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ORIGINAL ARTICLE

Comparative studies of leaf surface chemical biosynthesis in different tobacco cultivars

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Abstract

To understand the differences in the biosynthesis of leaf surface chemicals and their influence on aphid preference for different tobacco cultivars (*Nicotiana tabacum*), we analyzed the secretory characteristics of glandular trichomes of four commercial cultivars, K326 (flue-cured), Beinhart 1000-1 (cigar), Basma YNOTBS1 (oriental), and Dabai 1 (burley), and their parental species, *Nicotiana sylvestris* and *Nicotiana tomentosiformis*. Trichome-type observation showed that K326 and *N. sylvestris* have three kinds of glandular trichomes (non-glandular, long stalked glandular, and short stalked glandular trichomes), whereas Beinhart 1000-1, Basma YNOTBS1, Dabai 1, and *N. tomentosiformis* had two kinds of glandular trichomes (long and short stalked glandular trichomes). The gas chromatography–mass spectrometry profiles of leaf exudates indicated that *N. tomentosiformis* synthesized only labdanoids; *N. sylvestris*, K326 and Dabai 1 synthesized only cembranoids; and Beinhart 1000-1 and Basma YNOTBS1 synthesized cembranoids and labdanoids. Gene expression pattern analysis revealed that the labdanoid synthesis-related genes *NtABS* and *NtCPS2* were expressed in *N. tomentosiformis*, Beinhart 1000-1, and Basma YNOTBS1, whereas the cembranoid synthesis-related genes *NtCYC* and *NtCYP71D16* were expressed in *N. sylvestris* and all four commercial cultivars. Evolutionary analysis indicated that *NtCYC* and *NtCYP71D16* might be phylogenetically originated from *N. sylvestris*, whereas *NtABS* and *NtCPS2* expressed in Basma YNOTBS1 and Beinhart 1000-1 might be derived from *N. tomentosiformis*. In addition, aphid attraction (number of aphids) was significantly and positively correlated with the total glandular secretion ($r^2 = 0.9425$, $P \leq 0.05$), and it was significantly and positively correlated with amount of CBT-diol ($r^2 = 0.9224$; $P \leq 0.05$). These results provide new insights into the biosynthesis of diterpenoids and biotic stress resistance in tobacco.

Keywords *Nicotiana tabacum* · *Nicotiana sylvestris* · *Nicotiana tomentosiformis* · Glandular trichome · Diterpenoid · Aphid resistance

Introduction

Glandular trichomes on tobacco leaves secret high amounts of important biochemical compounds, such as sucrose esters, waxes, micro-elements, and diterpenoids (Johnson et al. 1985), which represent 10–30% of the leaf dry weight (Wagner 1991). Of these, diterpenoids are related to plant defense, since they have antifungal (Kennedy et al. 1992, 1995) and insecticidal (Lin and Wagner 1994) activity. In addition, diterpenoids, accounting for more than 50% of the foliar chemical composition under optimal conditions, are considered as the precursors of important compounds produced during leaf curing and significantly contribute to tobacco aroma (Wagner 1991; Wagner et al. 2004).

Two types of diterpenes have been found in cultivated tobacco, cembranoids and labdanoids (Wagner 1991).

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Mingyue Huang and Hongying Zhang contributed equally to this study.

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The major labdanoids are Z-abienol (Figs. 1, 2, 3, 4, and 5) and labdenediol (Figs. 1, 2, 3, 4, 5, and 6), whereas the major cembranoids are α- and β-cembratrien-diol (CBT-diols; Figs. 1, 2, 3 and Figs. 1, 2, 3, and 4) along with small amounts of their respective precursors, α- and β-cembratrien-ol (CBT-ols; Figs. 1 and 2) (Severson et al.

