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BIOLOGY

Floral scent components in *Rhododendron fortunei* and its regulation by gene expression of *S*-adenosyl-L-methionine: benzoic acid carboxyl methyl transferase (BAMT)

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ABSTRACT: *Rhododendron fortunei* belongs to a scented Rhododendron species native to China, which produces fragrant flowers of great ornamental and environmental values for landscaping or indoor beautification. However, the scents in *Rhododendron fortunei* have not yet been investigated, let alone the mechanism of the formation of these fragrance in the flowers. In this study, we measured the scents in terms of its volatile components and contents (VOC) in *Rhododendron fortunei* at four different flowering stages and in different tissues of the plant by headspace solid-phase micro-extraction combined (HS-SPME) with gas chromatography-mass spectrometry (GC-MS). Then the characteristic aromatic values, which reflects the degree of scent perception by human, of each VOC in the plant was calculated according to its respective aromatic thresholds. Results showed that three main VOCs measured from highest to lowest are methyl benzoates, terpenes and fatty acid derivatives. Their content increased after the flower bud opening and reached the highest at half to full blossom. In a flower most VOC contents were measured in petals and only trace amount in other tissues such as stamen, pistil. A small amount of VOCs was determined in leaves as well.All aromatic values were almost corresponded to the contents of three main VOCs, indicating that the flower fragrance arises truly from these VOC components. S-adenosyl-L-methionine: benzoic acid carboxyl methyl transferase (BAMT) catalyzes the final step to form methyl benzoates. To understand the mechanism of the formation of this main type fragrance and its regulation, we firstly isolate a gene of RfBAMT from petal of *Rhododendron fortunei* by using homologous cloning and RACE technology. The full length of its cDNA was 1383 bp， with an open reading frame of 1104 bp, encoding a total of 368 amino acids. The phylogenetic tree analysis showed that RfBAMT was the closest to the BSMT of Camellia japonica, belonging to methyltransferases family. Then we measured the expression level of RfBAMT again at four flower developmental stages and in different flower tissues and leaves. The results showed that the expression level of this gene was highly positively correlated with the emitted content of methyl benzoates in the flowering, implying that RfBAMT plays a pivotal role in the formation and regulation of methyl benzoates in Rhododendron fortune.This researchshowed that the RfBAMT was cloned and identified in our study and its expression level was highly positively correlated with the emitted content of methyl benzoates in the flowers and leaves, which indicated this gene may play an important role on regulation of methyl benzoate synthesis in *Rhododendron fortunei*.
Key words: *Rhododendron fortunei* , volatile compounds, BAMT, GC-MS, HS-SPME.

Componentes de aroma floral em *Rhododendron fortunei* e sua regulação pela expressão gênica de S-adenosil-L-metionina: ácido benzóico carboxil metil transferase (BAMT)

RESUMO: *Rhododendron fortunei* pertence a uma espécie de rododendro perfumada nativa da China, que produz flores perfumadas de grande valor ornamental e ambiental para paisagismo ou embelezamento de interiores. No entanto, os aromas em *Rhododendron fortunei* ainda não foram investigados, muito menos o mecanismo de formação dessas fragrâncias nas flores. Neste estudo, medimos os aromas em termos de seus componentes e conteúdos voláteis (VOC) em *Rhododendron fortunei* em quatro diferentes estágios de floração e em diferentes tecidos da planta por microextração em fase sólida *headspace* combinada com cromatografia gasosa-espectrometria de massa. Em seguida, foram calculados os valores aromáticos característicos, que refletem o grau de percepção olfativa pelo ser humano, de cada VOC na planta de acordo com seus respectivos limiares aromáticos. Os resultados mostraram que três COVs principais medidos do mais alto ao mais baixo são benzoatos de metila, terpenos e derivados de ácidos graxos. Seu conteúdo aumentou após a abertura do botão floral e atingiu o máximo na metade da floração total. Em uma flor, a maioria dos teores de COV foram medidos em pétalas e apenas traços em outros tecidos, como estame, pistilo. Uma pequena quantidade de COVs foi determinada nas folhas também. Todos os valores aromáticos foram quase correspondentes aos teores de três COVs principais, indicando que a fragrância da flor surge verdadeiramente desses componentes de COV. Para entender o mecanismo de formação deste tipo principal de fragrância e sua regulação, primeiramente isolamos um gene de RfBAMT da pétala de *Rhododendron fortunei* usando clonagem homóloga e tecnologia RACE. O comprimento total de seu cDNA era de 1383 bp, com um quadro de leitura aberto de 1104 bp, codificando um total de 368 aminoácidos. A análise da árvore filogenética mostrou que RfBAMT foi o mais próximo do BSMT de *Camellia japonica,* pertencente à família das metiltransferases. Em seguida, medimos o nível de expressão de RfBAMT novamente em quatro estágios de desenvolvimento da flor e em diferentes tecidos e folhas de flores. Os resultados mostraram que o nível de expressão deste gene foi altamente correlacionado positivamente com o conteúdo emitido de benzoatos de metila na floração, implicando que RfBAMT desempenha um papel fundamental na formação e regulação de benzoatos de metila em *Rhododendron fortune* foi clonado e identificado em nosso estudo e seu nível de expressão foi altamente correlacionado positivamente com o conteúdo emitido de benzoatos de metila nas flores e folhas, o que indicou que este gene pode desempenhar um papel importante na regulação da síntese de benzoato de metila em *Rhododendron fortunei*.
Palavras-chave: *Rhododendron, Rhododendron fortunei*, perfume floral, BAMT.

