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# Variation of Flavor Components on Leaf Surfaces of Tobacco Genotypes Differing in Trichome Density

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Three populations of tobacco-doubled haploids (DH) were evaluated to characterize the relationship between density of secreting glandular trichomes (SGT) and levels (micrograms per square centimeter of leaf surface) of three trichome exudate constituents and to identify DH lines with altered concentrations of *cis*-abienol, sucrose esters containing β-methylvaleric acid (BMVSE), and α- and β-duvatrienediols (DVT). In the two populations in which some DH lines had few or no SGT, density scores for SGT were correlated positively with concentrations of *cis*-abienol, BMVSE, and DVT. All plants in the third population of DH lines had SGT, but SGT scores and concentrations of exudate constituents were not correlated. Several DH lines with DVT levels 2–3 times greater than the parent genotypes were identified. The results suggest that in some populations selection for glandular trichome density could alter levels of flavor and aroma constituents on the leaf surface.

Trichomes on leaves of many tobacco (*Nicotiana tabacum* L.) genotypes terminate in multicellular glands that are known to have biosynthetic capability (Keene and Wagner, 1985). Depending on the genotype, the exudate or secretion product of glandular trichomes may contain chemical compounds that influence insect and disease resistances and directly, or via their degradation products, affect flavor and aroma of tobacco products (Severson et al., 1985b). Recent evidence (Kandra and Wagner, 1987; Keene and Wagner, 1985; Wagner, 1988) suggests that the biosynthesis of sucrose esters (6-*O*-Acetyl-2,3,4-tri-*O*-acyl-α-D-glucopyranosyl-β-D-fructofuranoside), α- and β-4,8,13-duvatriene-1,3-diols (DVT), and possibly the labdane, *cis*-abienol (12(*Z*)-labda-12,14-dien-8α-ol) occurs in head cells of secreting glandular trichomes (SGT). Nonsecreting glandular trichomes (NSGT) are present on leaves of some genotypes. These trichomes are morphologically similar to SGT except they lack exudate.

Relatively large quantities of sucrose esters and *cis*-abienol are typically found on the leaf surface of Oriental tobacco genotypes, but they are not present in significant quantities in the other two major types, burley and flue-cured tobaccos, used in cigarette manufacture (Severson et al., 1985b). Enzell (1976), Kallianos (1976), and Smeeton (1987) suggested that these compounds impart flavor and aroma properties characteristic of the Turkish or Oriental types of tobacco. DVT's are present in all three of these tobacco types with SGT, but DVT concentrations vary among tobacco types.

The inheritance of trichome exudate constituents and other trichome traits has been fairly well documented. A single gene influences the presence of *cis*-abienol in tobacco (Coussirat et al., 1983/84). Similarly, the production of sucrose esters containing β-methylvaleric acid (BMVSE) is controlled by a single gene (Gwynn et al., 1985). Coussirat et al. (1983/84) suggested that two genes control the presence of DVT's in tobacco. Other genetic studies have also shown that a single gene affects the secreting capability of glandular trichomes (Nielsen et al., 1982) and that the density of glandular trichomes is controlled by three genes (Johnson et al., 1988).

Breeding efforts designed to alter the concentration of *cis*-abienol, BMVSE, and DVT could be based on selection for density of SGT, if these traits are related. This would eliminate many chemical analyses and permit selection prior to flowering and seed production and would facilitate breeding progress. However, there is little direct evidence suggesting a relationship between the density of glandular trichomes and the level of these leaf surface compounds. Increasing the density of SGT in tobacco may increase the concentration of chemical constituents associated with these structures. Therefore, the main objective of this study was to determine the extent of the relationship between density of SGT and the levels of DVT's, *cis*-abienol, and BMVSE on the leaf surface. An additional objective was to characterize the extent of variation for leaf surface compounds in populations of doubled haploids developed from crosses among geno-

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types known to differ for trichome density and for production of the three constituents listed above.

