

# 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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## Research article

**Keywords:** Rhododendron, *Rhododendron fortunei*, floral scent, BAMT

**DOI:** <https://doi.org/10.21203/rs.3.rs-57054/v1>

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# Abstract

**Background:** *Rhododendron fortunei* belongs to a scented *Rhododendron* species native to China, which produces fragrant flowers of great ornamental and environmental values. However, the scents in *R. fortunei* have not yet been investigated.

**Results:** The 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. 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 *R. 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 *R. fortunei*.

**Conclusions:** This research showed 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 *R. fortunei*.

## Background

*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 China [1–3]. 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. *Rhododendron fortunei* 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 adaption 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 [4].

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 [5], we then cloned the BAMT gene from this species and study its expression pattern in relation to the accumulation of aroma compositions. We hope that our experiments could provide some data to explain the mechanism of the aroma formation and regulation in *R. fortunei* and eventually identify the gene targets for molecular breeding of fragrant *Rhododendron* cultivars.

## 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 1 and Fig. 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 (Fig. 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 found in plants [6], 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 [7]. However, contents of fatty acid derivatives were significantly lower than both the contents of benzenoids and terpenoids.

**Table 1** The contents of VOCs emitted from *R. fortunei* at four different flowering stages.

Number	Name of compound	RT	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.

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 (Fig. 2b and Table 2). 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.

**Table 2** 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	-

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 [8], 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 [9]. 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 1 and 3. Moreover, the aromatic value of each VOCs was calculated and shown in Table 2 and 4; Fig. 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.

**Table 3** The contents of VOCs emitted from *R. fortunei* at different tissues.

Number	Name of compound	RT	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.

**Table 4** 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	-	-	-

### 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-methionine: benzoic acid carboxyl methyl transferase (BAMT) catalyzes the final step to form methyl benzoates shown in Fig. 3a [5]. To understand the function of this enzyme in the formation of benzenoids 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 (Fig. 3b). These three sequences were spliced to obtain a sequence of 1383 bp which was named *RfBAMT* (Fig. 3c). *RfBAMT* contains an ATG start codon and a TGA 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 (Fig. 3c), whose theoretical molecular weight is 41.09 kDa with an isoelectric point 5.19 predicted by ProtParam. Conserved domains Research tool showed 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*.

### Expression pattern of *RfBAMT*

To clarify the role of *RfBAMT* on regulation of benzoid formation, we measured the expression of *RfBAMT* gene at four flowering stages 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 (Fig. 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 (Fig. 5b). Together with the results on the measurements of floral scent contents (Fig. 1b and Fig. 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 benzoid metabolism in *R. fortune*.

# Discussion

## Determination of floral compositions in *R. fortunei*

Floral scent is an important component part of plant volatile compounds [10], which is a complex mixture of many low-molecular-mass and volatile compounds [11]. 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 [7, 12, 13].

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 (Fig. 1b and Fig. 2b). Benzenoids are commonly found in plant VOC [14] such as in *Prunus mume* [15], European *Narcissus* [9] and especially in rose [16] that more than 50% of the total released VOC is benenoids. Therefore, our results are consistent with these above reports. However, Su et al [17] found 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 [18]. 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 [19]. As a benzenoid, methyl benzoate has a rich aroma of wintergreen and eucalyptus oil scent, and is the main aroma component of flowers [20]. Verdonk et al [21] found that the release of methyl benzoate was large in *Petunia*; the floral composition analysis of *Snapdragon* indicated that the relative content of methyl benzoate was as high as 60% [5]; Zhang et al [22] 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 (Fig. 1b and Fig. 2b ). As we mentioned above, these 3 classes of compounds contribute to the aroma value as well (Fig. 1c and Fig. 2c), which demonstrates 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 (Fig. 3a) [5]. The *BAMT* has been isolated from plants such as *Nicotiana suaveolens* [23], *Petunia hybrid* [23] and *Snapdragon* [5]. 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* (Fig. 4).

