The solvent was then evaporated by heating at 40 °C under an N_2 gas stream using a Pierce Reacti-therm heating module and Reacti-vap III manifold (Pierce Chemical Co., Rockford, IL). 100 μL of a 1:1 mixture of BSTFA:DMF (Pierce Chemical Co.) was added, the test tube was capped tightly under a head of N_2, and the sample was heated at 75 °C for 30 min. After cooling, 100 μL of a 1:1 BSA:pyridine solution was added to dissolve hydrocarbons. Samples were stored at −20 °C until analysis. Prior to analysis, samples were brought to room temperature and vortexed.

Gas chromatographic analysis was conducted using an Agilent HP 6890 GC-FID (Santa Clara, CA) and a 30 m DB-5 column (J & W Scientific, 0.32 mm i.d. and 0.25 μm film thickness). The carrier gas was Helium at a linear gas velocity of approximately 28 cm/s. The injector was set at 240 °C and the detector at 375 °C. The analysis consisted of a temperature program from 160 to 310 °C at 4 degrees per minute followed by a hold at 310 °C for 10 min.

The identity of the compounds of interest (groups III through VI sucrose esters, and *cis*-abienol) was verified by comparison of retention times to that of authentic standards and/or confirmed by GC−MS analysis using an HP 5890 GC equipped with an HP5972 series MSD detector. The GC−MS system was equipped with a 30 M DB-5MS column (0.25 mm i.d., 0.25 μm film thickness) and operated using the same temperature program used in the GC-FID analyses. Linear gas velocity for the GC−MS analyses was 38 cm/s, and the ionization voltage was 70 eV. GC−MS verification of the silylated sucrose esters and *cis*-abienol utilized diagnostic fragment ions previously reported in the literature (*18*) or from GC−MS analysis of standards. GC-FID quantification of compounds utilized an internal standard method. An authentic standard of *cis*-abienol was prepared in our laboratories via preparative low pressure liquid chromatography (*19*). Because the sucrose esters are a complex mixture for which no purified standard was available, quantification of sucrose esters was based upon an internal standard method calibration prepared using synthetic sucrose octa-acetate (Sigma-Aldrich, St. Louis, MO) as a surrogate standard. Data was collected and analyzed using Agilent ChemStation software (Agilent Technologies, Palo Alto, CA). Quantification of the sucrose esters was carried out using multiple peak summing to simplify reporting of the groups III through VI sucrose ester data. For lines accumulating either *cis*-abienol or groups III through VI sucrose esters, an analysis of variance appropriate for a randomized complete block design was conducted using PROC GLM of SAS (SAS Institute,Cary, NC) to determine if there were significant differences among the evaluated lines for accumulation of these compounds.

Linkage Mapping. DNA was isolated from single plants of each doubled haploid line and each parental line according to Johnson