## MATERIALS AND METHODS

Anther-derived doubled haploids were developed from individual $F_1$ plants of three crosses involving genotypes selected for their diverse trichome characteristics and different leaf surface constituents. The three parents were KY 14, a burley tobacco cultivar with an intermediate density of SGT, TI 1068, a tobacco type grown in Brazil with a high density of SGT, and TI 1406 (also known as Virgin A mutant), with an intermediate density of NSGT. The three $F_1$'s were KY 14 × TI 1406, KY 14 × TI 1068, and TI 1406 × TI 1068. Anthers from a plant of each cross were cultured on artificial media, and haploid plants were generated by standard techniques (Kasperbauer and Collins, 1972). An array of haploid plants from each cross was transferred to the greenhouse under supplemental fluorescent lighting (Johnson et al., 1988). Trichome density on the abaxial surface of a 20-cm leaf from each plant was counted under a light microscope. Haploid plants from each cross, selected to provide a range in density of glandular trichomes, had their chromosomes doubled by the technique of Kasperbaur and Collins (1972). The doubled haploid plants were grown in the greenhouse and self-pollinated to provide seed for the field evaluation phase.

Transplant production and field practices were used in accordance with those recommended for burley tobacco. The doubled haploid lines and their parents were grown in a split-plot experimental design with three replications in 1986 and 1987. The doubled haploid cross or population was the main plot, and individual doubled haploid lines were the subplots. Parents of each population were included in the appropriate main plot. Soil type in both years was a Maury silt loam, and 252 kg of N $ha^{-1}$ was applied as ammonium nitrate in a split application 1 week before and 3 weeks after transplanting. Application of 280 kg of $K_2O$ $ha^{-1}$ was also done 1 week before transplanting. Forty-five and 47 days after transplanting in 1986 and 1987, respectively, the first 20 (±1) cm long leaf below the apical meristem was removed from five plants in each plot. Half of the leaf was used to obtain estimates of glandular trichome density, and leaf disks were removed from the other half to provide material for determination of leaf surface chemical constituents.

Trichome density scores were obtained by cutting a small lamina section (0.5 $cm^{-}$) from a site on each leaf approximately 1 cm from the midrib and 7 cm from the leaf tip. Lamina sections were gently placed between two glass slides, and abaxial leaf surfaces were viewed under a light microscope at 100×. Densities of SGT and NSGT on the abaxial leaf surface was scored against a scale ranging from 0 (no trichomes) to 9 for both trichome types. Scores ranging from 1 to 8 corresponded to SGT densities ranging from 1 to 120 SGT/2.52 $mm^2$ and to NSGT densities ranging from 1 to 80 NSGT/2.52 $mm^2$. A score of nine reflected densities greater than 120 SGT/2.52 $mm^2$ and 80 NSGT/2.52 $mm^2$. Mean SGT and NSGT density scores for each plot were calculated by averaging the density scores of the five plants sampled in each plot.

Primary lateral veins were avoided when the 3.14-$cm^2$ leaf disks were cut from a site opposite to that used for the trichome estimates. Each disk was immersed 10 times in 10 mL of methylene chloride, contained in a scintillation vial, for approximately $^1/_2$ s each time to remove the chemicals from the leaf surface. Five disks corresponding to the five plants sampled in each plot were extracted in a single vial representing each plot. Vials were immediately placed on dry ice and subsequently stored in a freezer (−20 C) before analyses.

After being warmed to room temperature, 100 μL of internal standard solution [2.2 mg/mL of tricosanol (Analabs, North Haven, CT)] was added to each sample vial, which was then capped and agitated to ensure uniform mixing. A portion of the mixture (0.8 mL for tobaccos with trichome scores of 5 or higher and 1.5 mL for samples with scores of 4 or lower) was transferred to a tapered test tube and taken to dryness on a nitrogen blow-down apparatus at 40 °C. A 50-μL portion of 1:1 *N,O*-bis(trimethylsilyl)trifluoroacetamide and dimethylformamide (BSTFA/DMF; Pierce Chemical Co., Rockford, IL) was added to the residue, and the test tube was capped with a Teflon-lined cap and heated for 45 min at 76 °C. After cooling, the derivatized sample was transferred to a microautosampler vial, capped, and placed in an autosampler.