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 (Fig. 5) was highly corresponded to the content of methyl benzoate (MeBA) (Fig. 1b and Fig. 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 [16]. 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* [24].

## 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*.

## Materials

### 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 pistil at 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°C 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°C. 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°C 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°C; the injector was operated splitless at a temperature of 250°C with He as a carrier gas at 1.0mL/min. The following temperature program was used: initial temperature of 40°C (2min hold), increase to 160°C at 3°C/min, 10°C/min ramp to 200°C, followed by a 20°C/min ramp to 300°C (3min hold), with the port in splitless injection mode<sup>5</sup>.

Mass spectrometer conditions: ionization mode: EI; electron energy 70 eV; interface temperature: 250°C; ion source temperature: 230°C; quadrupole temperature 150°C; 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 [25]. 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) ×10<sup>-3</sup> / sample weight (kg). Then the aromatic value was calculated for each components respectively according to the threshold of odor [6].

### 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 NanoDrop™2000 and 1% agarose gel electrophoresis, it was reverse transcribed to first-strand cDNA (CWBI, 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 5). The PCR reaction procedure was determined as follows: pre-denaturation 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 5) 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 (Table1). *EF1a* was selected as internal reference gene for each sample (Table 5). qRT-PCR was carried out using the Bio-Rad real-time PCR system with ChamQ™SYBR®Color qPCR Master Mix. The program was: 95°C degeneration 3 sec, 40 cycles of 95°C for 10 s, 60°C for 30s, fluorescent signal acquisition in 60°C. 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 5 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	universal primer
	CTAATACGACTCACTATAGGGC	
BAMT-F2	AGAGAGAGAGAGATGGAAGTGTATCA	Full-length cDNA amplification
BAMT-R2	ATAAAACATCACAACAATACCAACAT	
BAMT-F	TCTACTGTCCCTTGGAGTGCCT	Primer for qRT-PCR
BAMT-R	TCATACCTCTGGAAAACCTTGTCTA	
EF1 $\alpha$ -F	TGTCATCGATGCTCCTGGAC	Reference Primer
EF1 $\alpha$ -R	TCTCGGGTCTGACCATCCTT	

## Abbreviations

BAMT: Benzoic acid carboxyl methyl transferase; BSMT: Benzoic acid/salicylic acid carboxyl; HS-SPME-GC/MS: Headspace: Solid-phase Microextraction and Gas Chromatography/Mass Spectrometer; MEP: 2-C-methyl-D-erythritol 4-phosphate Methyltransferase; MVA: Mevalonate; SAMT: Salicylic acid methyltransferase; VOC: volatile components and contents; MeBA: methyl benzoate.

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent to publication

Not applicable.

### Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests.

### Funding

This research was supported by major Public Welfare Agriculture Project in Ningbo (2014C11002). We thank the foundation for economic support. The funding organization provided the financial support to the research projects, and involved in the design of the study.

### Authors' contributions

YY W, XH X and YH J conceived and designed the experiments. CF Z, QH W and WJ W performed the experiments. CF Z, SJ L and F H analyzed the data. CF Z and ZH C wrote the article. All authors read and approved the manuscript.

## Acknowledgements

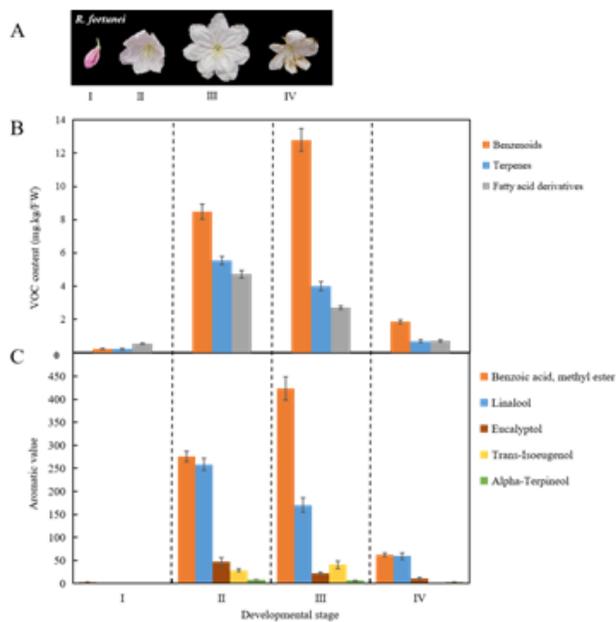
Not applicable.