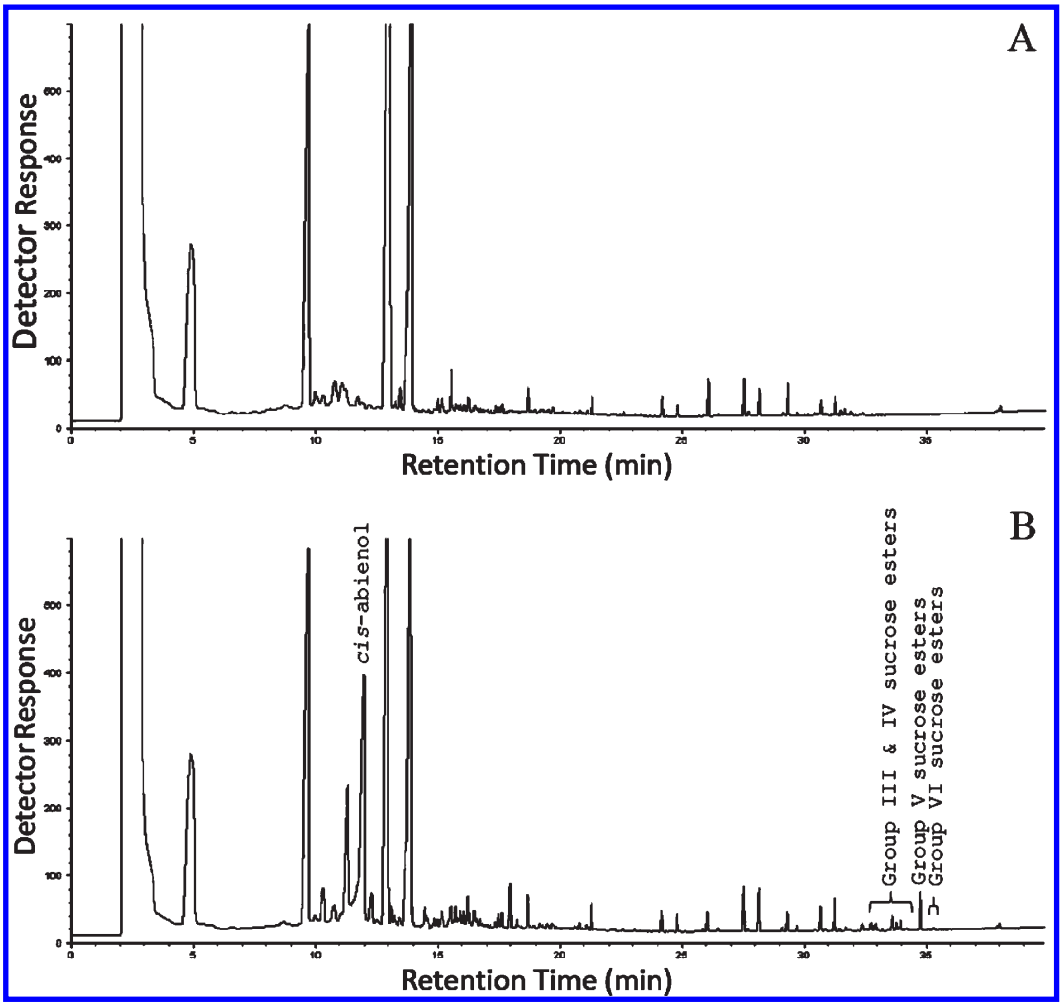

Figure 2. Gas chromatograms for leaf surface washes from (**A**) flue-cured tobacco cultivar, 'Hicks', and (**B**) cigar tobacco cultivar 'Beinhart-1000'.

et al. (*20*), except that a BIO 101 FastPrep machine (BIO 101, Inc., Vista, CA) was used for tissue grinding. A set of 433 microsatellite marker primer pairs were first screened for the ability to amplify polymorphic bands between Beinhart-1000 and Hicks. Some of these primer pairs were previously described by Bindler et al. (*14*), while others were previously unpublished and provided by Altria Client Services. Polymorphic markers were then genotyped on each of the 117 doubled haploid lines. PCR reactions for microsatellite detection were performed using the fluorescent M13-tail labeling procedure of Schuelke (*21*), which involved three primers: the microsatellite-specific forward primer with a M13(-19) tail at its 5′ end, the reverse microsatellite-specific primer, and a universal M13(-19) primer labeled with either IRD-700 or IRD-800 (LI-COR Biosciences, Lincoln, NE). Reactions were performed in 15 μL volumes containing 25 ng of DNA, 1× PCR buffer (20 mM Tris HCl pH 8.8, 2.0 mM $MgSO_4$, 10 mM KCl), 0.2 mM dNTPs, 0.015 μM forward primer, 0.06 μM reverse primer, 0.06 μM fluorescently labeled M13 primer, and 1 U Taq DNA polymerase (New England Biolabs, Ipswich, MA). Reaction conditions were conducted with an initial denaturation step of 94 °C for 5 min, followed by 30 cycles of 30 s at 94 °C, 45 s at 55 °C, 45 s at 72 °C, followed by 8 cycles of 30 s at 94 °C, 45 s at 53 °C, 45 s at 72 °C and a final extension at 72 °C for 10 min. Reaction products were mixed with 10 μL of formamide loading dye (95% deionized formamide, 20 mM EDTA, and 0.8 mg/mL bromophenol blue). The mixture was denatured for 3 min at 94 °C and then immediately chilled on ice for at least 10 min. Microsatellite alleles were resolved using 8.0% polyacrylamide gels and a LI-COR 4300 DNA Analysis System. IRDye 700- or 800-labeled molecular weight standards (50−350 bp) were loaded on each gel for SSR allele sizing. Scoring of gels was performed using AFLP Quantar 1.0 software (KeyGene Products B.V., Wageningen, The Netherlands).