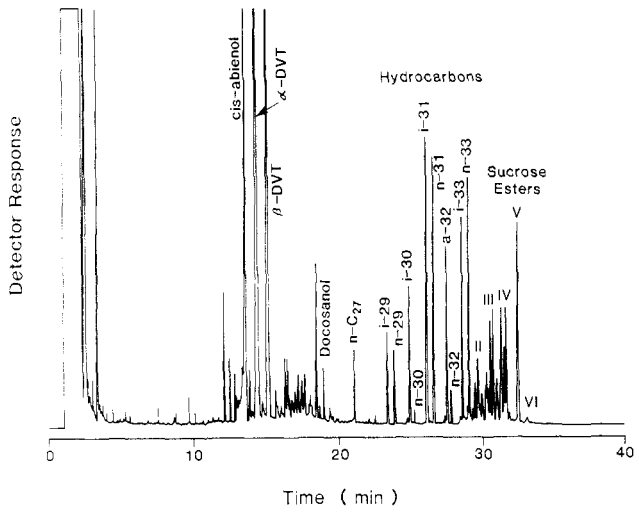


**Figure 1.** Capillary gas chromatogram of the silylated cuticular extract of TI 1068: n = normal chain; i = iso = 2-methyl-branched chain; a = anteiso = 3-methyl-branched chain.

The samples were analyzed on a Hewlett-Packard 5890 gas chromatograph (equipped with a split–splitless injection port, FID detector, 7673A autosampler and a 3396A integrator) using a 0.3 mm (i.d.) × 30 m immobilized thin film (about 0.1 μm) SE-54 fused silica WCOT column and splitless injection (purge activation time 2.0 min; temperature program of 100 °C for 2 min to 180 °C at 10 °C/min to 320 °C at 4 °C/min; 35 cm/s $H_2$ flow rate; 50 mL/min purge; injection port temperature 250 °C; FID temperature 325 °C). Columns were prepared from Hewlett-Packard fused silica tubing according to the procedures of Arrendale and Martin (1988). Levels (micrograms per square centimeter of leaf surface) of components were calculated by an internal standard quantitation method with chromatographic response factors obtained from standard mixtures of α-DVT, β-DVT, *cis*-abienol, SE, and tricosanol. Standard diterpenes and SE were isolated from the cuticular waxes of green tobacco as described by Severson et al. (1985a, 1989).

The cuticular components of the DH populations were confirmed by comparison of GC retention data to those of authentic standards. To further characterize the surface chemistries of the parent lines, TI 1068, KY 14, and TI 1406, plant tops were dipped into methylene chloride in 2-L beakers. After solvent removal the extracts were partitioned between hexane and 80% MeOH–$H_2O$ (Severson et al., 1985a, 1989). After treatment with BSTFA/DMF, GC/MS on an HP 5985B MS system modified for capillary GC/MS (Arrendale et al., 1984) verified the presence of *cis*-abienol in the hexane fraction of TI 1068 and TI 1406 and α- and β-DVT and sucrose esters in the MeOH/$H_2O$ fractions of all parents. KY 14 did not produce detectable amounts of BMVSE (groups III–VI; see Figures 1–3).

## RESULTS AND DISCUSSION

Capillary gas chromatograms of the parent lines are given in Figures 1 (TI 1068), 2 (KY 14), and 3 (TI 1406). TI 1068, with a high density of SGT, produced high levels of *cis*-abienol, α- and β-DVT's, and BMVSE (Table I). The major trichome product components in the cultivar waxes of KY 14 are the α- and β-DVT's. In contrast, analysis of the cuticular extracts of TI 1406, a tobacco with NSGT, revealed only trace levels of *cis*-abienol and very low levels of DVT's and BMVSE. The chromatogram of the TI 1406 chemistry was obtained with a 2× equivalent of cuticular extract relative to the other parents. The cuticular extract of all parents contained sim-

