Hedychium coronarium J. Koenig MYB132

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The R2R3-MYB transcription factors (TFs) play several key roles in numerous plant biological processes. *Hedychium coronarium* is an important ornamental plant well-known for its elegant flower shape and abundant aroma type. The floral aroma of *H. coronarium* is due to the presence of a large amount of terpenes and benzenoids. However, less is known about the role of R2R3-MYB TFs in the regulatory mechanism of floral aroma production in this breed. Herein, we isolate and functionally characterize the R2R3-MYB TF HcMYB132, which is potentially involved in regulating floral aroma synthesis. Sequence alignment analysis revealed that it includes a nuclear localization signal NLS(s) and a 2R, 3R motif signature in the sequences. A subcellular localization assay revealed that HcMYB132 protein localizes to the nucleus. Real-time qPCR assays showed that *HcMYB132* is specifically expressed in flowers and its expression pattern correlates with the emission of floral volatile compounds. In *HcMYB132-silenced* flowers, the levels of floral volatile compounds were significantly reduced, and the expression of key structural volatile synthesis genes was downregulated compared to control. Collectively, these results suggest that HcMYB132 might play a significant role in the regulation of terpenoid biosynthesis in *H. coronarium.*

floral scent | Hedychium coronarium | R2R3-MYB | structural genes | terpenes

1. Introduction

The floral aroma is one of the crucial characteristics of plants, which improves the economic and aesthetic values of ornamental plants. White ginger lily (*H. coronarium)* is famous due to its pure white color and butterfly flower shape. The *H. coronarium* flower emits a strong aroma, which is a combination of several floral volatiles including terpenes, benzenoids, and phenylpropanoids [1][2][3][4][5]. Monoterpenes and sesquiterpenes are the major floral volatile contents of this breed, and in our previous studies we identified several key volatile synthesis genes (*HcTPS1/2/3/5/7/8/10, HcBSMT1/2, HcIAA2/4, HcARF5 and HcPAL*) involved in floral aroma biosynthesis [6][7][8][9]. The identification of the genes, transcription factors (TFs), and proteins relevant to floral scent biosynthesis has been advanced. However, less is known about the regulatory mechanism of R2R3-MYB TFs in *H. corornarium.* In our previous RNA sequence and genome-wide data, we reported on a group of *HcMYB* genes potentially involved in the regulating mechanism of secondary metabolites [1][10]. Among them, *HcMYB132* is specifically expressed in flowers and its expression correlates with flower development and emission contents of floral volatiles. However, a detailed functional characterization of this transcription factor in *H. coronarium* has not yet been produced.

MYB TFs are vital regulators of secondary metabolites such as isoflavones and phenylpropanoids [11][12][13]. MYB TFs are classified into four groups based on the number of repeats (1R, R2R3, 3R, and 4R-MYB) [13]. Among

them, R2R3-MYB domain proteins are widely abundant in plants and play important role in several processes, including environmental stress, growth and development, secondary wall biosynthesis, and flavonoid/phenylpropanoid metabolism [14][15][16][17]. For example; *GbMYB5*, *AtMYB44* and *AtMYB60* induced drought tolerance in cotton and *Arabidopsis* [18][19]. *AtMYB33* and *AtMYB65* assist in the formation of viable pollen and produce high pollen fertility, while *AtMYBL2* functions as a transcriptional repressor, and prevents the accumulation of proanthocyanin in *Arabidopsis* [12][20]. In *Malus domestica*, *MdMYB3* modulates the production of anthocyanin via its effect on the various flavonoid pathway genes and assists in flower formation [21]. Similarly, *Arabidopsis AtMYBL2/4/7* and litchi R2R3-MYB showed their important role in the regulation of flavonoid and anthocyanin biosynthesis, respectively [12][22][23]. The soybean *GmMYB100*-and grape *VvMYB4-like* genes negatively regulate the production of flavonoids [24][25].