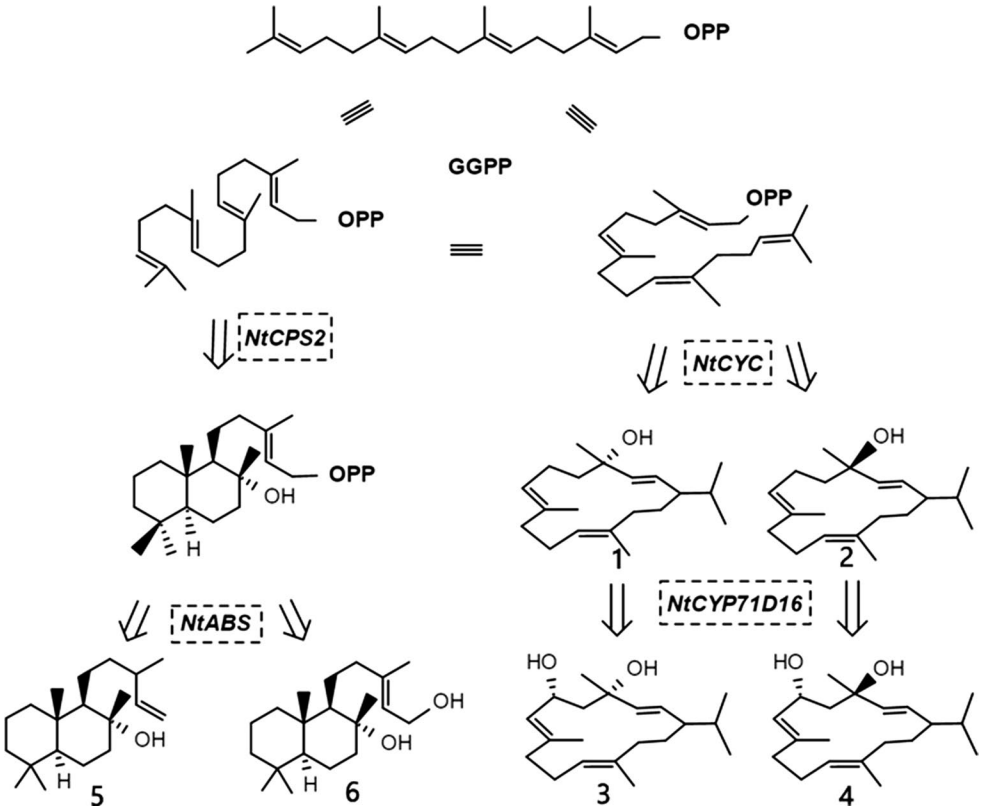

Fig. 1 Major diterpenoid biosynthesis pathway in glandular trichomes of tobacco. Four key genes controlling diterpenoid synthesis were divided into two groups: *NtCYC* and *NtCYP71D16* that control the synthesis of cembranoids, and *NtABS* and *NtCPS2* that control the synthesis of labdanoids. (1–6): α-CBT-ol, β-CBT-ol, α-CBT-diol, β-CBT-diol, cis-abienol, and labdenediol

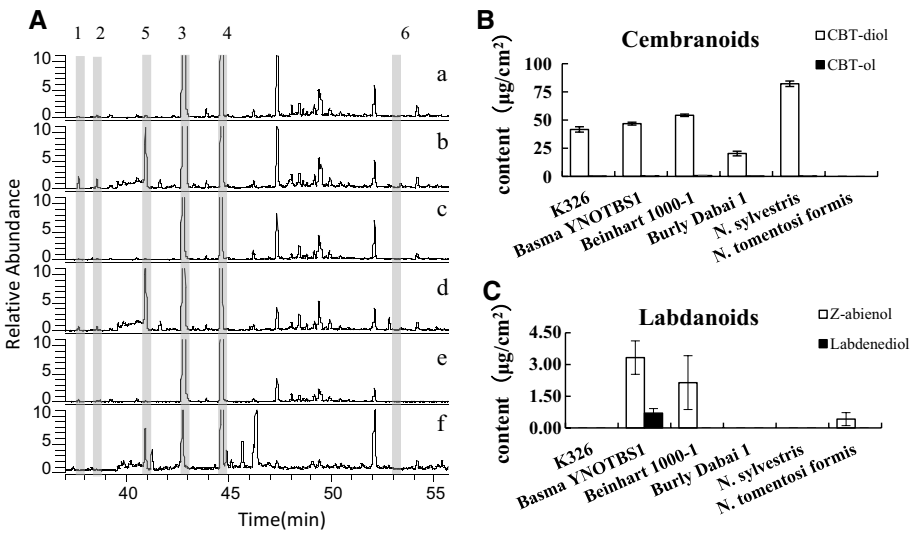

Fig. 2 **a** Gas chromatography–mass spectrometry (GC–MS) profiles (34–55 min) of leaf exudates in (a–d) four *Nicotiana tabacum* cultivars (K326, Beinhart 1000-1, Dabai 1, and Basma YNOTBS1), (e) *Nicotiana sylvestris*, and (f) *Nicotiana tomentosiformis*. (1–6) α-CBT-ol, β-CBT-ol, α-CBT-diol, β-CBT-diol, cis-abienol, and labdenediol. Note: the peak 3 and peak 4 in (f) are not α-CBT-diol, β-CBT-diol after mass spectrum analysis (figure S3). **b** Cembranoid components quantified by GC–MS and expressed in microgram per square centimeter (μg cm^{-2}) of fresh leaf. The data was shown as mean ± SD ($n=3$). **c** Labdanoid components quantified by GC–MS and expressed in microgram per square centimeter (μg cm^{-2}) of fresh leaf. The data were shown as mean ± SD ($n=3$)

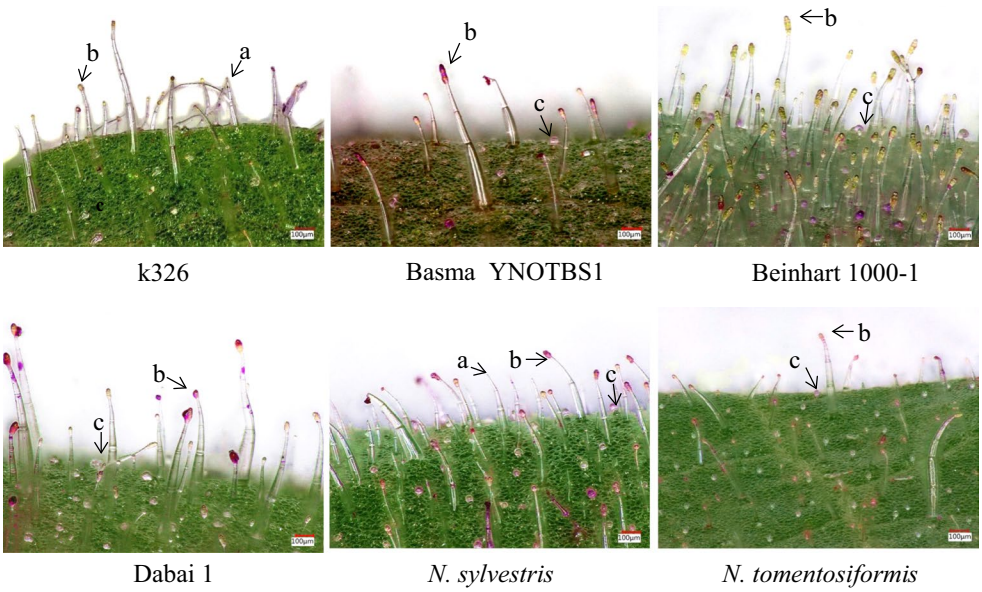

Fig. 3 Leaf trichome morphology of four *Nicotiana tabacum* cultivars (K326, Beinhart 1000-1, Dabai 1, and Basma YNOTBS1), *Nicotiana sylvestris*, and *Nicotiana tomentosiformis* at the five-leaf stage. **a** Non-glandular trichome (i.e., type III-like); **b** tall glandular trichome (i.e., types I-like, IV-like, and VI-like); **c** short glandular trichome (i.e., type VII-like)