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INTRODUCTION

Rhododendron is the largest genus in the family Ericaceae, with as many as 1024 species and amongst them 567 species representing 6 subgenera are native to Chinay (ZHANG et al., 2017 and QIAN et al., 2019). They are very popular grown as one of the major horticultural plants and mostly used for landscaping or indoor beautification. There is a plethora of varieties with different flower colors or shapes of Chinese *Rhododendron* but only a few wild species called the fragrant *Rhododendron* produce flowers of fragrance and habitat predominately in mountainous areas. The fragrant *Rhododendron* is obviously welcome in the flower plant market due to their strong aesthetic and emotional values. *Rhododendronfortunei* belongs to a fragrant *Rhododendron* species, which produces fragrant flowers. However, it is difficult to be popularized for commercial purposes because of its short flowering period and difficulties in adaptation to grow in low altitudes. It is plausible to breed fragrant *Rhododendron* by transferring the scent traits of *R. fortunei* to non-scent species through an approach of genetic engineering.

To explore the genetic material of *R. fortunei* for transforming floral scent in other *Rhododendron* species, we investigate the volatile constituents and the mechanism of it formation and regulation in *R. fortunei*. We firstly determined the scent volatile constituents in this species by headspace solid-phase micro-extraction combined with gas chromatography-mass spectrometry. The volatile components and relative contents in *R. fortunei* at four different flowering stages and in different tissues were measured and results shown that the main VOC constituent is benzenoid class compounds. As it is known that *S*-adenosyl-L-methionine: benzoic acid carboxyl methyl transferase (BAMT) catalyzes the final step to form methyl benzoates (DUDAREVA, N. et al., 2000), we then cloned the BAMT gene from this species and study its expression pattern in relation to the accumulation of aroma compositions.

MATERIALS AND METHODS

Plant material

The fresh flower petal during bud stage, middle opening stage, full opening stage and wilting stage (Fig. 1a) and the flower petal, stamen, and pistilat full opening stage and leaves (Fig. 2a) of *R. fortunei* were collected from Siming Mountain National Forest Park in Ningbo, China. Parts of them were directly used to determinate VOCs, and the remained parts were placed at -80 ℃ for storage. Wenguang Hu undertook the formal identification of the samples and provide details of specimens deposite in Flora of China. All *R. fortunei* material was obtained with permission.

Floral scent collection and analysis

The HS-SPME-GC-MS was used to collect the floral scent. Aging the 65μmol/L PDMS/DVB SPME fibers at injection port of GC and set the temperature at 250 ℃. Weigh 5g of the shredded sample rapidly and put it into an 8mL headspace bottle. And then insert the SPME injector into a sealed bottle. Headspace extraction for 1h at 30 ℃ water bath with 500 r/min stirring. After the extraction, insert the SPME fibers into injection port of GC and resolve for 5 min immediately. Three biological replicate measurements were performed on each sample.

Chromatographic conditions: Agilent lichrosorb 19091S-433 (30m x 250μm x 0.25μm); the temperature of column is 40 ℃; the injector was operated splitless at a temperature of 250 ℃ with He as a carrier gas at 1.0mL/min. The following temperature program was used: initial temperature of 40 ℃ (2min hold), increase to 160 ℃ at 3 ℃/min, 10 ℃/min ramp to 200 ℃, followed by a 20℃/min ramp to 300 ℃ (3min hold), with the port in splitless injection mode (DUDAREVA, N. et al., 2000).

Mass spectrometer conditions: ionization mode: EI; electron energy 70 eV; interface temperature: 250 ℃; ion source temperature: 230 ℃; quadrupole temperature 150 ℃; mass scan range: 15-500 AMU, solvent delay 2.6 min.

Preliminary identification of the VOCs was made by searching the NIST library and checked according to its retention index. The Identification results with a matching degree above 80 (maximum 100) are used (HANAA YAMANI et al., 2014). Benzyl benzoate (0.186g/L) was used as an internal standard to quantify the volatile compounds of the floral scent. 200μL of benzyl benzoate was placed in a headspace bottle with the sample for extraction. The content of each VOC (mg/kg) = [Peak area of each VOC/Peak area of internal standard] × the concentration of internal standard × the volume of internal standard (μL) $x10^{-3}$ / sample weight (kg). Then the aromatic value

was calculated for each components respectively according to the threshold of odor.

Isolation of the full-length cDNA

Total RNA was isolated from *R. fortunei* petal using the RNAprep Pure Plant Kit (TIANGEN, China). After passing the NanoDropTM2000 and 1% agarose gel electrophoresis, it was reverse transcribed to first-strand cDNA(CWBIO, China). Download the full length sequence of *BAMT* gene from plants similar to *Rhododendron* in GenBank, and then using ClustalW to find their conserved sequence. Finally, a pair of degenerate primers *BAMT-F1* and *BAMT-R1* were designed for PCR amplification by Primer 5.0 (Table 1). The PCR reaction procedure was determined as follows: predenaturation at 94 °C for 5 min, denaturation at 94 °C for 30s, annealing at 50 °C for 30s, extension at 68 °C for 1 min, a total of 35 cycles, and finally, 72 °C. Extend for 10 min and store at 4 °C (TRANS, China).

The PCR product was detected by 1.0% agarose gel electrophoresis. The fragment was extracted with plastic recycling kit, and then cloned to the vector pEASY-Blunt Zero (TRANS, China) and sequenced by Shanghai Sangon Biotech Company.

Gene-specific primers (5'GSP and 3'GSP, Table 1) were designed based on the sequence of middle segment. The SMARTer® RACE 5'/3'Kit (Takara) was used to isolate the 3' and 5' ends of the cDNA, and spliced a full-length cDNA sequence by DNAMAN.

Bioinformatics analysis

The open reading frame of nucleotide sequence and deduced amino acid sequence was analysed by ORF finder tool on the http://www.NCBI.com website. Conserved domains Research tool was used to predictive gene domain. Molecular weight and isoelectric point of the protein were predicted by the online software ProtParam (http://web.expasy.org/protparam/) on ExPASy. Homology evolution of amino acid sequence and members of the gene family were analysed by DNAMAN software, and constructed phylogenetic tree by MEGA 6.06 software.