Chi-square tests (*22*) were applied to each marker and the genes affecting *cis*-abienol and sucrose ester accumulation to test for segregation distortion. Estimation of recombination fractions and linkage map construction were performed using MAPMAKER/EXP 3.0 (*23*). Linkage was determined based on a minimum logarithm of odds (LOD) score of 3.0 and a linkage threshold of 40 cM using the Group command. Linkage order was established by first selecting a subset of eight markers based on LOD scores and pairwise linkages and then identifying the best order using the Compare command. Remaining markers were added using the Try command. The final marker order was checked using the Ripple command with the default log-likelihood threshold value of 2.0. Map distances (centimorgans, cM) were estimated from recombination fractions using the Haldane mapping function (*24*).

Microsatellite markers found to be linked to genes affecting *cis*-abienol and sucrose ester accumulation were also used to genotype six accessions of probable *N. tabacum* progenitor species *N. sylvestris* ('S' genome donor), and six accessions of *N. tomentosiformis* (possible 'T' genome donor) to generate data supporting the evolutionary origin of the chromosome segment carrying these genes.

RESULTS

Phenotypic Data. Leaf surface chemistry data was collected from a field experiment with two replications. Although quantitative variability was observed among entries and between replications, samples could easily be classified as producing or not producing *cis*-abienol or groups III through VI sucrose esters. For each line tested in the experiment, there was complete agreement between the two replications for this classification. For lines accumulating either of these compounds, simple *F*-tests revealed significant differences among them for levels of accumulation ($P < 0.0001$ for both compounds).

Neither *cis*-abienol nor groups III through VI sucrose esters were detected in the flue-cured tobacco parental line, Hicks (**Figure 2**). The mean production levels for *cis*-abienol and groups III through VI sucrose esters (quantities combined) for the parental line, Beinhart-1000, were 34.0 and 27.66 μg/cm^2, respectively. For the doubled haploid derivatives that synthesized these compounds, the mean accumulation levels were 28.4 and 28.8 μg/cm^2, respectively, with the ranges being 9.0−51.8 and 8.0−58.6 μg/cm^2 (**Figure 3**). Among doubled haploid lines accumulating both *cis*-abienol and groups III through VI sucrose esters, the correlations between the determinations was positive and significant ($P < 0.0001$).

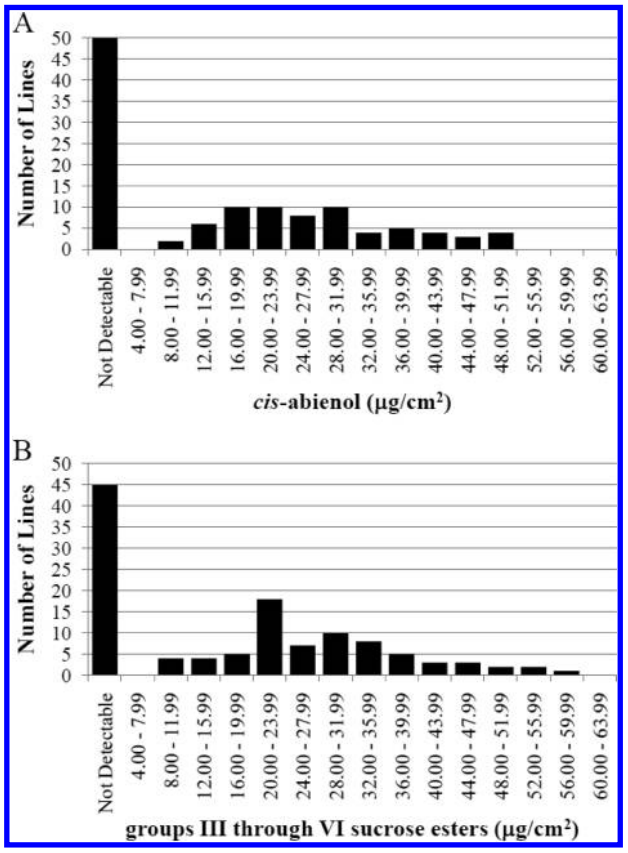

Figure 3. Frequency histograms for 117 doubled haploid lines derived from the cross Beinhart 1000 × Hicks for mean levels of (**A**) *cis*-abienol and (**B**) groups III through VI sucrose esters.

The doubled haploid lines could easily be categorized into one of four groups: (1) produced both *cis*-abienol and sucrose ester groups III through VI, (2) produced *cis*-abienol but not sucrose ester groups III through VI, (3) produced sucrose ester groups III through VI but not *cis*-abienol, and (4) did not produce *cis*-abienol or sucrose ester groups III through VI. The numbers of lines classified into these categories were 64, 4, 9, and 40, respectively. This segregation ratio clearly demonstrated linkage between the two genes responsible for synthesis of these two groups of molecules, *Abl* and *BMVSE*. The calculated recombination frequency resulting from genetic crossover events between the two genes was $r = 0.11$.