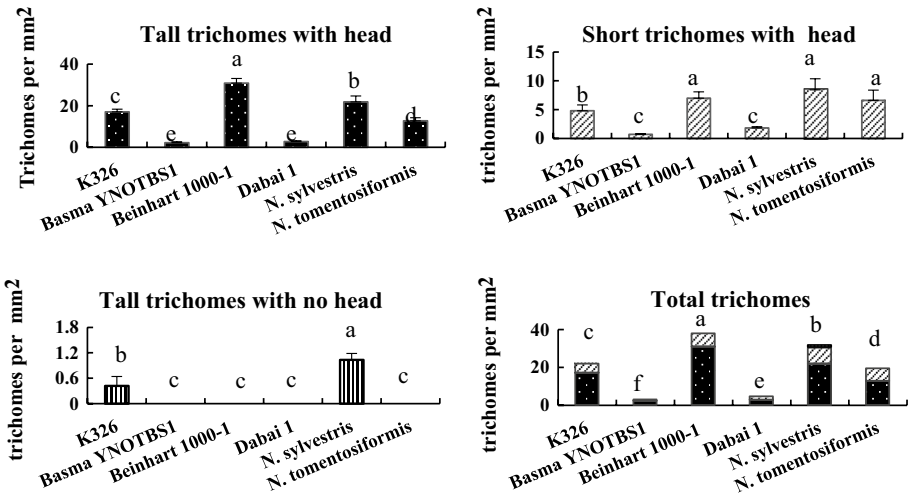

Fig. 4 Leaf trichome density of four *Nicotiana tabacum* cultivars (K326, Beinhart 1000-1, Dabai 1, and Basma YNOTBS1), *Nicotiana sylvestris*, and *Nicotiana tomentosiformis*. The data were shown as mean ± SD ($n = 3$). Different letters indicate statistically significant differences at $P \leq 0.05$

1984). The biosynthesis of cembranoids includes two steps: (1) the cyclization of the precursor geranylgeranyl diphosphate (GGPP) that is catalyzed by cembratrien-ol synthase (CBTS) and produces a mixture of α- and β-cembratrien-ols (CBT-ols) (Guo and Wagner 1995), and (2) the oxidation of CBT-ols in the presence of cytochrome P450 oxygenase (CYP450) (Wang et al. 2001). CBTS and CYP450 are encoded by *NtCYC* and *NtCYP71D16*, respectively (Ennajdaoui et al. 2010; Wang and Wagner 2003). As proposed in recent studies (Carman et al. 1993; Peters 2010), the biosynthesis of labdanoids also proceeds from GGPP. Accordingly, in the biosynthesis of Z-abienol, copalyl diphosphate synthase (CPS) catalyzes the synthesis of 8-a-hydroxy-copalyl diphosphate (8-OH-CPP), which is then converted to Z-abienol and labdenediol by ABS synthase (Sallaud et al. 2012). In addition, previous studies on *NtCPS2* and *NtABS* have shown that both genes are necessary and sufficient for the biosynthesis of Z-abienol and labdenediol from GGPP in glandular trichomes (Sallaud et al. 2012). The cognition of the biosynthetic pathway of terpenoids can help to elucidate their role in plant defense against insects and fungi, and thus, to improve tobacco resistance by genetic engineering. For instance, the suppression of *NtCYP71D16* in plant trichome glands increases the value of CBT-ol/CBT-diol, enhancing aphid resistance in tobacco (Wang and Wagner 2003; Wang et al. 2001).

