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IDENTIFICATION OF VOLATILE AROMA COMPOUNDS OF *TOONA SINENSIS* (A. JUSS) ROEM BUDS AND INVESTIGATION OF GENES EXPRESSION PROFILES CONFERING AROMA PRODUCTION

REN LIPING[1,2], WAN WENYANG[1], XIE YUNHAO, CHEN BEIBEI, WANG GUOSHU[3], YIN DANDAN[1], SU XIAOHUI[1] AND CAO XIAOHAN[1*]

[1]*Key Laboratory of Horticultural Plant Biology of Biological and Food Engineering School, Fuyang Normal University, Fuyang, Anhui 236037, China*
[2]*Fuyang Academy of Agricultural Sciences, Fuyang, Anhui 236000, China*
[3]*Forestry Nursery of Taihe County, Fuyang, Anhui 236600, China*
[*]*Corresponding author's email: caoxiaohan@163.com*

Abstract

Aroma is one of the important indexes of *Toona sinensis* (A. Juss) Roem quality that also determines its edible and commercial value. To detect the existing compounds of volatile aroma and related biosynthetic genes that the variety 'Heiyouchun' (named THToa) originated in the county of Taihe (Province of Chinese Anhui), confer aroma training in *Toona sinensis* was studied. By using the headspace solid-phase micro extraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS) methods, the chemical composition and relative content were analyzed. The gene expression of key enzymes including terpene synthase (*TsTPS*), farnesyl diphosphate synthase (*TsFPS*), 1-deoxy-d-ribulose 5-phosphate synthase (*TsDXS*), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*TsHMGR*) and two important regulators, *TsAP2/ERF* and *TsWRKY*, were also detected by real-time PCR (RT-PCR) at different developmental periods of leaves. The results showed that a total of 24 compounds were detected from the volatile oil of buds of THToa, which were mainly terpenes, esters, alcohols, ethers, aldehydes and thiophenes. Among them, α-caryophyllene (8.521%) was the most abundant, followed by prop-l-enyldithiopro (8.214%), 1-propyl-2-(4-thiohepth) (7.155%), aromadendrene (5.106%) and γ-elemene (6.928%). *TsDXS*, *TsHMGR1*, *TsTPS*, *TsAP2* and *TsWRKY47* showed first low expression, then elevated and then decreased, while *TsFPS2* showed early high expression during leaf growth and then gradually decreased tendency. It was speculated that the differential expression of these key genes during the growth of *Toona sinensis* leaves was closely related to the synthesis of aroma components. The results of this study may provide a foundation for elucidating the formation mechanism of *Toona* aroma, evaluating the quality of buds, as well as facilitating the molecular assisted breeding and development and utilization of *Toona Sinensis*.

Key words: *Toona sinensis*; Volatile components; Gene expression; Aroma.

Introduction

The aroma of plants consists of volatile components that are synthesized in a range of micro concentrations and have different types of functions through secondary metabolism pathways of a plant with a limited molecular weight and a heavy boiling point (Dudareva *et al.*, 2013). In terms of ecological value, plant aroma has played an important role in plant reproduction, since it can attract pollinators (Koeduka, 2018). Plants can also defend against insects and pathogenic microorganisms through volatile substances (Gray *et al.*, 2015). In the medical research field, researchers have found multiple functions of plant aroma in therapies (Buchbauer *et al.*, 1993). For example, the volatile aroma of lavender oil help alleviate the distressing symptoms of anxiety (Shaw *et al.*, 2007), the volatile aroma of Selaginella has a tranquilizing effect (Kagawa *et al.*, 2003), and application of plant aroma therapy to patients with dementia has proved to be curative (Thorgrimsen *et al.*, 2003; Fujii *et al.*, 2008). In terms of food consumption, the aroma of fruits and vegetables is an important indicator of their flavour quality. Consumers like fruits and vegetables with strong aroma. Therefore, improving the aroma of fruits and vegetables can not only increase their quality but also enhance market competitiveness (Kulkarni *et al.*, 2012). The growing market demand for fruit and vegetable quality has given more and more focus to the study of the formation of the aroma of fruit and vegetables.