However, only limited MYB TFs related to volatile biosynthetic pathways have been characterized from a few plant species, including snapdragon (*Antirrhinum majus*) and petunia (*Petunia* spp.), which are known as model floral scent species. The volatile phenylpropanoid/benzenoid metabolic pathway is regulated by *AmMYB305/340*, ODORANT 1 (*ODO1*), and EMISSION OF BENZENOID II (*EOBII*) in snapdragon [26][27] and petunia, respectively [28][29][30]. Likewise, *PpMYB15* and *PpMYBF1* exhibited a floral expression and participated in the biosynthetic control of flavanol from *Prunus persica* [31]. The production of phenylalanine and its metabolic flow to lignin biosynthesis are controlled by *MYB8* and ELONGATED HYPOCOTYL (*HY5*) in *Pinus pinaster* [32]. Until now, several reports of MYB TFs related to flavonoid biosynthesis in other species have been discussed, but still, there is a gap in knowledge of the role of MYB in *H. coronarium.*

2. Characterization of HcMYB132

In a previous genome-wide analysis, we identified a group of R2R3-MYB family members expressed specifically in flowers that increased in expression with flower development and floral volatile emissions [1]. Among them, *HcMYB132* is specifically expressed in flowers. The coding sequences of *HcMYB132* include open reading frames of 624 bp, encoding polypeptides of 207 amino acid residues with a molecular weight of 23.76 kilodaltons (kDa), isoelectric point (*pI*) 6.16, and the protein GRAVY −0.733. Further analysis revealed that *HcMYB132* contains two exons, and is located on chromosome 11. Prediction analysis of HcMYB132 protein sequences showed the presence of R2 and R3 repeat signatures at the N-termini, which is a key feature of R2R3 DNA-binding MYB proteins (**Figure 1**a).

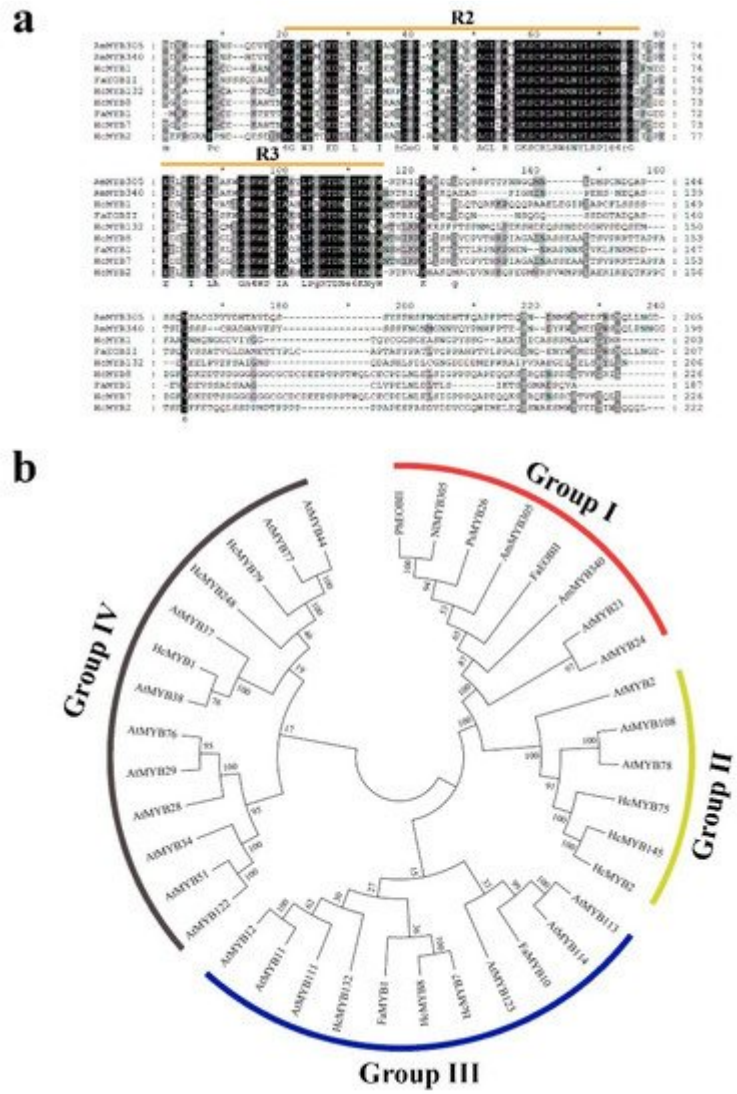

Figure 1. Sequence alignment and phylogenetic analysis of HcMYB132. (**a**) Multiple sequence alignment of HcMYB132 with R2R3-MYB proteins. Sequence alignment was performed by ClustalX 2.1 and shaded in GeneDoc. Amino acid residues are shaded in light gray, gray, and black showing 50, 70 and 100% identity, respectively, while dashes indicate gaps used for optimal alignment. R2R3 motifs are indicated by orange lines. (**b**) Phylogenetic analysis of HcMYB132 together with previously characterized R2R3-MYB proteins. The protein sequences were aligned by Clustal X 2.1 and the phylogenetic tree was built in MEGA X using the Nj method. All R2R3-MYBs are grouped into 4 subclades named G I–G IV. Genes used in phylogenetic tree and their accession numbers are listed in Table S2.