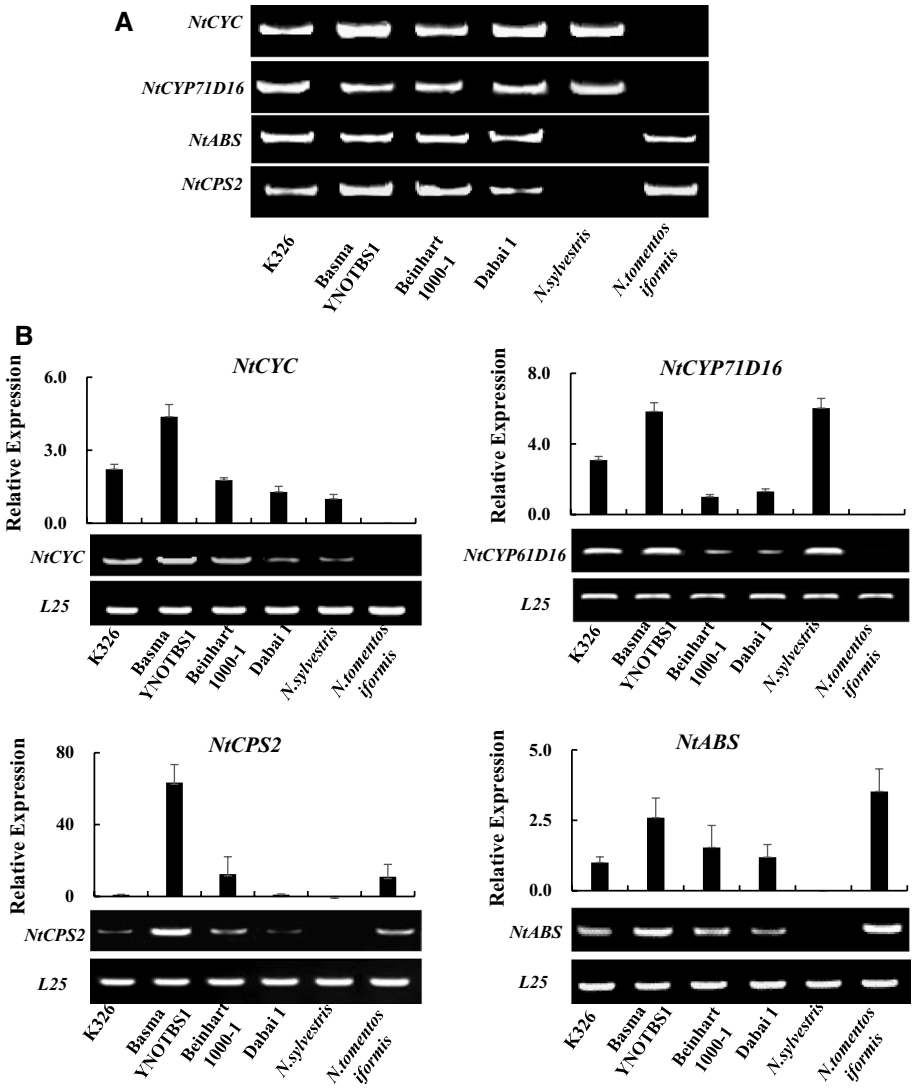

Fig. 5 Expression patterns of key genes involved in diterpenoid biosynthesis in trichomes of four *Nicotiana tabacum* cultivars (K326, Beinhart 1000-1, Dabai 1, and Basma YNOTBS1), *Nicotiana sylvestris*, and *Nicotiana tomentosiformis*. **a** General PCR for genetic analysis. **b** Expression level of *NtCYC*, *NtCYP71D16*, *NtABS* and *NtCPS2* was identified with both qRT-PCR (histograms) and semi-quantitative RT-PCR (gels). Top gels, semi-quantitative of *NtCYC* , *NtCYP71D16*, *NtABS* and *NtCPS2*; bottom gels, *L25* was used as an internal control. The data of qRT-PCR were shown as mean ± SD ($n = 3$)

The key genes that involved in diterpenoid biosynthesis are specifically expressed in the gland cells of tobacco trichome tissue (Cui et al. 2011; Brückner et al. 2014; Wang et al. 2002). Trichomes are specialized outgrowths of epidermal cells that exist on most leaves and stems of terrestrial vascular plants (Peng and Hu 2007) and cover approximately 30% of the aerial parts of vascular plants (the surface of stems or leaves) (Fahn 2000). Luckwill (1943) first examined the trichomes of the genus *Lycopersicon* and categorized them as types I–VII. Depending on their morphology, trichomes can be divided into non-glandular trichomes (i.e., types II, III, and V) and glandular trichomes (i.e., types I, IV, VI, and VII). Non-glandular trichomes that consist of a basal cell and stalk cells do not participate in the synthesis and accumulation of secondary metabolites and are present in most angiosperms, some gymnosperms, and bryophytes (Wagner et al. 2004); for example, the model plant *Arabidopsis* possesses only non-glandular trichomes (Mathur and Chua 2000; Schwab et al. 2000), which have been widely studied with respect to their development (Larkin et al. 2003). Previous studies showed that more than 30 genes are involved in non-glandular trichomes development (i.e., type III-like) in *Arabidopsis*, including genes that control the occurrence of trichomes and epidermal hair clusters. Of them, three transcription factors (GL1, GL3, and TTG1) regulate the occurrence and development of trichomes (An et al. 2011). Glandular trichomes consist of basal cells, stalk cells, and apical cells. The latter are responsible for the production of diterpenoids and other important foliar chemical substances. Glandular trichomes can be used as a bioreactor in the production of medical (Weathers et al. 2011) and chemical products (Lange et al. 2011; Schilmiller et al. 2008), as well as in the prevention and control of agricultural diseases and insect pests (Dayan and Duke 2003; Mellon et al. 2012). Tobacco glandular trichomes are divided into short trichomes with a unicellular stalk, possessing a multicellular head (i.e., type VII-like), and tall trichomes with a multicellular stalk, possessing uni- or multicellular heads