Toona sinensis (Juss) M. Roem is a deciduous tree plant of *Toona Sinensis* in the Meliaceae family. Its tender leaves and buds have a strong aroma, unique flavour, and rich nutrition. Taihe *Toona Sinensis*, which originated in Taihe county, Anhui province of China, has a long history of cultivation. There are many kinds of varieties of *Toona Sinensis* in Taihe, among which the 'Heiyouchun' variety (named THToa) has the best quality. The *Toona sinensis* sprouts are thick, fat and tender with the best oils, strong fragrances, crunchiness, good taste, and good quality before the grain rain is formed. Also, the tender bud is rich in protein, amino acids, soluble sugar, vitamins and other nutrients. As well as flavonoids, alkaloids and other active ingredients, which have high nutritional and health value and is loved by people (Qiao *et al.*, 2016). It's reported that the main volatile components were alkenes, aldehydes, sulfur, ketones, alcohols, esters, and hydrocarbons (Gao *et al.*, 2016).

In plants, terpenesynthase (TPS) are important genes regulating the synthesis of a wide range of terpenoids (Chen *et al.*, 2011). In *Picea sitchensis*, the synthesis of carene is found regulated by PsTPS (Hall *et al.*, 2011). 1-deoxy-d-ribulose 5-phosphate synthase (DXS) is the first key enzyme of the 2-c-methyl-d-erythritol-4-phosphate (MEP) pathway. In *Arabidopsis*, the DXS enzyme is the main limiting enzyme of the MEP pathway, and overexpression of the DXS gene increased isoprenoid products and photosynthetic rate (Wright *et al.*, 2014). 3-hydroxy-3-methylglutaryl coenzyme. A reductase (HMGR) is the rate-limiting enzyme in terpene biosynthesis in the cytosol (Liu *et al.*, 2018). Studies have shown that transcriptional controls of biosynthetic volatile compounds are not limited to the final biochemical stage, but co-regulated in one or more steps (Muhlemann *et al.*, 2012). Transcription factors can regulate the expression of multiple genes in a single biochemical pathway. ORCA3 in *Catharanthus roseus* is a transcription factor in AP2/ERF that controls the expression of genes involved in terpenoid indole alkaloid (TIA) synthesis (Zhang *et al.*, 2011).In *Artemisia annua*, the AaWRKY1 transcription factor is involved in the regulation of artemisinin synthesis, and ADS is a target gene of AaWRKY1 (Cai *et al.*, 2009).

Due to the difference in geographical distribution location and variety, different *Toona sinensis* cultivars have their unique taste and characteristics; also, *Toona sinensis* leaves have different intensities of aroma in different growth cycles, i.e. the forms and contents of volatile aroma compounds are different. Although the volatile composition analysis of *Toona sinensis* has been reported previously, few studies were made on THToa, the best variety with the best aroma, taste, and quality. In this study, HS-SPME / GC-MS was used to detect volatile compounds of THToa before grain rain coming and the differences in the expression levels of *TsDXS*, *TsFPS2*, *TsHMGR1*, *TsTPS*, *TsAP2* and *TsWRKY47* in different periods were analyzed by RT-qPCR. We hope to provide a theoretical basis for the identification of quality and the mechanism of aroma synthesis of *Toona sinensis*.

Materials and Method

Samples and processing: 'Heiyouchun' (THToa) was used as the experimental materials, which was harvested from young leaves three times in March-April 2017, located at Taihe County, Anhui Province of China. Tender fresh shoots of THToa with at least six branches and 5-10 cm in length, were collected and immediately placed in liquid nitrogen for fast freezing, then stored at -80°C for future use. Meanwhile, another THToa tender bud was picked on 10 April. A total of 2,0 g of tender bud was powdered and deionized in 20 ml of water and then converted into a 40 ml Brown silica pad container, which was sealed and then subject to a water bath at 40°C for 30 min, after this GC-MS analysis was performed and repeated 3 times.

GC-MS analysis: The SPME FIBER was placed in the GC-MS injection port and the samples were resolved at 250°C for 5 min for aroma composition analysis by GC-MS. GC conditions: HP-5 capillary column (30m×0.25 mm×0.25μm, Beijing Science and Technology Co., Ltd.), high-purity helium (He_2, 99.999%) as the carrier gas, and the flow rate was 1 ml·min^{-1} with splitless injection. The gas chromatography temperature program was as follows: initial temperature of 40°C, held for 1 min, increased to 80°C at a rate of 20°C·min^{-1}, increased again to 160°C at a rate of 20°C·min^{-1}, and finally increased to 250°C at a rate of 20°C·min^{-1}, holding for the end of 5 min.MS conditions: Shuttle line temperature was set at 250°C, electron ionization source, mass spectrometer ion source temperature was set at 230°C, quaternary bar temperature was set at 150°C, ion source excitation energy was set at 70eV. Full scanmode, ranging from 40-500 amu. The major ingredients in aroma have been established by computer search in accordance with standard mass spectrometry database, NIST 12 (National Institute of Standards and Technology, USA) and peak normalization of each composition has been used to define the relative percentage in volatile components.