Quantitative real-time PCR

To perform quantitative real-time PCR (qRT-PCR) analysis, total RNA was isolated from different flower developing stages and floral parts of *R. fortunei*. Primers for qRT-PCR were designed based on the *RfBAMT* cDNA sequence (Table 1). *EF1α* was selected as internal reference gene for each sample (Table 1). qRT-PCR was carried out using the Bio-Rad real-time PCR system with ChamQ™SYBR®Color qPCR Master Mix. The program was: 95 ℃ degeneration 3 sec, 40 cycles of 95 ℃ for 10 s, 60 ℃ for 30s, fluorescent signal acquisition in 60 ℃. All the qRT-PCR results were presented as means ± SD of three biological replicates. The relative expression level of genes were calculated by the $2^{-\Delta\Delta Ct}$ equation.

Table 1 - Primers for experiment.

Primers	Sequence (5'→ 3')	Purpose
BAMT-F1	GTTGTTGAWGTTCTTCACATGAATGG	Intermediate fragment amplification
BAMT-R1	ACTTCTKCTGGTGATGGTGTRTAYTGAGG	
5'GSP	GCTTGGTGGGCTATTGCTTCCGAT	5′-RACE amplification
3'GSP	GAATTGGTGACGGGTGGTCGCAT	3′-RACE amplification
UPM	TAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT CTAATACGACTCACTATAGGGC	universal primer
BAMT-F2	AGAGAGAGAGAGATGGAAGTGTATCA	Full-length cDNA amplification
BAMT-R2	ATAAAACATCACAACAATACCAACAT	
BAMT-F	TCTACTGTCCCTTGGAGTGCCT	Primer for qRT-PCR
BAMT-R	TCATACCTCTGGAAAACCTTGTCTA	
EF1α-F	TGTCATCGATGCTCCTGGAC	Reference Primer
EF1α-R	TCTCGGGTCTGACCATCCTT	

Table 2 - The contents of VOCs emitted from *R. fortunei* at four different flowering stages.

Number	Name of compound	Retention time	----------release content (mg/kg)----------			
			Bud	Middle opening	Full opening	Wilting
1	Bicyclo[3.1.1]hept-2-ene, 3,6,6-trimethyl-	9.035	0.06±0.01	0.36±0.02	0.54±0.04	-
2	Tricyclo[2.2.1.0(2,6)]heptane, 1,3,3-trimethyl-	9.319	0.37±0.02	0.55±0.06	-	-
3	Benzaldehyde	10.443	0.15±0.02	0.34±0.12	0.38±0.03	0.09±0.03
4	Cyclohexene, 4-methylene-1-(1-methylethyl)-	11.116	0.12±0.01	0.64±0.08	0.71±0.02	-
5	Eucalyptol	13.646	-	0.56±0.11	0.27±0.02	0.13±0.03
6	Benzoic acid, methyl ester	16.683	0.08±0.02	7.72±0.33	11.85±0.72	1.76±0.11
7	Linalool	17.084	-	1.55±0.08	1.02±0.1	0.36±0.04
8	2,6-Nonadienal, (E,Z)-	19.531	-	0.14±0.02	0.09±0.01	-
9	2-Nonenal, (E)-	19.842	-	0.17±0.02	0.08±0.02	-
10	Benzoic acid, ethyl ester	20.321	-	0.12±0.02	0.15±0.05	-
11	(3R,6S)-2,2,6-Trimethyl-6-vinyltetrahydro-2H-pyran-3-ol	20.521	-	0.49±0.04	0.56±0.05	0.15±0.03
12	alpha-Terpineol	21.3	-	2.52±0.23	2.11±0.18	0.2±0.04
13	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, acetate, (1S-endo)-	25.621	-	0.13±0.01	-	-
14	Cyclohexene, 4-ethenyl-4-methyl-3-(1-methylethenyl)-1-(1-methylethyl)-, (3R-trans)-	27.802	-	1.98±0.17	0.72±0.06	0.55±0.06
15	gamma-Elemene	31.99	0.11±0.03	0.58±0.03	0.31±0.06	-
16	trans-Isoeugenol	32.652	-	0.29±0.03	0.4±0.08	-
17	gamma-Muurolene	33.758	0.1±0.03	0.33±0.05	0.27±0.06	-
18	Benzene, 1,2-dimethoxy-4-propenyl-, (Z)-	34.704	-	0.24±0.03	-	-

Note: Values as average ± SD of measurements with triplicate samples.

RESULTS

VOC constituents and related aromatic values of compounds emitted from the flowers of R. fortunei

Eighteen VOCs were detected in *R. fortunei*, which can be divided into three classes: benzenoids, terpenoids, and fatty acid derivatives (Table 2 and Figure 1b). The content of benzenoids increased after the flower bud opening and reached the highest at full blossom from 0.23 to 12.79 mg/kg·FW; while terpenoids and fatty acid derivatives increased and reached the highest at half blossom from 0.21 to 5.53 mg/kg·FW, and for from 0.55 to 4.72 mg/kg·FW, respectively (Figure 1b). Among them 4 kinds of benzenoids derived from the shikimic acid pathway accounted for the highest content of VOC in each sample, which indicated that the benzenoids were the most dominant VOC components in floral scent of *R. fortunei*. A total of 5 terpenoids derived from the MEP and MVA pathways were also detected in this study. Terpenoids are the secondary abundant metabolites commonly reported in plants (DUet al., 2019), most of which have a strong sweet, floral and woody aroma. Fatty acid derivatives are the most diverse compounds having 15 species detected including alkanes, alkenes, alcohols, aldehydes and other compounds, derived from the lipoxygenase pathway (DUDAREVAet al., 2013). However, contents of fatty acid derivatives were significantly lower than both the contents of benzenoids and terpenoids.