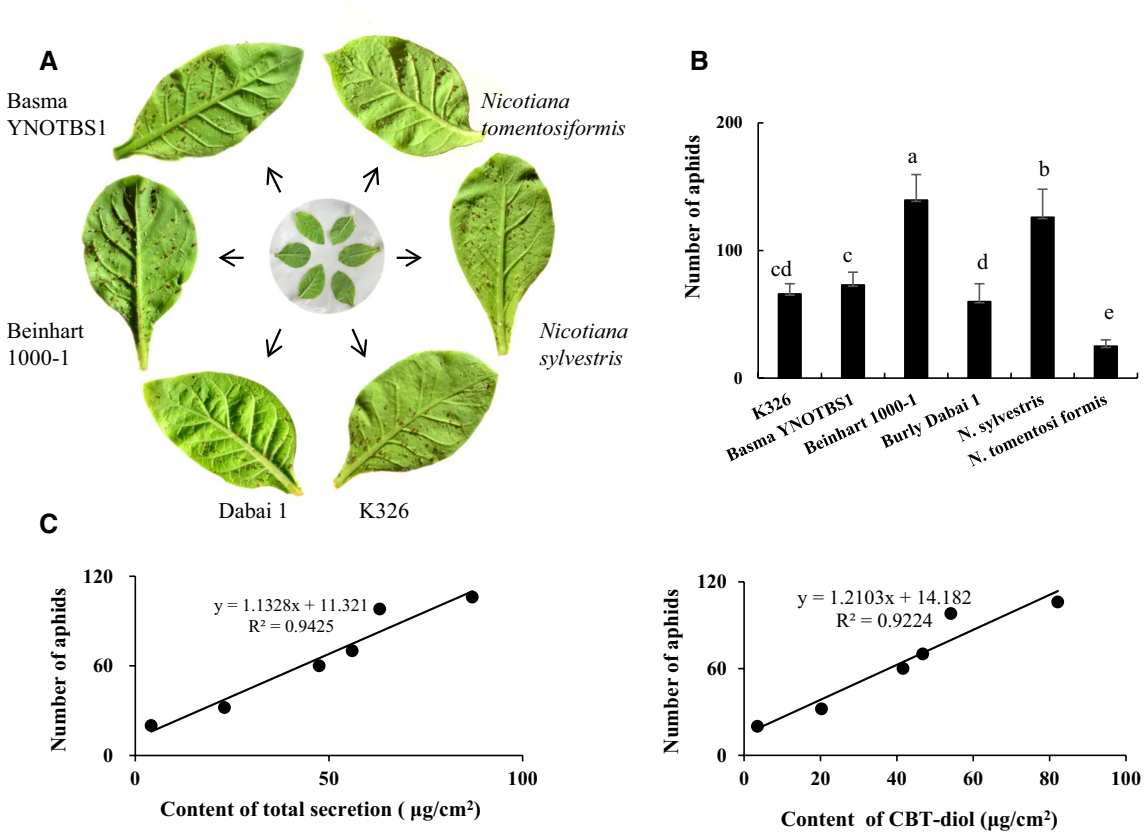

Fig. 6 Aphid infestation detection and aphid numbers. **a** Lower side of aphid-infected leaf blade; **b** Numbers of aphids collected on the leaves; and **c** correlation analysis of aphid number and content of total secretion, and content of CBT-diol. The data were shown as mean ± SD ($n = 3$). Different letters indicate statistically significant differences at $P \leq 0.05$

(i.e., types I-like, IV-like, and VI-like); each kind secretes different components (Meyberg et al. 1991).

Cultivated tobacco (*Nicotiana tabacum*), which is natural allopolyploid that phylogenetically originated from the hybridization of two species, *N. sylvestris* Speg. & Comes and *N. tomentosiformis* Goodsp. (Glas et al. 2012). Here, we studied the differences of leaf trichomes, including their morphology and density, kinds of secreted substances, and expression of genes related to the synthesis of diterpenoids of four cultivars belonging to different types of tobacco and the parental species.

Materials and methods

Plant material

In this study, we used four tobacco cultivars, K326 (flue-cured), Basma YNOTBS1 (oriental), Dabai 1 (burley), and Beinhart 1000-1 (cigar), with different aromas during curing (Table S1) and two wild species *N. sylvestris* and *N. tomentosiformis*. Seeds were sown in mixed soil (1:1 vermiculite: humus) under greenhouse conditions (temperature of 22 °C, relative humidity of 70%, natural diurnal rhythm, and natural illumination), and the seedlings were transplanted to the experimental fields of Henan Agricultural University, Xuchang, Henan Province, China. Fertilization and irrigation were applied as required.

Microscopic observation of trichome morphology and density

For trichome morphology analysis, five plants were randomly selected for each cultivar. Leaves at different growing stage were sampled. At five-leaf stage the third leaf from the stem base were used, while at mature stage, the 16th–18th (upper) and 10th–12th (middle) leaves from the stem base were chosen. The bottom four leaves of each tobacco plant were removed at the beginning of the mature stage.

Leaves at the five-leaf stage were also used for trichome density analysis. Leaves between the main vein and the left leaf margin located between the third and fourth branch vein from the leaf apex to leaf base were sampled. Twenty squares of the sampled leaves were cut out (area, 1 × 1 cm).

Samples were observed under a real-time and 3D digital microscope (VHX-5000; Keyence Corporation, Osaka, Japan). The mean number of trichomes per mm^2 of leaf area was calculated.

RNA extraction and RT-PCR

Total RNA was extracted from the roots, stems, and leaves without trichomes, as well as from trichomes using RNeasy kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Trichomes were harvested by freezing three or four mature leaves (20 cm in length) on an aluminum sheet over a liquid nitrogen bath. Once frozen, each leaf was brushed with a paintbrush over a liquid nitrogen-cooled mortar to collect trichomes, whereas the rest of the tissue was collected as leaf without trichomes. Trichomes and leaves were frozen immediately with liquid nitrogen and stored at − 80 °C for RNA extraction. To remove traces of genomic DNA, RNA samples were treated with DNase (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA quantity was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and its quality was analyzed using 1% agarose gel electrophoresis stained with ethidium bromide. For reverse transcription (RT), each RT reaction consisted of 0.5 µg RNA, 2 µl of PrimerScript Buffer, 0.5 µl of oligo dT, 0.5 µl of random 6-mers, and 0.5 µl of PrimerScript RT Enzyme Mix I (TaKaRa, Tokyo, Japan) in a total volume of 10 µl and performed with a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA) for 15 min at 37 °C, followed by heat inactivation for 5 s at 85 °C. The RT reaction mix was then diluted tenfold in nuclease-free water and stored at − 20 °C.

Gene expression pattern analysis

For gene expression pattern analysis, we designed primers based on mRNA sequences obtained from the SOL Genomics Network (SGN; https://www.sgn.cornell,edu) database and synthesized by Generay Biotech (Shanghai, China) (the primer sequences are presented in Table 1). Four genes (*NtCYC, NtCYP71D16, NtABS* and *NtCPS2*) were amplified in the four tobacco cultivars and two parental species.