RT-qPCR analysis: Sequences of the following genes, *TsDXS*, *TsFPS2*, *TsHMGR1*, *TsTPS*, *TsAP2,* and *TsWRKY47* were obtained from Genbank *Toona sinensis* genome database (Ji *et al.*, 2021). RT-qPCR primers were designed by Primer5.0 software. β-actingene of *Toona sinensis* was selected as the reference gene (Zhao *et al.*, 2017). The primers were synthesized by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China) (Table 1).

RNA extraction, cDNA synthesis and qRT-PCR program: Total RNA extraction from *Toona sinensis* leaves was performed according to the manufacturer's instructions, its quality and concentration were checked by agarose gel electrophoresis, and then stored at - 80℃ for further use. RNA was used as template to reverse transcribe into cDNA first strand by PrimeScript™RT reagent Kit with gDNA Eraser (Perfect Real Time) kit (Takara, Japan). Amplification conditions: pre-denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 1 min and final extension at 72°C for 10 min. The CT values of the reactions were collected using Light Cycler ® 480 software. Each sample was repeated 3 times and the final result was calculated using the $2^{-\Delta\Delta CT}$ method.

Table 1. Listed primer for RT-qPCR.

Gene	Primer F (5′-3′)	Primer R (5′-3′)
TsTPS	CTGGGATGATCTGGGAACT	CGATGAAGGCAATAATGTA
TsDXS	AAAACCCTTTCCTACACGC	TCCAGTACCACCACCCATT
TsFPS2	TTGAGGGTGTATTTATGGA	TCACTTCTGCCTCTTGTAT
TsHMGR1	GAAATCTTTGGACGGAATG	TGTTTGTACTCGCCACCAG
TsAP2	GGACCGAGGTCGAGAAGTT	GCATGGGCAGTGTCAAATC
TsWRKY47	CACCATTTCCCACCGTTAC	GCATGTCGTTGCCCTAATT
TsActin	TATGGTTGGTATGGGTCAGA	GTGTGATGCCAGATTTTCTC

Results and Analysis

Dominant aroma components and contents: As detected by GC-MS analysis, a total of 24 compounds were identified in the aroma composition of THToa tender buds, accounting for 82.44% of the total components (Fig. 1). In addition, a few other compounds were also present, but these could not be identified so far. The 24 identified compounds were mainly terpenes, esters, ethers, thioethers, thiophenes, aldehydes and alcohols, in which terpenes were the most abundant. The peak numbers, egress times, retention indices, compound names and peak areas for each of the 24 compounds are shown in Table 2. The main compounds identified were a-Caryophyllene (8.521%), Prop-l-enyl, dithiopro (8.214%), 1-Propyl-2-(4-thiohepth) (7.155%), γ-Elemenne (6.928%), Thiophene, 2, 4-dimethyl (5.951%), Aromadendrene (5.106%). Additionally, Bicyclo [4.4.0] dec-2-ene-4-ol, 2-methyl-9, 1-Cyclohexane, 1.1-Dodecanediol, diacetate, 2, 6, 10-Dodecatrien-1-ol, 3, 7, 11-trimethyl-, acetate. (E, E), α-Cubebene、Acetic acid 6, 6-dimethy were also found. The chemical structures of the dominant aroma compound components are shown in Fig. 2.

Expression profiles of aroma synthesis related genes in different development stages of *Toona sinensis* leaves: The expression levels of *TsDXS*, *TsFPS2*, *TsHMGR1*, *TsTPS*, *TsAP2* and *TsWRKY47* in leaves of THToa were measured by RT-qPCR. As shown in Fig. 3, the expression levels of *TsDXS*, *TsHMGR1*, *TsTPS*, *TsAP2,* and *TsWRKY47* genes increased first and then decreased during leaf growth and reached the peak on April 10. Among them, the expression levels of *TsHMGR1*, *TsAP2,* and *TsWRKY47* were the lowest on April 23; the expression levels of *TsTPS* and *TsDXS* were the lowest on March 28; while the expression levels of *TsFPS2* were higher in the early stage of leaf growth and then decreased gradually.