The phylogenetic analysis of HcMYB132 was performed with the previously characterized R2R3-MYB proteins involved in secondary metabolism derived from *H. coronarium* and other plant species. All R2R3-MYBs were clustered into 4 distinct groups (G I–G IV) (**Figure 1**b). Among them, subgroup G II included the least number of R2R3-MYB members (6), while subgroup G IV constituted the largest group, holding 13 R2R3-MYB members. HcMYB132 clustered into subgroup III, which included FaMYB1/10 (*Fragaria* × *ananassa*), HcMYB7/8 (*H. coronarium*), and AtMYB11/12/111/113/114/123 (*Arabidopsis thaliana*).

3. Subcellular Localization of HcMYB132

Nuclear localization prediction tools predicted that HcMYB132 is located in the nucleus. To verify the prediction results, we generated HcMYB132-GFP constructs driven by a CaMV *35S* promoter and transferred them to *N*. *benthamiana* leaves via agroinfiltration, followed by visualization using confocal laser scanning microscopy (Zeiss, Jena, Baden-Württemberg, Germany). The results verified that HcMYB132 protein was localized to the nucleus (**Figure 2**).

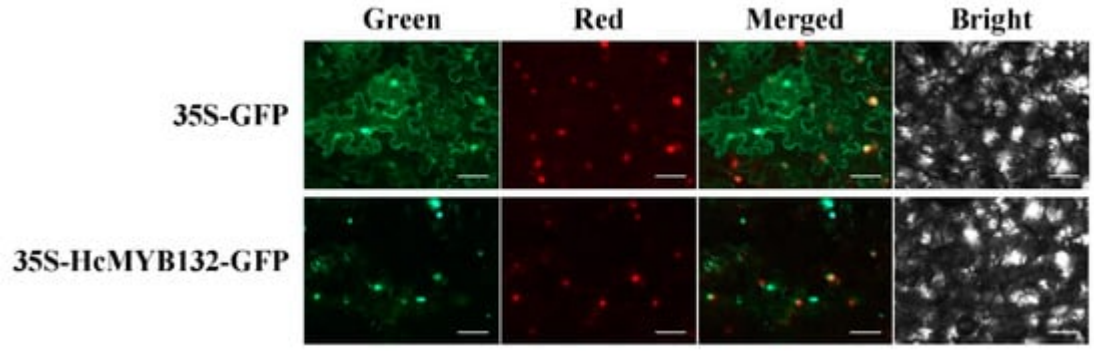

Figure 2. Nuclear localization of *H*. *coronarium* MYB132 protein in *N*. *benthamiana* leaves. Green: GFP fluorescence, red: mcherry as NLs marker, merged: merged green and red channels and bright field. Bars, 50 μM.

2.3. Expression Pattern of HcMYB132

Previous research indicated that the accumulation of floral volatiles increases with flower development [1][2][7]. To analyze the aforementioned process, flower development was divided into four stages (**Figure 3** and **Figure 4**).