The VOCs in different tissues and leaves were also measured and showed that most amount of VOCs were released from the petal and trace amount from stamen, pistil and even from leaves. The total release of VOCs from flower petal were 5.89-fold, 15.73-fold and 6.6-fold higher than from stamen, pistil and leaf, respectively (Figure 2b and Table 3). Again the compositions from high to low in all tissues and leaves was benzenoids the highest, followed by the terpenoids and then the fatty acid derivatives.

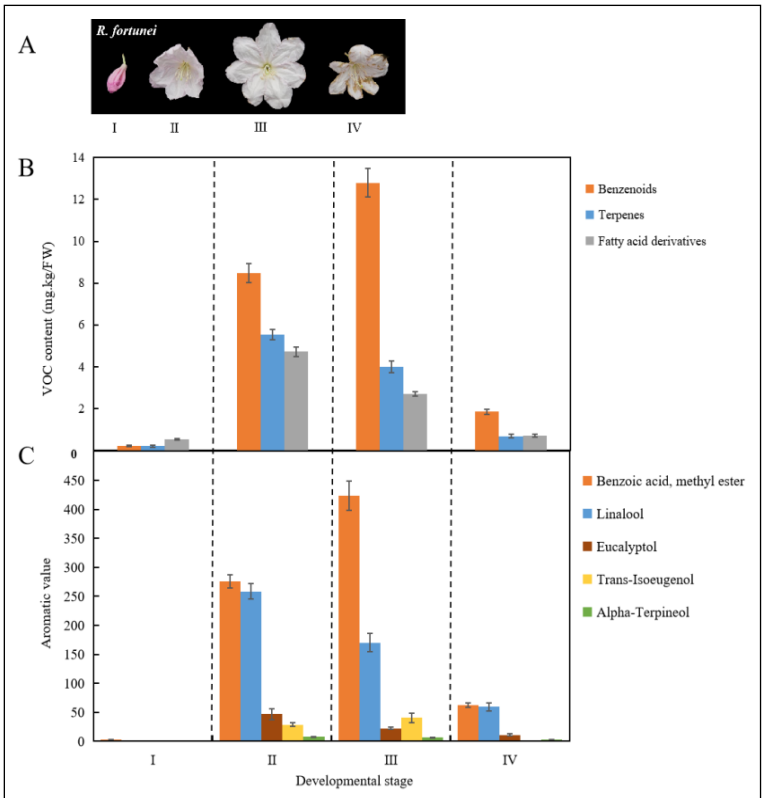

Figure 1 - VOCs content and related aromatic values of components emitted from *R. fortunei* at different flowering stages. (A) Flowers at different stages. IBud stage IIMiddle opening stage III Full opening stage IV Wilting stage. (B) The content of VOCs. (C) The characteristic aroma values of components. Data represent means and standard deviations of measurements with triplicate samples.

The aromatic value or odor unit of a specific released compounds were calculated based on the content of the VOCs dividing by its respective odor threshold, which reflects the degree of scent olfactory perception by human. If the odor unit is more than 1, we regard it as a characteristic aroma component. Since benzaldehyde and ethyl benzoate has a high aroma threshold, the odor unit is less than 1, so it is not a characteristic aroma component. According to this criteria the characteristic aroma components in flowers at different developmental stages and tissues of *R. fortunei* were listed in table 2 and 4. Moreover, the aromatic value of each VOCs was calculated and shown in table 3 and 5; figure 1c and 2c. The data shows that *all aromatic values were almost corresponded to the contents of three main VOCs,* indicating the flower fragrant odor arisen truly from these VOC components.

Cloning and sequence analysis of RfBAMT

We learned from above analysis that benzenoids are the main floral scent in *R. fortune*. It is also known that *S*-adenosyl-L-methionine: benzoic acid carboxyl methyl transferase (BAMT) catalyzes the final step to form methyl benzoates shown in Fig. 3a (DUDAREVA, N. et al., 2000). To understand the function of this enzyme in the formation of benenoids in the flowers of *R. fortune*, we first clone this gene. The petal cDNA of *R. fortunei* was used as a template for RT-PCR amplification, and obtained the middle segment sequence by cloning. And then the 3' and 5' ends fragments were cloned by RACE technology respectively. The conserved region was 806 bp, the 3' ends was 788 bp, and the 5' ends was 614 bp (Figure 3b). These three sequences were spliced to obtain a sequence of 1383 bp which was named *RfBAMT* (Figure 3c). *RfBAMT* contains an ATG start codon and a TGA