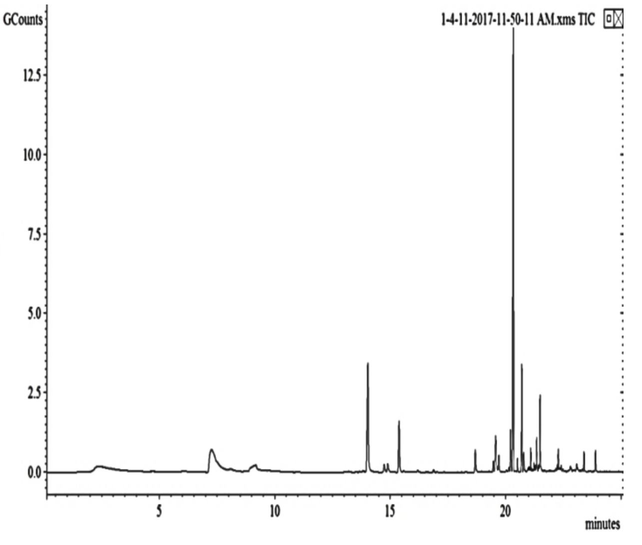

Fig. 1. Total ion chromatograms of aromatic components in tender buds of *Toona sinensis* in THToa.

Table 2. Chemical composition of *Toona sinensis* tender buds.

No.	Retention time	Compounds	Molecular formula	Matching degree	Area %
1.	2.600	Thiirane methyl-	C_3H_6S	748	1.727
2.	7.366	Thiophene, 2, 4-dimethyl-	C_6H_8S	940	5.951
3.	9.1O6	1H-lmidazole	$C_4H_4N_2$	961	2.169
4.	14.761	Allyl dithiopropanoate	$C_6H_{10}S_2$	858	1.905
5.	14.949	Prop-l-enyl dithiopro	$C_6H_{10}S_2$	849	8.214
6.	16.185	1.1-Dodecanediol, diacetate	$C_{16}H_{30}O_4$	863	3.511
7.	16.862	*cis*-3-Penten-1-ol	$C_{11}H_{20}O_2$	859	0.681
8.	19.459	1-Cyclohexane-	$C_{15}H_{24}O$	868	3.557
9.	20.215	δ-Gurjunene	$C_{15}H_{24}$	906	1.581
10.	20.320	β-Caryophyllene	$C_{15}H_{24}$	947	1.703
11.	20.513	Aromadendrene	$C_{15}H_{24}$	929	5.106
12.	20.702	Bicyclo[4.4.0] dec -2-ene-4-ol, 2-methyl-9	$C_{15}H_{24}$	929	4.173
13.	20.788	γ -Elemenne	$C_{15}H_{24}$	946	6.928
14.	21.085	α-Caryophyllene	$C_{15}H_{24}$	953	8.521
15.	21.285	1H-Cycloprop[e]azulen-4-ol	$C_{15}H_{26}O$	849	2.665
16.	21.348	Copaene	$C_{15}H_{24}$	912	1.131
17.	21.427	Naphthalene, 1, 2, 4a, 5, 6 ,8a-hexahydro-4 7-dimethyl-1-	$C_{15}H_{24}$	906	2.115
18.	21.495	α-Cubebene	$C_{15}H_{24}$	930	2.915
19.	22.173	1-Hydroxy-1, 7-dimethl	$C_{15}H_{26}O$	921	1.164
20.	22.233	1H-Cycloprop[e]azulen	$C_{15}H_{26}O$	800	2.196
21.	22.279	1-Propyl-2-(4-thiohepth)	$C_9H_{18}S_3$	817	7.155
22.	22.406	Acetic acid 6,6-dimethy	$C_{16}H_{24}O_4$	794	2.787
23.	22.489	10, 11-Dioxatricyclo[6.2.2.0(1.6)]	$C_{17}H_{14}N_4O_2S$	719	1.102
24.	23.849	2, 6, 10-Dodecatrien-1-ol, 3, 7, 11-trimethyl- acetate.(E,E)-	$C_{17}H_{28}O_2$	875	3.482
		Total			**82.44**