Mapping of Linked Microsatellite Markers. A total of 17 markers were found to be associated in a single linkage group that also carried *Abl* and *BMVSE* (**Table 1**, **Figure 4**). Nine of these markers exhibited segregation distortion ($P < 0.05$) (**Table 1**). *BMVSE* also exhibited significant segregation distortion ($P = 0.007$). Segregation for *Abl* was close to being significantly distorted ($P = 0.079$). For these two genes and all linked markers, we observed a greater fraction of Beinhart-1000 alleles in the doubled haploid population.

The genetic distance between *Abl* and *BMVSE* was estimated to be 8.5 cM. *BMVSE* exhibited complete cosegregation with microsatellite markers PT30209 and PT20315, and was flanked by markers PT30354 and PT52061 + PT61362 at distances of 3.9 cM and 1.3 cM, respectively. *Abl* exhibited complete cosegregation with microsatellite marker PT30124 and was flanked by markers PT55091 and PT61373 at distances of 2.2 cM and 0.6 cM, respectively.

The A chromosome of the tobacco genome and the ability to synthesize *cis*-abienol and sucrose esters was previously predicted to have been donated by a member of section *Tomentosae* (*1*, *25*), likely a species closely related to modern-day *N. tomentosiformis*. Multiple accessions of *N. sylvestris* and *N. tomentosiformis* were genotyped with each of the 17 microsatellite markers found to reside in the linkage group presented in **Figure 4**. Based on the presence/absence of bands in these species accessions, we were able to confirm these predictions (**Table 2**). Fifteen of

Table 1. Segregation Distortion Statistics for Genes Controlling *cis*-Abienol and Sucrose Ester Accumulation and for Microsatellite Markers Linked to These Genes

marker/gene	forward primer	reverse primer	$\chi^{2\,a}$	*P*-value
PT30272	5′-GAACCTAACCTCGCTCCACA-3′	5′-AAATGGTAGCTGCGAGGAGA-3′	0.08	0.7778
PT30224	5′-CTTGCAGCAACAATCTCAA-3′	5′-CACTTGGCTAGGCTAAATAAGCA-3′	0.21	0.6439
PT30223	5′-TCAAATGAAGATGTACAATGGAAA-3′	5′-TTCATATGGCCATCGAATAAC-3′	0.04	0.8501
PT30354	5′-TCGGTTTCTGCTCCAATTTC-3′	5′-TGTTCTACTGCTGGGTCGAA-3′	16.08	0.0001
PT30209	5′-CTTATGTTGAGACGGGCCAC-3′	5′-AGGTGTGACTGTTGGGCTTT-3′	6.23	0.0126
BMVSE			7.19	0.0073
PT20315	5′-ACACGACTTTTCATCTCCC-3′	5′-CGCATGAAATTGTAAGGG-3′	6.88	0.0087
PT52061	5′-AGTGTCATCACGACGCATGT-3′	5′-GCACCAACAGTTTAATTTGAGTTT-3′	6.76	0.0093
PT61362	5′-TTCACAATGTGCAAAGTCCC-3′	5′-CAGCTTTGTTGGCATTTGAG-3′	8.04	0.0046
PT51311	5′-ACGTCTCTGTTGAAACCTGC-3′	5′-GCCATCCCAAGAGAAACAAA-3′	7.63	0.0058
PT60146	5′-AAACCTGGAAAGCAACATCA-3′	5′-GACCACGTTGTGTAGTTGTATTCTT-3′	4.52	0.0335
PT55091	5′-CACATCAATGAGCAGGAGAGAG-3′	5′-AAAGTGAAGGTGGTTCCCAA-3′	4.68	0.0305
PT30124	5′-TCCTCCAACCAAACTCAAGC-3′	5′-TTTCTGTTCGCGTTTCAAAT-3′	2.51	0.1129
Abl			3.09	0.0790
PT61373	5′-GCGGGATAAACATGGGTAAA-3′	5′-ACCCAAATAACCGCTCACAT-3′	2.51	0.1129
PT51164	5′-CAAGGACCACTCCAATGCTT-3′	5′-TTCCAATGTTGTTTCTGTGTCTG-3′	1.07	0.3008
PT50962	5′-CTCGAATTCAATCGCGTCTT-3′	5′-CCATTATGTATTTGCGTTTGGA-3′	12.67	0.0004
PT20343	5′-GGAACACCACCACCATAA-3′	5′-GGAGCTCAGGTTCCAATG-3′	0.55	0.4576
PT30346	5′-ATCCAAATCGGATCCTCCAT-3′	5′-TCCCTGCTTTATTCACGTCC-3′	0.69	0.4054