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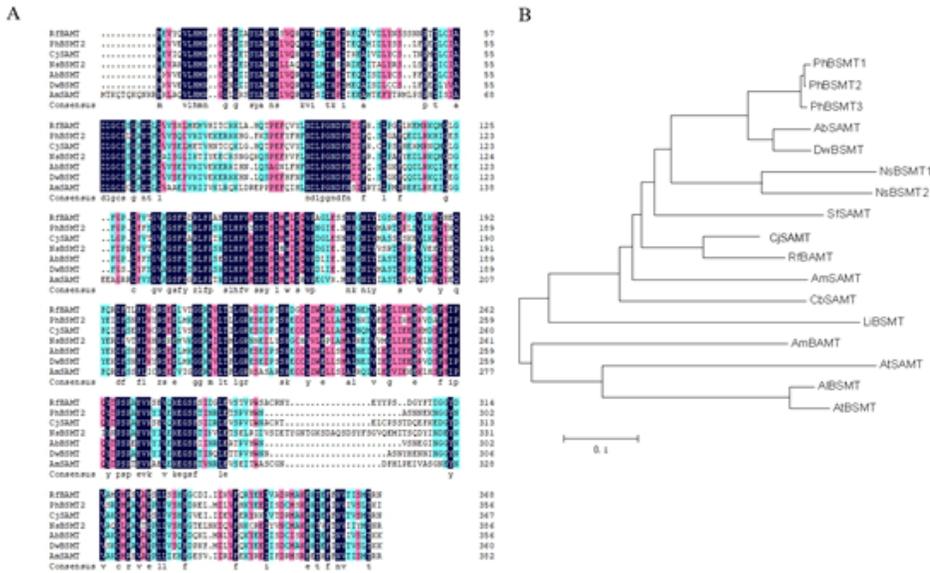
## Figures



**Figure 1**

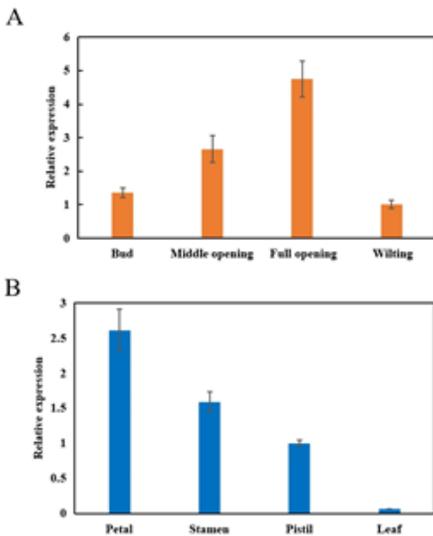
VOCs content and related aromatic values of components emitted from *R. fortunei* at different flowering stages. a Flowers at different stages. □ Bud stage □ Middle opening stage □ Full opening stage □ Wilting stage. b The content of VOCs. c The characteristic aroma values of components. Data represent means and standard deviations of measurements with triplicate samples.





**Figure 4**

BAMT identification. a Multiple alignment of amino acids of RfBAMT and BSMT-SAMT homologs from other plant species. b Phylogenetic tree of B. AMT. Accession number in GenBank: 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), AIBSMT(Arabidopsis lyrata subsp. Lyrata, AY224596.1), AtBSMT(Arabidopsis thaliana, AAY25461.1)



**Figure 5**

Expression of B. AMT 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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