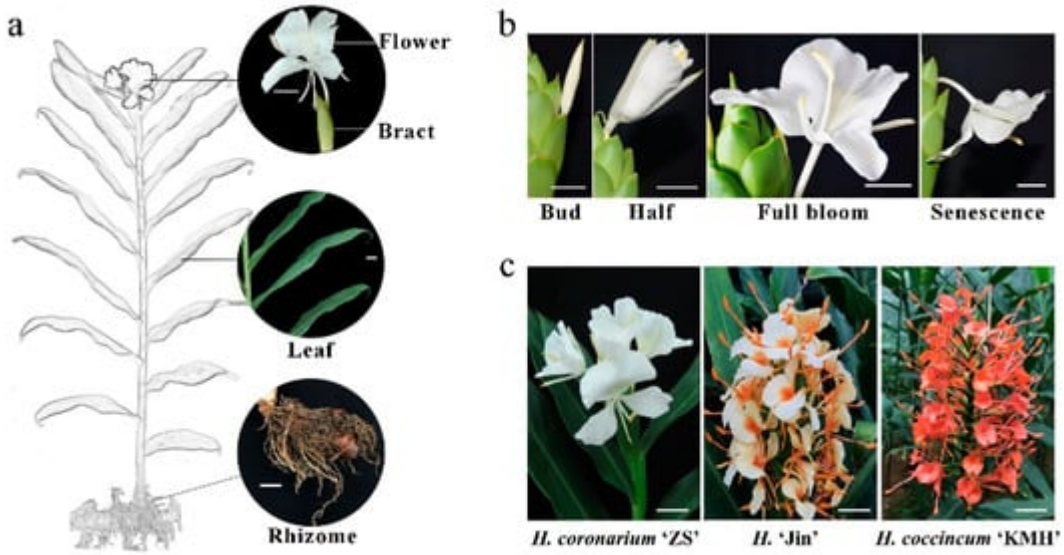

Figure 3. A pictorial view of labeled *H*. *coronarium* tissues. (**a**) Figure representation of *H*. *coronarium* flower, bracts, leaves, and rhizome; (**b**) figure illustration of different flower developmental stages (bud stage, half bloom,

full-bloom and senescence stage); (**c**) pictorial representation of three different *Hedychium* accessions. Scale bar indicates 2 cm.

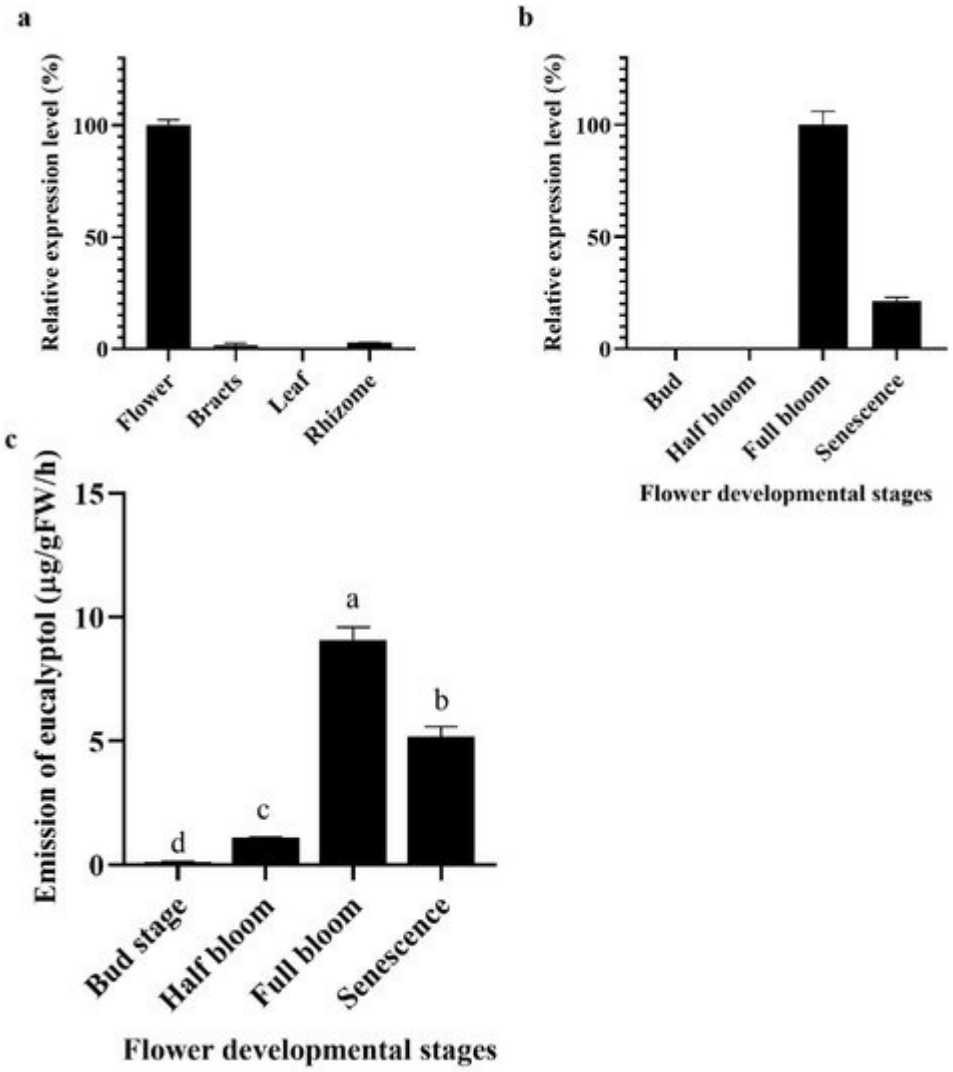

Figure 4. Expression analysis of *HcMYB132* in different tissues. (**a**) Relative expression level of *HcMYB132* in different parts; (**b**) different flower development stages of *H. coronarium,* results are shown as a percentage with a maximum value set to 1 (100%); (**c**) emission level of eucalyptol during flower development stages, data are shown as ± SEM of three to five repeats. Lowercase letters represent statistically significant differences at $p < 0.01$, according to least significant difference (LSD).