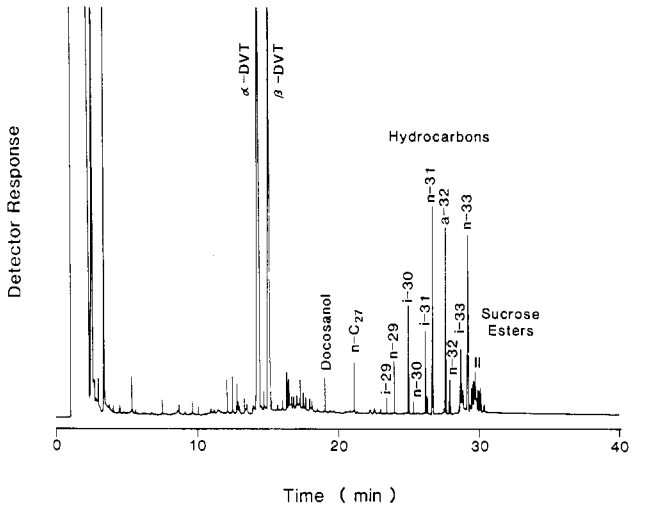


**Figure 2.** Capillary gas chromatogram of the silylated cuticular extract of KY 14: n = normal chain; i = iso = 2-methyl-branched chain; a = anteiso = 3-methyl-branched chain.

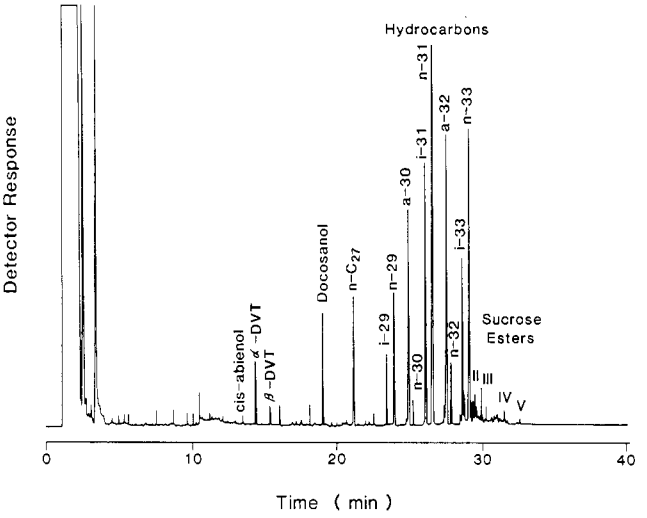


**Figure 3.** Capillary gas chromatogram of the silylated cuticular extract of TI 1406: n = normal chain; i = iso = 2-methyl-branched chain; a = anteiso = 3-methyl-branched chain.

ilar levels and distributions of non-trichome-produced normal- and branched-chain aliphatic hydrocarbons (Severson et al., 1984, 1985b).

All parents produced measurable levels of sucrose esters having a sucrose molecule with a 6-*O*-acetyl-2,3,4-triacyl-substituted glucose moiety (Severson et al., 1985a). TI 1068 and TI 1406 have β-methylvaleric acid and produce a sucrose esters function with six major groups (I–VI) differing in molecular weight by 14 atomic units. Major acid moieties for each grouping are group 1, $C_2$ $C_4$ 2 $C_8$; Group II, $C_2$ 3 $C_5$; group III, $C_2$ 2 $C_5$ $C_6$; group IV, $C_2$ $C_5$ 2 $C_6$; group V, $C_2$ 3 $C_6$; and group VI, $C_2$ 2 $C_6$ $C_7$, the major $C_5$ acids being 2- and 3-methylbutyric and $C_6$ acid being 3-methylvaleric. KY 14 lacks the β-methylvaleric acid trait and produces only very low levels of groups I and II sucrose esters (Severson et al., 1985b).