[a] Chi-square value for test of 1:1 segregation.

the seventeen microsatellite primer pairs amplified bands in the six *N. tomentosiformis* accessions, but no band in the six *N. sylvestris* accessions. One primer pair amplified bands in three *N. tomentosiformis* accessions and no *N. sylvestris* accessions. Only one primer pair (PT30346) produced bands in all accessions of both species.

DISCUSSION

cis-Abienol and the sucrose esters are important natural products because of their possible involvement in plant defense against insects and pathogens, and also because of the contribution of their breakdown products to aromatic and flavor properties of cured tobacco leaves (*1*). The presence of these compounds and their breakdown products in Oriental tobacco is the reason for inclusion of cured leaf of this tobacco type in many tobacco products. The transition toward contract tobacco growing in many parts of the world may provide opportunities for development and commercial use of tobacco types with novel combinations of specific leaf chemistries and agronomic characteristics. Increased knowledge of the genetics underlying biosynthesis of important leaf chemistry compounds is thus important, as is the development of technologies that might contribute to selection of these genes in breeding programs.

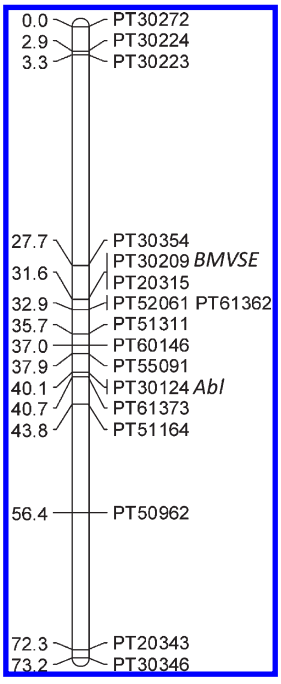

Figure 4. Linkage map containing 17 microsatellite markers and genes controlling *cis*-abienol (*Abl*) and sucrose ester (*BMVSE*) accumulation.

Genes responsible for the accumulation of *cis*-abienol and the 3-methylvaleric acid-containing sucrose esters were previously localized to chromosome A (*9*, *13*). In the current study, we determined that the two genes *Abl* and *BMVSE* were linked at a genetic distance of 8.5 cM (centimorgans). This genetic distance is intermediate in length, and extraordinarily large population sizes would therefore not be required to identify individuals possessing one or the other trait. We have also reported here the identification of several closely linked microsatellite markers that might be used in a comprehensive tobacco breeding program involving marker-assisted selection. Because of the very close proximity of these markers to *Abl* and *BMVSE*, one could identify individuals possessing the desired leaf chemistry trait(s) with a high degree of confidence as soon as plants develop to a size to permit DNA isolation. This would speed selection of novel lines and limit the need for chromatographic analysis of the plant materials. The markers could be used to introduce one or both of these genes into superior genetic backgrounds that contribute to higher yields and greater levels of disease resistance relative to currently available Oriental tobacco cultivars. The markers might also be valuable for selecting against this genomic region when using cigar tobacco varieties such as Beinhart-1000 as a source of other traits of interest, such as disease resistance, for flue-cured or burley tobacco improvement (*26*, *27*).

Previous researchers had suggested that the ability to synthesize *cis*-abienol and the sucrose esters was contributed to the amphidiploid species *N. tabacum* by the 'T' genome donor, likely a species closely related to modern-day *N. tomentosiformis* (*1*). Genotyping of probable progenitor species, *N. sylvestris* ('S' genome donor) and *N. tomentosiformis* ('T' genome donor), with the microsatellite markers found to be linked to *Abl* and *BMVSE* supports this hypothesis. By inference, we can thus assign the linkage group identified in this research to chromosome A of the *N. tabacum* genome. This confirms the origin of this chromosome

Table 2. Genotypes of Accessions of *N. sylvestris* and *N. tomentosiformis* for Seventeen Microsatellite Markers Linked to Genes Controlling Accumulation of *cis*-Abienol and Sucrose Esters