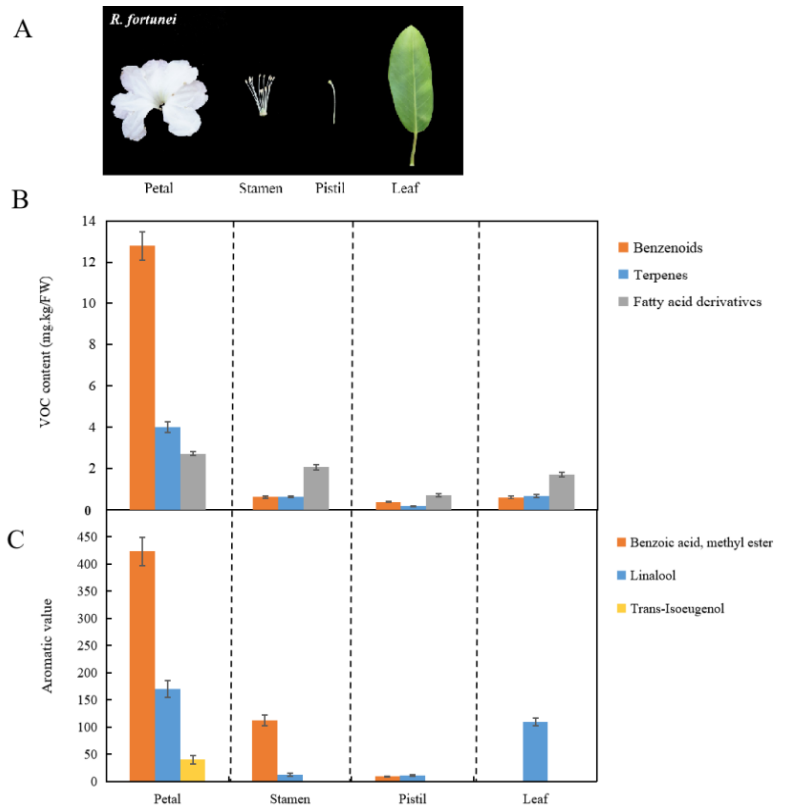

Figure 2 - VOCs content and related aromatic values of components emitted from different tissues of *R. fortunei*. (A) the flower tissues and leaf. (B) The content of VOCs. (C) The characteristic aroma values of components. Data represent means and standard deviations of measurements with triplicate samples.

stop codon, a tail signal AATAAA and a 26 bp polyA tail. Furthermore, the gene contains an ORF of 1104 bp, and an untranslated region with 5' UTR of 12 bp and 3'UTR of 267 bp. The deduced protein of the coding region containing 368 amino acid residues (Figure 3c), whose theoretical molecular weight is 41.09 kDa with an isoelectric point 5.19 predicted by ProtParam. Conserved domains Research tool showed

Table 3 - The characteristic VOCs and their aromatic value of the compounds emitted from *R. fortunei* at four different flowering stages.

Number	Floral component	Aromatic thresholds (mg/kg)	Characteristic aroma	Bud	Middle opening	Full opening	Wilting
1	Benzaldehyde	1.5	almond scent	0.1±0.01	0.23±0.08	0.26±0.02	0.06±0.02
2	Eucalyptol	0.012	camphor scent	-	46.67±9.17	22.5±1.67	11.11±2.1
3	Benzoic acid, methyl ester	0.028	wintergreen and eucalyptus oil scent	2.74±0.55	275.71±11.61	315.95±25.54	62.98±3.79
4	Linalool	0.006	woody scent	-	258.33±13.23	170±15.9	59.44±6.74
5	Benzoic acid, ethyl ester	300	fruit scent	-	0±0	0±0	-
6	alpha-Terpineol	0.33	clove scent	-	7.63±0.7	6.39±0.55	0.61±0.11
7	trans-Isoeugenol	0.01	clove scent	-	28.67±3.06	40.33±7.77	-

Table 4 - The contents of VOCs emitted from *R. fortunei* at different tissues.

Number	Name of compound	Retention time	Petal	Stamen	Pistil	Leaf
1	(E)-2-Hexenal	5.529	-	-	-	0.53±0.19
2	Bicyclo[3.1.1]hept-2-ene, 3,6,6-trimethyl-	9.035	0.54±0.04	-	0.09±0.01	-
3	Tricyclo[2.2.1.0(2,6)]heptane, 1,3,3-trimethyl-	9.319	-	0.72±0.13	0.43±0.02	-
4	Benzaldehyde	10.443	0.38±0.03	0.11±0.02	0.12±0.01	0.6±0.06
5	1-Octen-3-ol	11.024	-	-	0.11±0.03	1.17±0.09
6	Cyclohexene, 4-methylene-1-(1-methylethyl)-	11.116	0.71±0.02	0.15±0.04	-	-
7	beta-Pinene	11.227	-	-	0.05±0.01	-
8	Eucalyptol	13.646	0.27±0.02	0.05±0.01	-	-
9	Ethyl2-(5-methyl-5-vinyltetrahydrofuran-2-yl)propan-2-yl carbonate	16.416	-	-	0.07±0.01	-
10	Benzoic acid, methyl ester	16.683	11.85±0.72	0.5±0.04	0.25±0.03	-
11	Linalool	17.084	1.02±0.1	0.08±0.02	0.07±0.01	0.66±0.07
12	2,6-Nonadienal, (E,Z)-	19.531	0.09±0.01	-	-	-
13	2-Nonenal, (E)-	19.842	0.08±0.02	-	-	-
14	Benzoic acid, ethyl ester	20.321	0.15±0.05	-	-	-
15	(3R,6S)-2,2,6-Trimethyl-6-vinyltetrahydro-2H-pyran-3-ol	20.521	0.56±0.05	0.12±0.01	-	-
16	alpha-Terpineol	21.3	2.11±0.18	0.09±0.02	0.05±0.01	-
17	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, acetate, (1S-endo)-	25.621	-	-	-	-
18	Cyclohexene, 4-ethenyl-4-methyl-3-(1-methylethenyl)-1-(1-methylethyl)-, (3R-trans)-	27.802	0.72±0.06	1.07±0.06	-	-
19	gamma-Elemene	31.99	0.31±0.06	-	-	-
20	trans-Isoeugenol	32.652	0.4±0.08	-	-	-
21	(+)-epi-Bicyclosesquiphellandrene	33.135	-	0.05±0.01	-	-
22	gamma-Muurolene	33.758	0.27±0.06	0.3±0.02	-	-
23	Benzene, 1,2-dimethoxy-4-propenyl-, (Z)-	34.704	-	-	-	-
24	alpha-Muurolene	34.72	-	0.07±0.01	-	-

Note: Values as average ± SD of measurements with triplicate samples.

that the position of 38-366 amino acids contained a Methyltransf-7 superfamily's conserved region which is the characteristic domain of methyltransferases; therefore, identified as a true *BAMT* in *Rhododendron*.