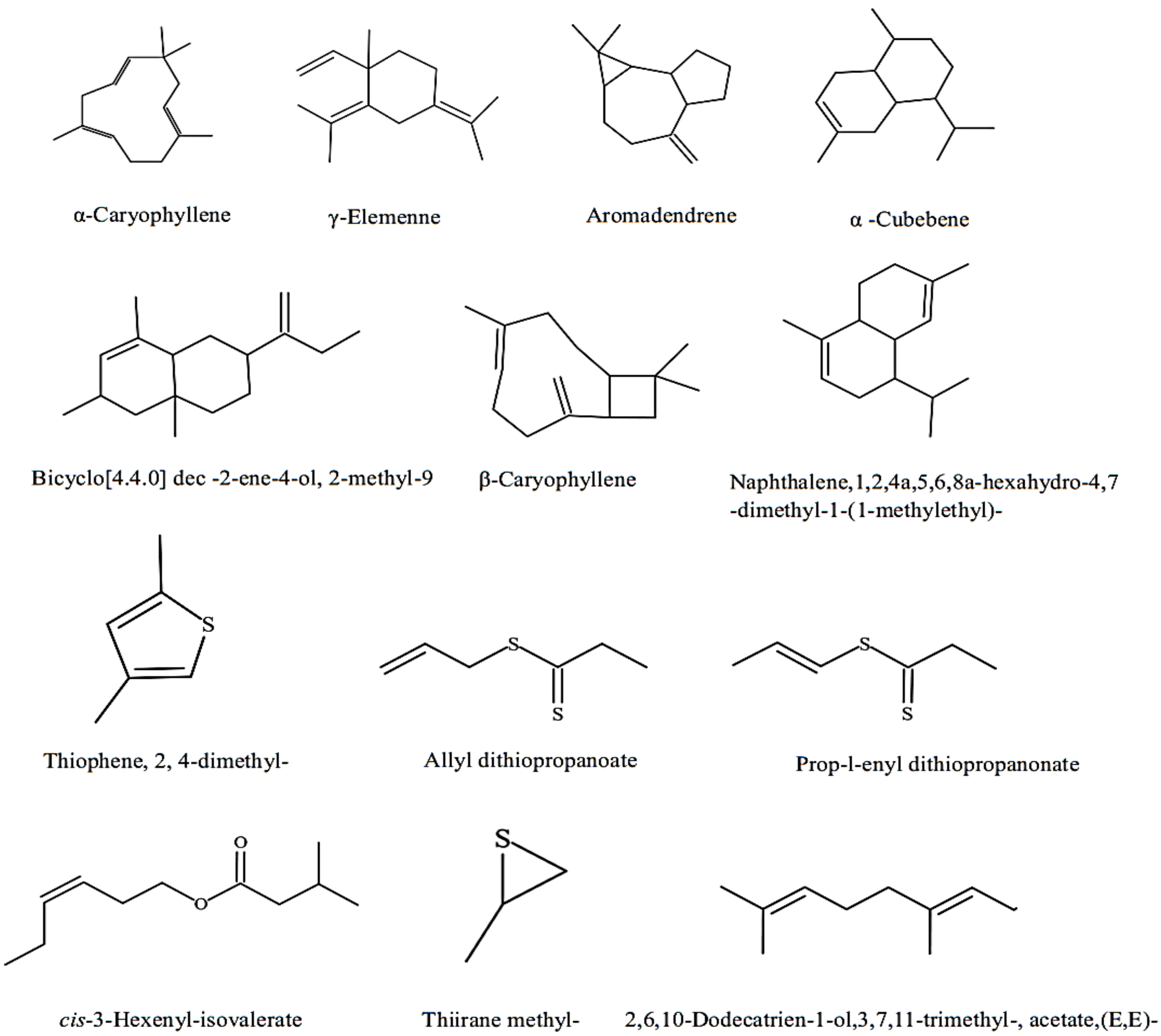

Fig. 2. Chemical structures of major phytoconstituents of *Toona sinensis* tender buds.

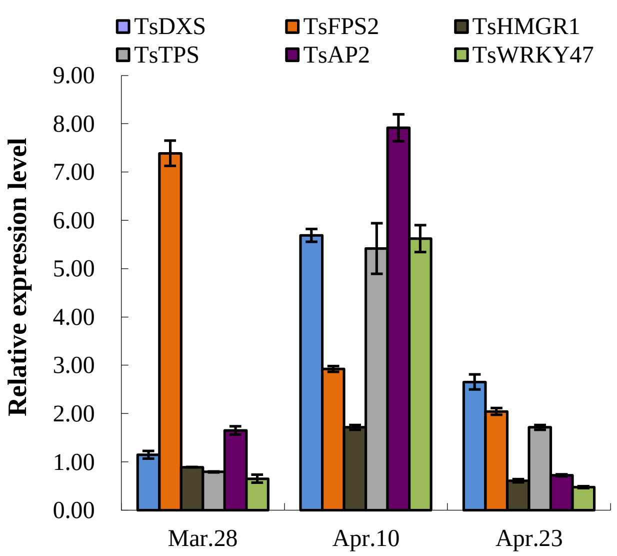

Fig. 3. Different expression patterns of *TsDXS*, *TsFPS2, TsHMGR1, TsTPS, TsAP2* and *TsWRKY47* in *Toona sinensis* tender buds.