The data showed that *HcMYB132* was specifically expressed in flowers, while negligible expression was measured in the rhizome and bracts (**Figure 4**a). Furthermore, the mRNA transcript levels of *HcMYB132* were abundant in the full-bloom stage, and low during senescence (**Figure 4**b). A similar pattern was observed in the emission level of eucalyptol contents; low during the bud stage, peaking during full bloom, and decreasing thereafter (**Figure 4**c).

4. Discussion

H. coronarium is popular in tropical and subtropical parts of the world due to its appealing strong aroma type and medicinal properties [3][33]. R2R3-MYB TFs are the main regulators of terpenes and phenylpropanoids [34][35]. However, less is known about the transcriptional regulatory mechanism of floral aroma production. Until now, a few MYB TFs have been reported that control the regulatory network of floral scent production [29][30][36][37]. Herein, we identified and functionally characterized a R2R3-MYB TF (HcMYB132) that is potentially involved in floral aroma synthesis in *H. coronarium*.

Multiple sequence analyses of HcMYB132 revealed the existence of 2R and 3R repeats in the sequences (**Figure 1**a). Several previous findings suggest that the R2 and R3 signature motifs are highly conserved and regulate various aspects of plant secondary metabolites [13][38][39][40]. We generated a phylogenic tree using the previously characterized R2R3-MYB TFs involved in the regulatory network of secondary metabolism, together with HcMYB132 (**Figure 1**b). HcMYB132 was classified into Group III with FaMYB1, FaMYB10, and AtMYB11/12/111/113/114/123. The functional characterization of aforementioned genes revealed their role in the regulation of the flavonoid/phenylpropanoid metabolism [14][41][42][43], indicating that *HcMYB132* might play a significant role in secondary metabolism. It has been reported that MYB TFs in same subclade have identical functions [13][35]. The structure analysis revealed that the *HcMYB132* contains two exons, which are in line with the previous reports [44]. A subcellular localization assay revealed that HcMYB132 protein is localized to the nucleus, which is consistent with the previous findings [1][7][13][45].

The process of floral scent production is interrelated with flower development [46][47][48]. Our previous studies revealed that production and emission of floral volatile compounds and the expression of key structural volatile biosynthesis genes were low during the bud stage and peaked during the full bloom stage [7][8][9][10]. Previous studies also showed that volatile emission content was significantly larger from the flower than from the rhizome and leaf, which is consistent with the expression pattern of *HcMYB132* [7]. In the current findings, it was revealed that *HcMYB132* was mainly expressed in the flowers and its expression pattern increased with flower development, peaked during the fully bloomed stage, and dropped down thereafter (**Figure 4**a,b), implying that it might potentially be involved in the floral aroma production and emission mechanism. A similar expression pattern was observed in *Fragaria ananassa EOBII*, *EOBI*, and *ODO1*, and was involved in the regulatory network of eugenol [15][29]. Likewise, *Prunus persica MYBF1* and *MYB15* showed the highest expression in the flower and were involved in flavanol biosynthesis regulation [31]. In *Lilium hybrid*, ODO1 TF had highest expression in the flower and plaedy a crucial role in the regulation of phenylpropanoid/ benzenoid volatile production [49]. These results suggest that *HcMYB132* potentially regulates the process of floral scent production.