Besides, the homology analysis of protein sequence revealed 70% homology with the amino acid ofBAMT、SAMT、BSMT in other plants by DNAMAN (Figure 4a). The results of cluster analysis of phylogenetic tree constructed by MEGA 6.06 appeared that the amino acid sequence of *RfBAMT* was related to *CjSAMT*, and they were clustered into the same subgroup (Figure 4a).

PhBSMT1 (*Petunia x hybrida*, AAO45012.1), PhBSMT2 (*Petunia x hybrida*, AAO45013.1), PhBSMT3 (*Petunia x hybrida*, ABF50941.1), AbSAMT (*Atropa belladonna*, BAB39396.1), DwBSMT (*Datura wrightii*, ABO71015.1), NsBSMT2 (*Nicotiana suaveolens*, ACZ55217.1), NsBSMT1 (*Nicotiana suaveolens*, CAF31508.1), SfSAMT (*Stephanotis floribunda,* CAC33768.1), CjSAMT (*Camellia japonica*, AGC11863.1), RfBAMT, AmSAMT (*Antirrhinum majus*, AAN40745.1), CbSAMT (*Clarkia breweri*, AAF00108.1), LiBSMT (*Lilium hybrid cultivar*, AIG92833.1), AmBAMT (*Antirrhinum majus*, AAF98284.1), AtSAMT (*Arabidopsis thaliana*, NP_001318222.1), AlBSMT (*Arabidopsis lyrata subsp. Lyrata*, AY224596.1), AtBSMT (*Arabidopsis thaliana*, AAY25461.1).

Expression pattern of RfBAMT

To clarify the role of RfBAMT on regulation of benezoid formation, we measured the expression of *RfBAMT* gene at four flowering stages

Table 5 - The characteristic VOCs and their aromatic value of the compounds emitted from *R. fortunei* at different tissues.

Number	Floral component	Aromatic thresholds (mg/kg)	Characteristic aroma	Petal	Stamen	Pistil	Leaf
1	Benzaldehyde	1.5	almond scent	0.26±0.02	0.08±0.02	0.08±0.01	0.4±0.04
2	beta-Pinene	0.006	floral scent	-	-	7.78±0.96	-
3	Eucalyptol	0.012	camphor scent	22.5±1.67	4.17±0.83	-	-
4	Benzoic acid, methyl ester	0.028	wintergreen and eucalyptus oil scent	315.95±25.54	12.36±9.42	9.05±0.9	-
5	Linalool	0.006	woody scent	170±15.9	12.78±2.55	11.11±0.96	109.44±6.58
6	Benzoic acid, ethyl ester	300	fruit scent	0±0	-	-	-
7	alpha-Terpineol	0.33	clove scent	6.39±0.55	0.28±0.05	0.16±0.02	-
8	trans-Isoeugenol	0.01	clove scent	40.33±7.77	-	-	-

and in different flower tissues in comparison to in leaves of *R. fortunei*. The results showed that the expression increased first from the flower buds stage and reached the peak of 3.48 fold increase at the full opening stage and then decreased after the flowers started to wilt (Figure 5a). *RfBAMT* was widely expressed in these tissues and leaves. However, the expression level in the petal was significantly 16.67 fold 1.64 fold and 1.59 higher than that in stamen, pistil and leaf, respectively (Figure 5b). Together with the results on the measurements of floral scent contents (Figure 1b and Figure 2b), it was revealed that this gene expression was highly correlated to the content of floral VOCs in the floral tissues and at different flowering stages, suggesting *RfBAMT* function in the regulation of benezoid metabolism *in R. fortune*.

DISCUSSION

Determination of floral compositions in R. fortunei

Floral scent is an important component part of plant volatile compounds (HU et al., 2017), which is a complex mixture of many low-molecular-mass and volatile compounds (INNA et al., 2002). So far, more than 1,700 floral volatile organic compounds (VOCs) have been identified from 90 different families of plants, most of which belong to terpenoids, benzenoids, and fatty acid derivatives (DU et al., 2019 and PICHERSKY et al., 2016).

In this study, three classes of the floral volatile compounds were detected in *R. fortunei* and among them more than 55% of VOC were benzenoids and thus it was the dominant scent constituent in the released compounds of flowers at the flowering stages and different tissues (Figure 1b and Figure 2b). Benzenoids are commonly found in plant VOC such as in *Prunus mume* (ZHANG et al., 2019), European *Narcissus* and especially in rose (VAN SCHIE et al., 2006) that more than 50% of the total released VOC is benenoids. Therefore, our results are consistent with these above reports. However, Su et al reported that the relative content of benzenoids in *Rhododendron* was only 16.6% of the total VOC released from the flowers which was lower than we measured in *R. fortunei*. This discrepancy could be caused by many factors such as plant material, cultivation environment and analysis conditions. Methyl benzoate belongs to benzenoids and full of strong wintergreen and eucalyptus oil scent which can be used to formulate rose flavor. In addition, it is also used as an additive for cosmetics and foods. As a benzenoid, methyl benzoate has a rich aroma of wintergreen and eucalyptus oil scent, and is the main aroma component of flowers. VERDONK et al., 2013found that the release of methyl benzoate was large in *Petunia*; the floral composition