Gene expression quantification was performed by semi-quantitative interpretation and real-time quantitative PCR (qPCR). Each semi-quantitative interpretation was same with PCR reaction. Each RT-qPCR reaction consisted of 1 µl of cDNA, 5 µl of 2 × LightCycler® 480 SYBR Green I Master (Roche, Basel, Switzerland), 0.2 µl of forward primer (Table 1), 0.2 µl of reverse primer, and 3.6 µl of nuclease-free water in a total volume of 10 µl and performed with a LightCycler® 480 II Real-time PCR (Roche). PCR conditions were as follows: 10 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C. Each sample was tested in triplicate. Melting curve analysis was performed to validate the specific amplification of the expected PCR product. The *L25* gene was used as an internal control for data normalization, and the expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Gas chromatography–mass spectrometry (GC–MS) analysis

Five healthy plants with strong growth, no pests, and similar height were selected from each tobacco cultivar in the field, and their middle leaves (10th to 12th leaf from the stem base) were collected at maturity stage for extraction and determination of leaf exudates. Leaf sample preparation and pretreatment were performed using the leaf disk method as described by Han (1995). Briefly, leaf disks of 10 cm in diameter were cut out along both sides of the midrib, and 60 disks were obtained and divided into three repeats. The leaf disks were immersed in dichloromethane successively for extracting leaf exudates. Each immersion was repeated eight times of 2 s each. A total of 1 ml of internal standard solution (mixture of 2.020 mg ml^{-1} sucrose octaacetate and 2.542 mg ml^{-1} *n*-heptadecane alcohol) was added to each extract, mixed, concentrated in a rotary evaporator, and subjected to silylation treatment. Sample solutions were analyzed by GC–MS using TRACE GC ULTRA-DSQ II (Thermo Fisher Scientific, Waltham, MA, USA) as described by Wang et al. (2013); the profiles were compared qualitatively using the NIST12 spectral library.

Table 1 Primer sequences used in qPCR analysis

Gene	NCBI accession no.	Forward (5′–3′)	Reverse (5′–3′)
L25	L18908	CCCCTCACCACAGAGTCTGC	TTCTAACTCCTGTTGTTGTGGGAATGTACTGCTAAGACTTATGA
NtCYP71D16	AF166332	AAGACTTATGA	GGTTTCGAGTTCGTCCATT
NtCYC	AF401	CGACTTGCGAGGCAACAAGG	GAGCGAATTCCATGTTCAATGAC
NtABS	HE588140.1	AGTTTGCCGTTTAGAATGGTAC	TTATGGGGAATATTGATTGAGTG
NtCPS2	HE588139.1	CAGTAGTGCCTTTCCTCTCTCA	ATGTTATTTCAGAATTACTGTCAGC

Aphid preference to different leaves

One young leaf (10–15 cm in length) from each cultivar was placed in a closed container at room temperature; they were arranged evenly in a circle. Repeat 3 times. Horizontal clapboard with small holes was placed in the container; water was added under the clapboard, and gauze and filter papers were placed on the clapboard. To prevent wilting and keep the surfaces dry, a small plastic disk was placed on the filter paper. The blades of the leaves were placed on the plastic film, whereas the leaf base was coated with absorbent cotton outside the filter paper. A few wingless aphids were released in the center of the circle to migrate to the leaves of preference. The number of aphids on each leaf was counted 2 d after the release.

Statistical analysis

One-way analysis of variance (ANOVA) in conjunction with the least significant difference multiple comparison test was performed to identify differences among the cultivars. Pearson's correlation coefficients between aphid numbers and content of total secretion, content of CBT-diol were also calculated. All analyses were performed using SPSS 19 software (IBM, New York, NY, USA).

Results

Diversity of foliar chemical profiles

The GC–MS profiles showed that the two parental species differed in the composition and content of trichome secretions, especially for diterpenoids (Fig. 2a). The glandular trichomes of *N. sylvestris* could synthesized and secreted cembranoids, but not labdanoids (Fig. 2b), whereas the glandular trichomes of *N. tomentosiformis* could synthesized and secreted small amount of labdanoids, but not cembranoids (Fig. 2c). The trichomes of all four tobacco cultivars could synthesize and secrete cembranoids (Fig. 2b); however, only the trichomes of Beinhart 1000-1 and Basma YNOTBS1, but not that of K326 and Dabai 1, could synthesize and secrete labdanoids (Fig. 2c). The quantity of diterpenoids for each cultivar is presented in Table 2. The results showed that the content of diterpenoids was significantly different among cultivars. The total diterpenoids content in *N. sylvestris* was the highest, followed by Beinhart 1000-1, Basma YNOTBS1, K326, and *N. tomentosiformis.* The cembranoids content in *N. sylvestris* was the highest, followed by Beinhart 1000-1, Basma YNOTBS1, and K326; while the labdanoids content in Basma YNOTBS1 was the highest, followed by Beinhart 1000-1, and *N. tomentosiformis.*

Table 2 Leaf exudates of four *Nicotiana tabacum* cultivars, *Nicotiana sylvestris*, and *Nicotiana tomentosiformis*

No.	Time (min)	Compound	Content (μg cm^{-2})					
			A	B	C	D	E	F
1	37.67	α-Cembratriene-ol	0.03	0.4	0.16	0.01	0.2	–
2	38.56	β-Cembratriene-ol	0.06	0.29	0.14	0.01	0.06	–
3	40.94	Cis-abienol	0	2.14	3.33	–	–	0.42
4	42.81	α-Cembratriene-diol	30.2	38.26	33.91	14.8	58.17	–
5	44.64	β-Cembratriene-diol	11.44	15.92	12.92	5.49	23.99	–
6	43.91	Isomers of cembratriene-diol	0.27	0.23	0.22	0.07	0.18	–
7	47.32		2.41	2.28	1.02	1.06	1.59	–
8	48.04		0.31	0.43	0.28	0.1	0.41	–
9	48.42		0.35	0.4	0.67	0.26	0.29	–
10	49.16		0.31	0.41	0.35	0.16	0.44	–
11	49.38		1.63	1.86	1.75	0.88	1.27	–
12	49.92		0.28	0.31	0.41	0.16	0.23	–
13	50.43		0.14	0.17	0.15	0.05	0.19	–
14	53.88	Labdenediol	–	–	0.7	–	–	–
		Total cembranoids	47.43^{d}	60.96^{b}	51.98^{c}	23.05^{e}	87.02^{a}	0^{f}
		Total labdanoids	0^{d}	2.14^{b}	4.03^{a}	0^{d}	0^{d}	0.42^{c}
		Total diterpenoid	**47.65^{d}**	**63.1^{b}**	**56.01^{c}**	**23.05^{e}**	**87.02^{a}**	**0.42^{f}**