	N. sylvestris accession						*N. tomentosiformis* accession					
marker	TW 136	TW 137	TW 138	NIC 6	NIC 37	AUS 303276	914750065	ITB 645	ITB 646	ITB 647	TW 142	NIC 479
PT30272	–[a]	–	–	–	–	–	137[b]	137	137	133	133	137
PT30224	–	–	–	–	–	–	92	92	92	114	114	92
PT30223	–	–	–	–	–	–	220	222	222	226	226	226
PT30354	–	–	–	–	–	–	173	173	173	166	166	173
PT30209	–	–	–	–	–	–	145	145	145	145	145	145
PT20315	–	–	–	–	–	–	178/187	178/187	178/187	170/182	170/182	178/187
PT52061	–	–	–	–	–	–	218	–	218	218	–	–
PT61362	–	–	–	–	–	–	210	210	210	210	210	210
PT51311	–	–	–	–	–	–	149	149	149	157	157	149
PT60146	–	–	–	–	–	–	117	117	117	110	110	117
PT55091	–	–	–	–	–	–	222	222	222	222	222	222
PT30124	–	–	–	–	–	–	225	225	225	225	225	225
PT61373	–	–	–	–	–	–	238	238	238	211	211	238
PT51164	–	–	–	–	–	–	144	144	144	110	110	144
PT50962	–	–	–	–	–	–	190	190	190	202	202	190
PT20343	–	–	–	–	–	–	278	278	278	280	280	278
PT30346	201	216	211	216	211	211	143	143	143	151	151	143

[a] "–" indicates absence of marker band. [b] Number indicates size (bp) of PCR amplification product(s).

as proposed by Clausen and Cameron (*25*), and is the first published case of assigning a microsatellite linkage group to a physical chromosome of the tobacco genome.

In the current investigation, we treated the accumulation of *cis*-abienol and sucrose esters as qualitative traits. Absolute amounts recovered from leaf surfaces are likely influenced by quantitative genetic factors, however, as genetic variation for trichome density and type, synthesis, and secretion probably exists (*8*, *18*−*30*). This was not studied in the present research. It would have been difficult to map genes potentially involved in the control of these characters because they are impacted by plant developmental stage and/or environmental conditions. Because of the extreme range of plant type, flowering time, and leaf morphology of doubled haploid lines from the diverse cross that was used, it would have been very difficult to obtain leaf disks from leaves at the same stalk position at the same stage of development. Further genetic analyses in which the plant materials are produced and assayed under highly controlled conditions might identify genomic regions that contribute to quantitative variability for these leaf chemistries.

Although *Abl* and *BMVSE* have now been positioned on the *N. tabacum* genetic linkage map, the specific function of the two genes remains unknown. Closely linked markers might be valuable in future efforts to characterize these genes. *Abl* may encode for *cis*-abienol synthase (*3*). Because many tobacco types produce only significant amounts of sucrose ester groups I and II, it appears that the esterification process for these chemical species may be controlled by one gene, and that the composition of the acyl groups is influenced by an array of other genes (*1*). *BMVSE* is involved in the biosynthesis of tobacco sucrose esters containing 3-methylvaleryl substituents (groups III through VI) (*7*), but the precise role of this gene is unknown. Research has suggested that the acyl moieties of sucrose esters in *Nicotiana* are derived through the α-ketoacid elongation pathway, a modified form of branched-chain amino acid metabolism (*11*, *31*). It is possible that, in groups III through VI nonproducers, certain enzymes in this pathway are absent, modified, or active at very low levels resulting in low availability of 3-methylvaleryl groups for esterification (*11*). Alternatively, these nonproducers may contain a gene encoding for a specific 3-methylvaleryl-CoA dehydrogenase which could catabolize this molecule (*11*). In tomato, esterification of acyl moieties with glucose to form glucose ester natural products has been reported to occur by UDP glucose fatty acid transglucosylation and transacetylation (*32*).

Motivation exists to understand the ultimate genetic control of these biochemical pathways. Once the relevant genes and enzymes involved in the pathways are characterized, it should be possible to modify *N. tabacum* to produce greater quantities of these compounds. Upregulation of these genes could increase natural pest resistance or enhance flavor and aroma properties. There has also been interest in large-scale isolation of tobacco leaf surface compounds for commercial use as organic pesticides, cosmetic ingredients, additives for tobacco products, or chemical intermediates in industrial synthesis (*3*, *33*−*39*).

ABBREVIATIONS USED

LOD, logarithm of odds.

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