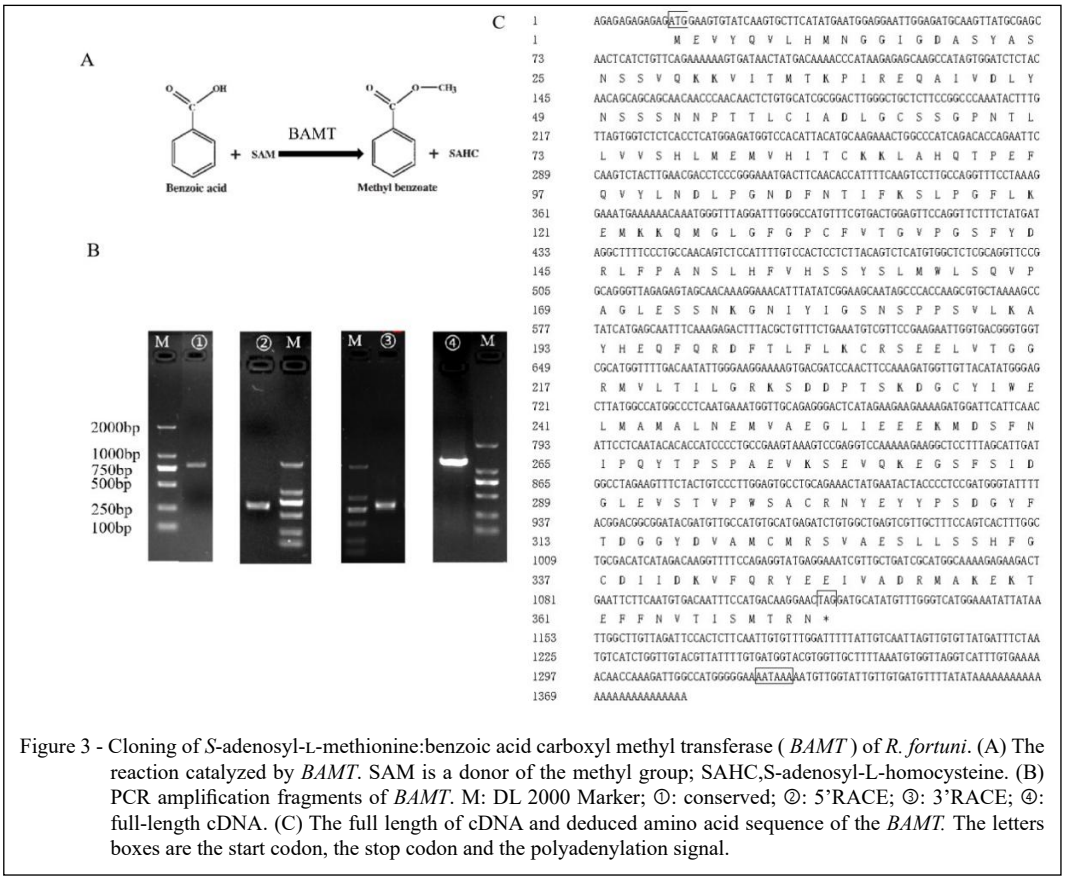

Figure 3 - Cloning of *S*-adenosyl-L-methionine:benzoic acid carboxyl methyl transferase (*BAMT*) of *R. fortuni*. (A) The reaction catalyzed by *BAMT*. SAM is a donor of the methyl group; SAHC,S-adenosyl-L-homocysteine. (B) PCR amplification fragments of *BAMT*. M: DL 2000 Marker; ①: conserved; ②: 5'RACE; ③: 3'RACE; ④: full-length cDNA. (C) The full length of cDNA and deduced amino acid sequence of the *BAMT.* The letters boxes are the start codon, the stop codon and the polyadenylation signal.

analysis of *Snapdragon* indicated that the relative content of methyl benzoate was as high as 60% (DUDAREVA et al., 2000); ZHANG et al.,(2013) found that the release amount of methyl benzoate in scented *Lilium* was higher, but it could not be detected in light-scented *Lilium*. This study demonstrated that the content of methyl benzoate was high in the flowering stage and different tissues in *R. fortunei*, and the aroma threshold was also low (0.028 mg/kg). It can be inferred that methyl benzoate identified as a key aroma component of *R. fortunei*.

Terpenoids and fatty acid derivates were also abundantly detected in the *R. fortunei* flowers, accounting for 10.43% and 8.69% of the total VOC emitted from the full opening flowers (Figure 1b and Figure 2b). As we mentioned above, these 3 classes of compounds contribute to the aroma value as well (Figure 1c and Figure 2c), which demonstrated the fragrance comes indeed from the combined three classes of VOCs.

Regulation of floral scent formation by RfBAMT in R. fortunei

As above shown that benzenoids are the dominant scent compounds in *R. fortunei*, we then focused on the biochemistry mechanism of its synthesis. Methyl benzoate is the main compound in benzenoid class and it is synthesized by benzoic acid/ salicylic acid carboxyl methyltransferase, catalyzing the transfer of the methyl donor benzoic acid to corresponding acids (Figure 3a) (DUDAREVA et al., 2000). The *BAMT* has been isolated from plants such as *Nicotiana suaveolens* (MARCELLA et al., 2004), *Petunia hybrid* (JULIAN et al., 2005) and *Snapdragon* (DUDAREVA et al., 2000). In this experiment, the full-length cDNA sequence of *BAMT* in *R. fortunei* was isolated by homologous and RACE cloning technology and identified as *RfBAMT* which could code putatively the enzyme of BAMT in *R. fortunei* (Figure 4).