Secreting glandular trichomes were present on leaves of all doubled haploid (DH) lines in the population derived from KY 14 × TI 1068, but 8 and 15 of the DH lines in the populations from KY 14 × TI 1406 and TI 1406 × TI 1068, respectively, had SGT scores of less than 1.0 and were classified as nonsecreting genotypes. For these 23 genotypes mean concentrations of DVT's and *cis*-abienol were less than 1.0 μg $cm^{-2}$ and the mean BMVSE concentration was less than 2.5 μg $cm^{-2}$. One parent, TI 1406, is homozygous recessive for the *te* allele and does not produce a sticky exudate (Nielsen et al., 1982). The 23 nonsecreting DH lines probably possessed the *te* allele and, thus, are not capable of synthesizing or secreting chemical constituents often found in the exudate of genotypes with SGT. In these two populations all of the DH lines with SGT scores of 1.0 or greater produced one of the three measured leaf surface chemical constituents in concentrations greater than 10 μg $cm^{-2}$. This provides indirect evidence supporting the role of glandular trichomes in the synthesis and secretion of *cis*-abienol in addition to the known production (Kandra and Wagner, 1987; Keene and Wagner, 1985) of DVT's and BMVSE in these structures.

The fact that some DH lines from the KY 14 × TI 1406 had BMVSE and *cis*-abienol plus evidence of trace levels of these compounds from TI 1406 suggested that TI 1406 possesses genes for production of BMVSE and *cis*-abienol. It is known that KY 14 does not produce those compounds; therefore, the presence of the *te* allele in TI 1406 limits greatly the expression of the genes for BMVSE and *cis*-abienol production.

In the populations derived from crosses between TI 1406 and the other two cultivars, density of SGT was correlated significantly and positively with the concentrations of DVT, *cis*-abienol, and BMVSE (Table II). Correlation coefficients between densities of NSGT and SGT were −0.65 and −0.87 for the two DH populations, KY 14 × TI 1406 and TI 1406 × TI 1068, respectively. These correlations suggested that selection for increased density of SGT in these populations would decrease NSGT and increase levels of DVT, BMVSE, and *cis*-abienol.

Minimum values for SGT, DVT, *cis*-abienol, and BMVSE of the doubled haploid lines derived from KY 14 × TI 1068 were similar to values found for KY 14, and maximum values for SGT, *cis*-abienol, and BMVSE but not DVT were similar to those for TI 1068. Of the 24 DH lines in this population, 8 had DVT concentrations significantly greater than that found for TI 1068. Determining the inheritance of DVT, SE, and *cis*-abienol was not a major objective of this research. Yet, the 24 DH lines segregated in a 1:1 ratio for presence:absence of BMVSE and in a 7:5 ratio for presence:absence of *cis*-abienol. The expected segration ratio for a single dominant gene in a haploid genetic model is 1:1; therefore, the data suggested that TI 1068 has a single dominant gene affecting the production of BMVSE and *cis*-abienol and that the two genes are not linked. The inheritance pattern for these compounds was the same as that reported elsewhere in studies using TI 1068 or other genotypes (Gwynn et al., 1985).

The number of DH lines in the population from KY 14 × TI 1068 was not large enough to estimate genetic models beyond a single gene. That no DH lines had DVT concentrations less than that found for KY 14 suggested that these two parental genotypes had similar alleles at a minimum of one locus. Since there were eight DH lines with DVT concentrations greater than that of TI 1068, genes affecting DVT production in KY 14 may differ from that of TI 1068 at one or more loci. None of the DH lines had SGT scores significantly greater than that of TI 1068, and SGT density did not greatly influence the high DVT values for these DH lines. Thus, recombination or possibly mutation may have resulted in those DH lines with DVT values greater than that found in TI 1068.