To reveal the role of *HcMYB132* in floral aroma production in *H. coronarium*, the activity of *HcMYB132* was repressed via gene silencing. The data showed that the volatile contents of eucalyptol were substantially decreased in *HcMYB132*-silenced flowers compared to control flowers. Furthermore, in *HcMYB132*-silenced flowers, the transcript levels of key eucalyptol volatile biosynthesis genes (*HcTPS1* and *HcTPS3*) were significantly decreased (**Figure 5**). Likewise, strawberry *MYB10* regulates the expression of numerous key genes involved in the flavonoid and phenylpropanoid biosynthesis process [14]. In petunia *ODO1*-suppressed plants, the mRNA levels of several scent-related genes were downregulated [29]. Similarly, litchi *MYB5* activates the transcript levels of key

genes involved in the synthesis of anthocyanin [23]. In *HcMYB1/2/7/8/75/79/145/238/248*-silenced flowers, the emission of floral volatiles and the expression of structural genes were significantly decreased [1][7]. Moreover, the emission of eucalyptol and the expression of *HcMYB132* were influenced by auxin treatments, which are consistent with previous findings [7][50]. These data endorse the previous findings that R2R3-MYB TFs are involved in the regulation of volatile formation in *H. coronarium*.

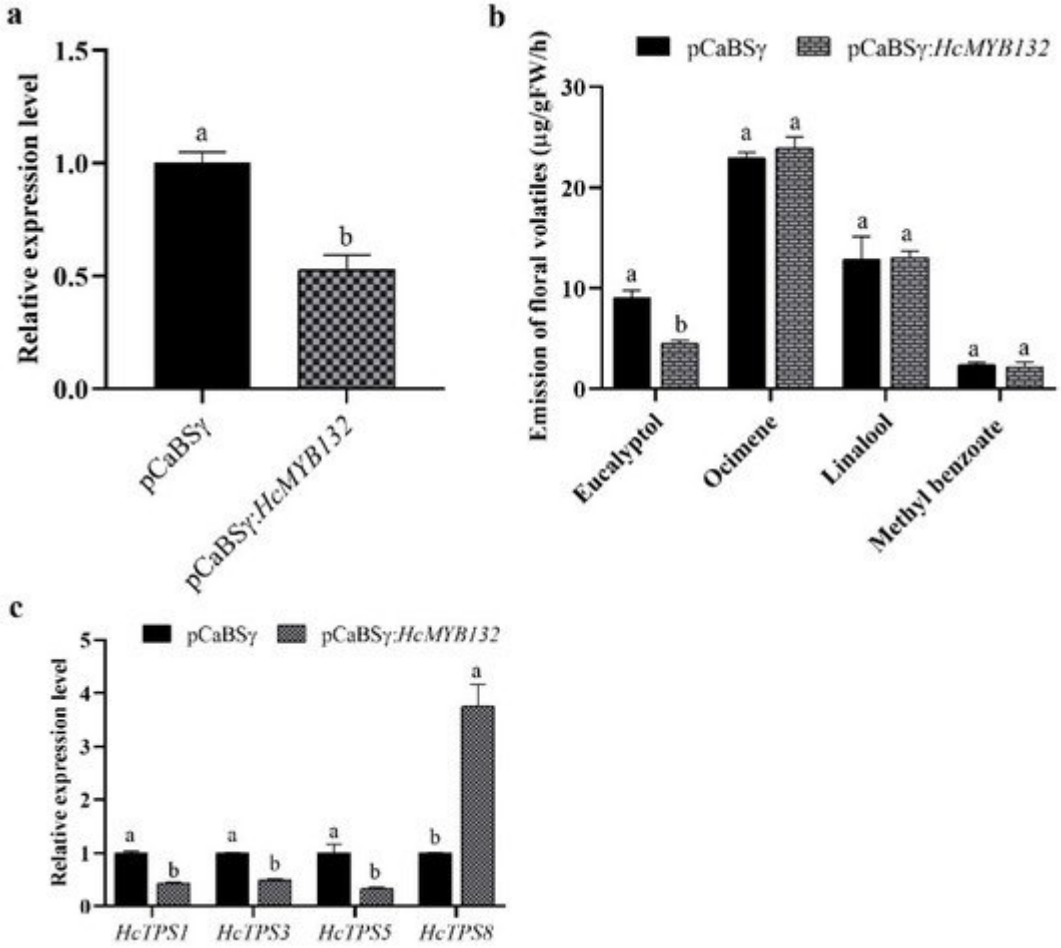

Figure 5. Suppression of *HcMYB132* in *H. coronarium* flowers. (**a**) RT-qPCR assay of *HcMYB132* transcript levels in *HcMYB132*-silenced and control flowers; (**b**) GC-MS analysis of floral volatiles in *HcMYB132*-silenced and control flowers; (**c**) transcript levels of key structural genes in *HcMYB132*-silenced and control flowers. Data are shown as ± SEM of three to five repeats. Lowercase letters represent statistically significant differences in LSD test ($p < 0.01$).

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