Discussion

HS-SPME has been widely used as an effective method for the identification of volatile components of *Toona sinensis* owing to its advantages of simple operation, less sample consumption and good repeatability (Gao *et al.*, 2016). In this paper, HS-SPME and GC-MS were used to analyze and identify the volatile components in the buds of *Toona sinensis* variety 'Heiyouchun' collected before grain rain. The results showed that a total of 24 compounds were identified, including terpenes, esters, ethers, thiophenes, aldehydes, and alcohols. Gao *et al.*, identified 36 substances from the leaves of *Toona sinensis*, accounting for 55.3% of the total components and the main compounds were terpenes, thiophenes, aromatics, alcohols, ketones and phenols etc Terpenes among which were the largest, consistent with our findings (Sun *et al.*, 2019). Liu et al. analyzed the volatile components of three *Toona sinensis* varieties by GC-MS (Jie & Changjin, 2013). Their results showed that the highest content of volatile components in Shandong 'ximuhong'variety was thiophene compounds, followed by terpene compounds; the highest content of volatile components in Henan 'Jiaozuohong'variety was terpene compounds, followed by thiophene compounds; the main volatile components in Anhui Taihe'heiyouchun' variety were terpene compounds. They also showed that *Toona sinensis* from different areas had different tastes and characteristics owing to their different volatile components and unique odor. They found that terpenes and esters were the main components of volatile components in Taihe 'Heiyouchun' in Anhui Province which was consistent with the results of this study. However, the specific types and contents of compounds were different, which might be related to different harvest times and different tissues. In addition, sulfur compounds and terpenes were active, easily affected by temperature or others.

The DXS regulates the synthesis of terpenoids in plants as the first limiting enzyme in the MEP pathway, controling the supply of their precursors. Therefore, the DXS gene plays an important role in the synthesis of terpenoids (Xiang *et al.*, 2007). The expression of TsDXS has demonstrated a growing pattern in the *Toona sinensis* leaves growth stage, which was consistent with the highest expression trend of the DXS gene in the early stage of leaf development in *Pelargonium hortorum* (Jadaun *et al.*, 2017). FPS is an isopentyltransferase and a major isoprenoid pathway regulator. The expression of FPS in plants increases with the content of isoprenoid derivatives. In *Albizia julibrissin*, five TPSs are involved in the synthesis of its volatile terpenoids (Liu *et al.*, 2021). Many experiments have shown that HMGR is an important regulatory point in the MVA pathway, and genetic manipulation of HMGR can increase the content of terpenoids in plants. Transgenic *Lavandula angustifolia* expressing *Arabidopsis* HMGR gene accumulated more essential oils, which were composed of monoterpenes and sesquiterpenes (Muñoz-Bertomeu *et al.*, 2007). WRKYs transcription factors can act alone or in combination with other regulators to activate or inhibit transcription, thereby regulating genes involved in terpenoid synthesis (Schluttenhofer & Yuan, 2015). In *Gentiana scabra*, overexpression of CbWRKY24 and up-regulation of key genes in the MVA pathway led to the increase of total saponin content (Sun *et al.*, 2018).

Toona sinensis is not only a kind of medicinal material but also a vegetable with a unique flavor. Its volatile components have certain pharmacological effects. β-caryophyllene, one of the terpenoids, has a certain antiasthmatic effect and is one of the effective components in the treatment of senile chronic bronchitis. The biosynthesis of DNA, RNA, polysaccharide and ergosterol in fungi may be inhibited by copaene (Xia *et al.*, 1999). Most of the other volatile components of *Toona sinensis* also have pharmacological effects. The research on the volatile components can further expand the application in cosmetics, food additives and drug production so that *Toona sinensis* has greater development and utilization value.

In short, volatile components of the *Toona sinensis* variety Taihe 'Heiyouchun' were investigated through solid phase head space microextraction and the composition of the volatile components and relative contents of tender buds were clarified, which may help to understand the aroma formation mechanism of *Toona sinensis* and may also provide a basis for further development and utilization.

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