The ranges in SGT scores, 0–5.4 and 0–6.3, in the first two populations were greater than that found in the DH population derived from KY 14 × TI 1068, which had a

**Table I. Two-Year Means (Standard Error) of the Three Parental Genotypes and Mean Minimum and Maximum Values of Three Doubled Haploid Populations of Tobacco for SGT and NSGT Density Scores and Concentrations ($\mu g\ cm^{-2}$) of Three Leaf Surface Chemicals**

| parent or DH population | $N^a$ | score | | | | |
|---|---|---|---|---|---|---|
| | | SGT | NSGT | α-, β-DVT | *cis*-abienol | BMVSE |
| KY 14 | | 3.5 | 0.8 | 38.1 | ND[c] | ND |
| | | (0.4) | (0.2) | (2.4) | | |
| TI 1406 | | ND | 5.3 | 0.2 | ND | 0.9 |
| | | | (0.6) | (<0.1) | | (0.6) |
| TI 1068 | | 5.8 | 0.2 | 75.1 | 26.1 | 56.6 |
| | | (0.4) | (0.1) | (5.6) | (3.3) | (5.2) |
| KY 14 × TI 1406 mean (15) | | 1.4 | 3.9 | 21.2 | 2.6 | 5.9 |
| | | (0.2) | (0.3) | (3.2) | (1.0) | (2.3) |
| min[b] | 7 | 1.0 | 0.3 | 16.5 | ND | ND |
| max | 7 | 5.4 | 3.6 | 92.6 | 21.0 | 71.8 |
| TI 1406 × TI 1068 mean (43) | | 3.4 | 2.6 | 50.6 | 8.4 | 18.8 |
| | | (0.2) | (0.2) | (3.7) | (0.8) | (1.7) |
| min | 29 | 2.4 | ND | 0.5 | ND | ND |
| max | 28 | 6.3 | 3.4 | 190.0 | 33.7 | 78.1 |
| KY 14 × TI 1068 mean (24) | | 4.6 | 0.3 | 84.0 | 11.4 | 19.0 |
| | | (0.1) | (0.1) | (3.2) | (1.2) | (2.4) |
| min | 24 | 3.4 | ND | 33.0 | ND | ND |
| max | 24 | 5.9 | 1.5 | 140.0 | 31.8 | 57.5 |

[a] Number of doubled haploid lines. [b] Data for minimum and maximum values were only from those DH lines with SGT scores greater than 1.0. [c] None detected, less than 0.1 $\mu g/cm^2$.

**Table II. Simple Correlation Coefficients between Density of Two Types of Glandular Trichomes and Level of Three Leaf Surface Chemicals in Three Doubled Haploid Populations**

| population | glandular trichome type | leaf surface chemicals | | |
|---|---|---|---|---|
| | | α-, β-DVT | *cis*-abienol | BMVSE |
| KY 14 × TI 1406 | SGT | 0.80** | 0.39** | 0.56** |
| | NSGT | -0.45** | -0.23* | -0.30** |
| TI 1406 × TI 1068 | SGT | 0.59** | 0.34** | 0.35** |
| | NSGT | -0.62** | -0.41** | -0.39** |
| KY 14 × TI 1068 | SGT | -0.11 | 0.06 | 0.06 |
| | NSGT | -0.09 | 0.04 | 0.05 |

[a] Key: *, **, significant at the 0.01 and 0.05 levels, respectively.

**Table III. Concentrations ($\mu g\ cm^{-2}$) of *cis*-Abienol, DVT, and BMVSE on the Leaf Surface of Selected DH Lines from Three DH Populations**

| cross | DH line no. | *cis*-abienol | α-, β-DVT | BMVSE |
|---|---|---|---|---|
| KY 14 × TI 1068 | 909 | ND[a] | 140.0 | 28.8 |
| | 913 | ND | 134.0 | ND |
| KY 14 × TI 1406 | 926 | 21.0 | 92.6 | 71.8 |
| TI 1406 × TI 1068 | 949 | 33.8 | 109.0 | ND |
| | 950 | 20.1 | 0.7 | 65.9 |
| | 959 | 25.6 | 3.5 | 69.0 |
| | 960 | ND | 191.0 | ND |

[a] None detected, less than 0.1 $\mu g/cm^2$.

range in SGT scores of 3.4–5.9. In this population there were no significant positive correlations between density of SGT or NSGT and levels of DVT, *cis*-abienol, and BMVSE (Table II). In fact, there was a slight negative correlation between SGT density and DVT concentrations. Obviously, in this population factors other than trichome density may affect the concentration of the leaf surface components. These factors may include the size of the trichome head responsible for biosynthesis of the different compounds, the rate of biosynthesis of these compounds in the different genotypes in this population, and perhaps, differences in the rate of plant growth and development.