– Not detected

A, K326; B, Beinhart 1000-1; C, Basma YNOTBS1; D, Dabai 1; E, *Nicotiana sylvestris*; F, *Nicotiana tomentosiformis*

Trichome morphology and density

The morphology of the glandular trichomes varies greatly between the two parental species (Fig. 3). The leaf surface of *N. sylvestris* contained three kinds of trichomes: long-stalked glandular trichomes (i.e., types I-like, IV-like, and VI-like), short-stalked glandular trichomes (i.e., type VII-like), and non-glandular trichomes (i.e., type III-like), whereas the leaf surface of *N. tomentosiformis* did not contain any non-glandular trichomes (i.e., type III-like). Additionally, the leaf surface of K326 contained three kinds of trichomes (i.e., types I-like, III-like, IV-like, VI-like, and VII-like), whereas the leaf surface of Beinhart 1000-1, Basma YNOTBS1, and Dabai 1 did not contain any non-glandular trichomes (i.e., type III-like). In addition, according to the observations of the morphology of glandular trichomes on different leaves in same adult plants of four *Nicotiana tabacum* cultivars and two parental species,the morphology of trichomes did not show any changes with plant growth and leaf position (Fig. S1).

The total trichome density per mm^2 in *N. sylvestris* (31.30) was significantly higher than that in *N. tomentosiformis* (19.33) ($P \leq 0.05$). The total trichome density in the four cultivars differed greatly, with the highest in Beinhart 1000-1 (37.86), followed by K326 (22.27), and relatively low in Dabai 1 (4.54) and Basma YNOTBS1 (2.91). All of them were significantly different with the two parental species ($P \leq 0.05$) (Fig. 4).

Expression of key genes involved in diterpenoid biosynthesis

Two key genes responsible for cembratrien biosynthesis, *NtCYC* and *NtCYP71D16*, were expressed in *N. sylvestris*, but not in *N. tomentosiformis*. However, two key genes for labdanoid biosynthesis, *NtABS* and *NtCPS2*, were expressed in *N. tomentosiformis*, but not in *N. sylvestris* (Fig. 5a). The four genes were highly expressed in Basma YNOTBS1 and a bit less in Beinhart 1000-1. For K326 and Dabai 1, *NtABS, NtCYC* and *NtCYP71D16* had high expression patterns, while *NtCPS2* was expressed at a markedly low level (Fig. 5b).

Aphid preference assay and correlation with glandular secretions

Aphid preference assay revealed that aphid attraction and host preference were different between tobacco species and cultivars (Fig. 6a). The number of aphids on the leaves of *N. sylvestris* (126) was significantly higher than that of *N. tomentosiformis* (25) ($P \leq 0.05$). Additionally, the number of aphids on the leaves of Beinhart 1000-1 (140) was significantly higher than that of Basma YNOTBS1 (73), K326 (66), and Dabai 1 (60) ($P \leq 0.05$) (Fig. 6b). The number of aphids on the leaves of Beinhart 1000-1 was not different from that on the leaves of *N. sylvestris*, but significantly higher than that on the leaves of *N. tomentosiformis* ($P \leq 0.05$); the number of aphids on the leaves of Basma YNOTBS1 and K326 was significantly lower than that on the leaves of *N. sylvestris*, but significantly higher than that on the leaves of *N. tomentosiformis* ($P \leq 0.05$); and the number of aphids on the leaves of Dabai 1 was significantly higher than that on the leaves of *N. tomentosiformis*, but significantly lower than that on the leaves of *N. sylvestris*.

Correlation analysis showed that insect attraction and host preference were significantly and positively correlated with the total amount of glandular secretions ($r^2 = 0.9425$; $P \leq 0.05$) (Fig. 6c) and the CBT-diol amount ($r^2 = 0.9224$; $P \leq 0.05$) (Fig. 6c).

Discussion

In this study, the surface of K326 leaves contained three kinds of trichomes (i.e., types I-like, III-like, IV-like, VI-like, and VII-like), whereas that of Beinhart 1000-1, Basma YNOTBS1, and Dabai 1 leaves did not contain any non-glandular trichomes (i.e., type III-like). The parental species *N. sylvestris* and *N. tomentosiformis* also showed different glandular morphology: the surface of *N. sylvestris* leaves contained both glandular and non-glandular trichomes (i.e., types I-like, III-like, IV-like, VI-like, and VII-like), whereas that of *N. tomentosiformis* leaves contained only glandular trichomes (i.e., types I-like, IV-like, VI-like, and VII-like). Therefore, the non-glandular trichomes (i.e., type III-like) of K326 probably originated from *N. sylvestris*.

In tobacco, the trichome density is affected by environmental conditions such as illumination, temperature, fertilization, and irrigation (Liang et al. 2009; Shi et al. 1999; Weng et al. 2009; Wilkens et al. 1996; Zhang et al. 2012), therefore, the trichome density of mature leaves might be biased by agronomic and environmental factors (Zhang et al. 2015). That is why we used young leaves for trichome density analysis, and to minimize the influence of external environment, the growing young plants were kept at a certain environment that was controlled by human.

Our results were in agreement with those reported by Simmons and Gurr (2006), which found that *N. sylvestris* produces only cembranoids, whereas *N. tomentosiformis* produces labdanoids only; however, they were in disagreement with those reported by Glas et al. (2012), which found that *N. tomentosiformis* produces both labdanoids and cembranoids, whereas *N. sylvestris* produces only the latter type of diterpenoids.