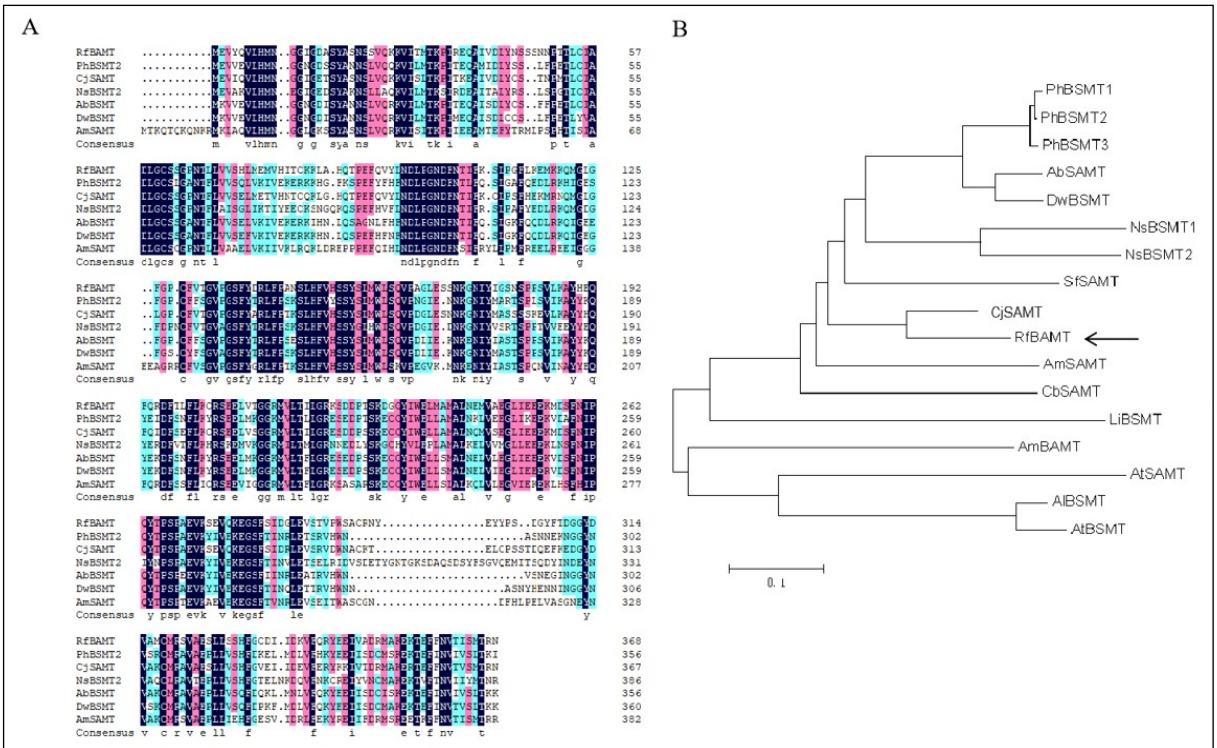

Figure 4 - BAMT identification. (A) Multiple alignment of amino acids of RhBAMT and BSMT、SAMT homologs from other plant species. (B) Phylogenetic tree of BAMT. Accession number in GenBank.

Then we examined its gene expression patterns both in different flowering stages and in different floral tissues and leaves of *R. fortunei* and our results showed that the expression level (Figure 5) was highly corresponded to the content of methyl benzoate (MeBA) (Figure 1b and Figure 2b), implying RfBAMT function in regulation of MeBA biosynthesis. However, to prove this function, we think that it is still required to measure the enzyme activity or protein amount of BAMT translated from its transcripts by biochemical assay or Western blot. Then we can make transgenic plants by knocking out or overexpressing the *RfBAMT* gene to assess its distinct role in the metabolism of benzenoids in *R. fortunei* and clarify whether the regulation of benzenoid biosynthesis is precursor-regulated when this enzyme only partial contributes to the total amount of MeBA content (VAN SCHIE et al., 2006). Furthermore, the transcriptomic approach could be used to address the floral scent mechanism in *Rhododendron* by comparing the scented *R. fortunei* with the non-scented *R. hybridae*, in hope that any transcription factor could be found, like Myb transcription factor ODORANT1 in *petunia* (JULIAN et al., 2005).

CONCLUSION

In summary, methyl benzoate was the dominant scent components emitted from the flowers of *R. fortunei*. At present, only the *RfBAMT* was cloned and identified in our study and its expression level was highly positively correlated with the emitted content of methyl benzoates in the flowers and leaves, which indicated this gene may play an important role on regulation of methyl benzoate synthesis in *R. fortunei*. To understand further the molecular mechanism of the regulation of the floral scent synthesis in *R. fortunei*, studies on other key genes involved in the biosynthesis of other main VOC components such as terpenoids and fatty acid derivatives are evidently warranted to illustrate the regulation network of floral scent metabolism and eventually identify the genetic targets for breeding fragrant varieties of *Rhododendron.*

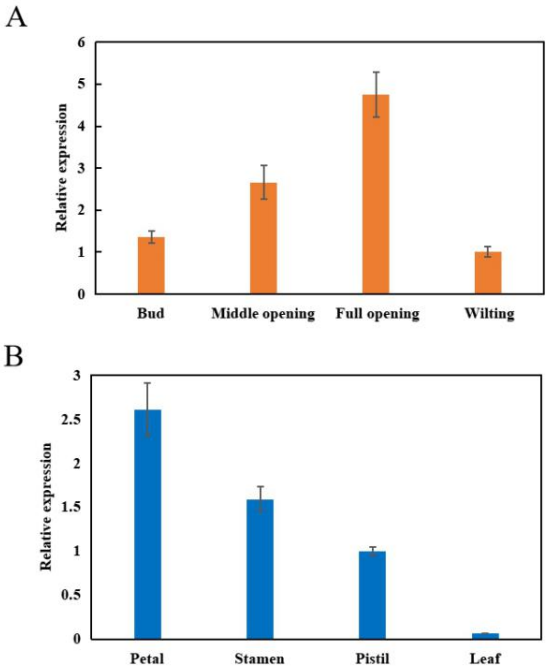

Figure 5 - Expression of *BAMT* in the flowers of *R. fortunei*. (A)At different flower developing stages: bud, middle opening, full blossom and wilting. (B)In different flower tissues and leaves. Data represent means and standard deviations of measurements with triplicate samples.

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