A number of DH lines (Table III) with greater levels of BMVSE and DVT were identified. One DH line, DH 960, had the highest DVT concentration we have encountered and three lines, DH 926, DH 950, and DH 959, had high BMVSE levels. These lines have no immediate agronomic utility but may be useful in further studies of trichome exudate constituents of tobacco, in providing biosynthetic sources of *cis*-abienol, DVT, and BMVSE and in tobacco-breeding programs.

The three flavor components evaluated in this study are typically found in Oriental tobacco, and *cis*-abienol and BMVSE are, in part, responsible for flavor characteristics distinguishing Oriental tobacco from most flue-cured and burley tobaccos (Severson et al., 1985b). The current study is part of an overall effort to develop a burley tobacco with an Oriental flavor and aroma characteristics in addition to the chemical and physical characteristics of burley tobacco. The highly significant correlations between SGT density and levels of *cis*-abienol, DVT, and BMVSE suggest that in certain breeding populations density of SGT could be used as a selection criterion to increase the levels of these flavor and aroma constituents. Barrera and Wernsman (1966) found no relationship between trichome density and tobacco aroma. However, exudate constituents were not actually measured in that study, and genotypes with high densities of SGT may have lacked genes necessary for the synthesis of compounds analyzed in our study. While selection for SGT in early generations should be effective, it should be followed by quantitative determinations of the leaf surface constituents of advanced breeding lines. Variability among the DH lines suggested that genetic recombination or possibly mutations of genes resulted in high levels of *cis*-abienol, DVT, and BMVSE. These DH lines could serve as parental sources for increasing the levels of these flavor and aroma constituents in commercial cultivars.

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# Volatile Constituents of Apricot (*Prunus armeniaca*)

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Volatile constituents of fresh apricots (*Prunus armeniaca*) of the Blenheim variety were analyzed by capillary gas chromatography and gas chromatography–mass spectrometry. The fruit was sampled by simultaneous vacuum steam distillation–extraction. A total of 49 components were identified in the extract, including 25 constituents reported for the first time in apricot. Linalool, lactones, and $C_6$ lipid peroxidation products were the major constituents in the extract. Odor unit values, calculated from concentration and odor threshold data, indicate that the following compounds are major contributors to blended apricot aroma: β-ionone, linalool, γ-decalactone, hexanal, (*E*)-2-hexenal, (*E*,*E*)-2,4-decadienal, (*E*)-2-nonenal, and γ-dodecalactone. Headspace analyses of the intact fruit led to the identification of 83 components, 60 of which had not been previously reported in apricot. Esters were the dominant constituents in the headspace samples.

The first significant studies on apricot flavor were performed by Tang and Jennings (1967, 1968) who utilized direct extraction, vacuum steam distillation, and charcoal adsorption to isolate the volatiles from the Blenheim variety. A number of terpene hydrocarbons, terpene alcohols, and lactones were identified by gas chromatographic retentions and infrared spectroscopy. Rodriguez et al. (1980) studied the variety Rouge du Roussillon and identified constituents such as camphene, γ-terpinene, hexanol, benzaldehyde, γ-butyrolactone, and nerol for the first time in apricot. Later studies on the same variety led to the identification of damascenone, β-ionone, dihydroactinidiolide, rose oxide, and nerol oxide (Chairote et al., 1981). These authors felt that the apricot aroma was dependent on several constituents such as lactones, terpene alcohols, and benzaldehyde. Guichard and Souty (1988) compared the relative concentrations of various volatiles present in six different apricot varieties (Precoce de Tyrinthe, Palsteyn, Moniqui, Rouge du Roussillon, Polonais, Bergeron) grown in the south of France. A total of 82 compounds were identified, 58 of which had not been previously reported in apricot. The most abundant constituents were $C_6$ lipid degradation products, lactones, terpene alcohols, and ketones. Sharaf et al. (1989) identified 31 components

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