According to previous research on the tobacco trichome function, the tall glandular trichomes are the main synthesis

and secretion site of diterpenoids (Meyberg et al. 1991), and the short glandular trichomes are the main synthesis and secretion site of the surface-localized defensive protein phylloplanins controlled by the gene *T-phylloplanin* (Shepherd et al. 2005); thus, the secretory function of trichomes differs in relation to their morphology (Yong et al. 2012). Wahlberg and Enzell (1987) reported that the biochemical profile of diterpenoids differs among the tobacco types. Oriental tobacco normally contains both types of diterpenoids, whereas burley tobacco contains only cembranoids. Additionally, Leffingwell (1999) revealed that cigar tobacco also contains two kinds of diterpenoids, whereas flue-cured tobacco contains only cembranoids. Our results are in line with the above findings, the gas chromatography–mass spectrometry profiles of leaf exudates indicated that Beinhart 1000-1 (cigar) and Basma YNOTBS1 (oriental) synthesized cembranoids and labdanoids; and K326 (flue-cured) and Dabai 1 (burley) synthesized only cembranoids.

The expression analysis of genes related to diterpenoid biosynthesis in the two parental species showed that the two key genes responsible for cembratrien biosynthesis, *NtCYC* and *NtCYP71D16*, were expressed in *N. sylvestris*, but not in *N. tomentosiformis*; however, two key genes involved in labdanoid biosynthesis, *NtABS* and *NtCPS2*, were expressed in *N. tomentosiformis*, but not in *N. sylvestris*. These results might explain the reason why *N. sylvestris* synthesizes only cembranoids, whereas *N. tomentosiformis* synthesizes only labdanoids (Simmons and Gurr 2006). In addition, the four key genes mentioned above, *NtCYC, NtCYP71D16*, *NtABS*, and *NtCPS2,* were highly expressed in Basma YNOTBS1 and Beinhart 1000-1. The genes responsible for cembratrien biosynthesis, *NtCYC* and *NtCYP71D16*, and the gene involved in labdanoid biosynthesis, *NtABS*, were highly expressed in K326 and Dabai 1, whereas *NtCPS2* was expressed at a markedly low level. These results might explain why Basma YNOTBS1 and Beinhart 1000-1 synthesize both types of diterpenoids, whereas K326 and Dabai 1 synthesize only cembranoids. Thus, *NtCYC* and *NtCYP71D16* expressed in four tobacco cultivars might be phylogenetically originate from *N. sylvestris*, whereas *NtABS* and *NtCPS2* expressed in Basma YNOTBS1 and Beinhart 1000-1 might be derived from *N. tomentosiformis*.

Our results showed that the relationship between the leaf trichome density and secretion amount was quite complex. For example, our results show that *N. sylvestris* synthesized and excreted more diterpenoids, although its trichome density was lower than that of Beinhart 1000-1. Our results were in agreement with those reported by Peng-Fei (2008) that showed a positive correlation between the trichome density and secretion accumulation. Therefore, at the molecular level, the content of trichome exudates is determined by both the density of glandular trichomes and the intensity of trichome metabolism. The expression level of *NtCYC* and *NtCYP71D16* in Basma YNOTBS1 was significantly higher than that in Beinhart 1000-1, whereas the trichome density followed the opposite trend. These results showed that, at the transcriptional level, Basma YNOTBS1 had a relatively higher ability to synthesize cembranoids than Beinhart 1000-1. Therefore, the high expression level of key genes might explain the high secretory volume of trichomes.

Labdanoids were detected in Beinhart 1000-1 and Basma YNOTBS1; labdenediol was detected only in Basma YNOTBS1, whereas Z-abienol was detected in both cultivars. Furthermore, the content of Z-abienol was the highest in Basma YNOTBS1. To further analyze the results, we examined the expression level of key genes involved in labdanoid biosynthesis. The results showed that, although the expression levels of *NtABS* were all high among the four cultivars, *NtCPS2*, the upstream regulation gene of *NtABS* in the labdanoid synthesis pathway was highly expressed only in Beinhart 1000-1 and Basma YNOTBS1. These results might explain the molecular mechanism of the foliar chemical characteristics at the transcriptional level in Basma YNOTBS1. The relationship between the expression of *NtCPS2* and the content of secreted labdanoids confirmed that the role of *NtCPS2* in labdanoid biosynthesis is critical and that it is possible to improve the aroma quality of K326 by restoring the labdanoid synthesis pathway.

Many studies have confirmed the role of diterpenoids in insect resistance (Ennajdaoui et al. 2010; Kennedy et al. 1995; Lin and Wagner 1994; Wang et al. 2001). Our results indicated that the aphid resistance of Beinhart 1000-1 and *N. sylvestris* was lower than that of the other studied materials. We also identified a significantly positive correlation between aphid preference and total glandular secretion as well as CBT-diol components. No relationship was found between aphid preference and other diterpene components (Fig. S2). Thus, further studies are needed to the better understand the contribution of diterpenoids to insect resistance.

Diterpenoids are important precursors of tobacco aroma substances and also influence insect resistance; thus, identifying the factors involved in the diterpenoid synthesis and secretion of glandular trichomes might help to further improve the aroma of tobacco and the biotic stress resistance of hairy plants. Our results showed that glandular trichome secretion was affected by the density of glandular hairs and the expression of key genes, and thus, they might be useful for improving abiotic and biotic stress resistance in crop plants.

Author contribution statement Mingyue Huang and Hongying Zhang performed the experiments. Zhaojun Wang participated in data analysis. Dexin Niu performed the qRT-PCR experiments, and Yanhua Li projected design and supervision.

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