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ORIGINAL ARTICLE

Functional characterization of the *CfAOC* and *CfJMT* gene promoters related to MeJA biosynthesis in *Cymbidium faberi*

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Abstract

Oriental orchids of the genus *Cymbidium* are famous for their flower fragrance and have great value in the horticultural market. To investigate the regulatory mechanisms of fragrance biosynthesis in *Cymbidium faberi*, the promoters of the *CfAOC* and *CfJMT* genes in the methyl jasmonate biosynthesis pathway were cloned and analyzed. Notably, the *CfAOC* promoter was transcriptionally active in all vegetative organs, which was not true for the *CfJMT* promoter, indicating that the latter is tissue-specific for the flowers of *C. faberi*. The dual-luciferase assay showed that the fragment between –629 and –1038 nt of the *CfAOC* promoter, as well as the fragment between –960 and –1121 nt of the *CfJMT* promoter, displayed specific interaction with the CfbHLH transcription factors. These results provide a theoretical basis for the genetic modification of the flower fragrance of *Cymbidium* based on specific CfbHLH transcription factors.

Keywords Methyl jasmonate · Allene oxide cyclase · Jasmonic acid carboxyl methyltransferase · Promoter · Orchid · Dual-luciferase assay

Introduction

Orchids are one of the largest families of angiosperms, and they have been widely spread from the tropics to polar regions on all continents except Antarctica. Nevertheless, horticultural cultivars are mainly classified into tropical and oriental orchids. In south Asia, tropical orchids are popular in the flower market, especially during the traditional festivals, since orchids are a symbol of wealth and prosperity (Zhang et al. 2018). In contrast, the oriental orchids are mainly spread in the mainland of China, Japan, and Korea where the latitudes are comparably higher and the temperatures are lower (Choi et al. 2020; Wei et al. 2020). Although most of the oriental orchids do not have colorful petals, the variable flower shapes and strong flower fragrance explain their great value in the orchid market (Ramya et al. 2019). *Cymbidium faberi* is one of the oldest varieties of oriental orchids, with a subdued flower color and strong flower fragrance, which could attract pollinating insects in the wild (Hossain et al. 2010, 2013). To investigate the biosynthesis of flower fragrance in *C. faberi*, RNA-seq analysis and yeast hybridization libraries were conducted, which provide great convenience for genetic research on the molecular level (Xu et al. 2019; Xu et al. 2020b).

Based on solid-phase microextraction (SPME) and GC/MS, the main component of the flower fragrance of *C. faberi* is methyl jasmonate (MeJA), which is also an important plant hormone (Zhou et al. 2018). The biosynthetic pathway of MeJA was elucidated in many model plants. MeJA is derived from α-linolenic acid in the chloroplasts where 12-oxo-phytodienoic acid (OPDA) is synthesized by a lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC). Then, OPDA is transferred to the peroxisomes to form the final product of jasmonic acid (JA) (Guan et al. 2019; Yang et al. 2019). JA can be converted into many derivatives, such as JA-isoleucine (JA-Ile), 12-hydroxyjasmonic acid (12-OH-JA) and MeJA. MeJA is synthesized by jasmonic acid carboxyl methyltransferase

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(JMT), which is the rate-limiting enzyme in the biosynthetic pathway (Seo et al. 2001). Among these enzymes, AOC is the only one responsible for the correct enantiomeric structure of JA (Riemann et al. 2013).

The promoter is a DNA fragment upstream to the coding region of a gene responsible for the specifical recruitment of the RNA polymerase when a gene is activated. In eukaryotes, many regulators can specifically recognize the cis-acting elements in the promoter sequences and induce the plant to rapidly respond to internal signals or the external environment. In general, the promoters can be categorized into three classes: constitutive, inducible and specific promoters (Chen et al. 2014). To identify how and where the MeJA biosynthetic pathway is regulated in *C. faberi*, the promoter sequences of the *CfAOC* and *CfJMT* genes were cloned from the genomic DNA and used to ectopically express a reporter gene in tobacco in this study.

To finely regulate the growth, development, and stress responses of the plant, MeJA is often interconnected with other plant hormones and controlled by many pivots of transcription factors (Ku et al. 2018; Yang et al. 2019). For example, the hormonal signals gibberellin (GA) and jasmonate (JA) act either antagonistically or synergistically to regulate diverse aspects of plant growth, development, and defense (Qi et al. 2014). Both DELLAs and Jasmonate ZIM-domain (JAZ) proteins interact with the WD-repeat/bHLH/MYB complex to mediate the synergism between GA and JA signaling in regulating trichome development. The antagonism between JA and ethylene (ET) during apical hook development has been intensively studied in Arabidopsis (Zhang et al. 2014). In banana fruit, the jasmonate signaling regulator MaMYC2s was found to physically interact with MaICE1 to mediate MeJA-induced chilling tolerance (Zhao et al. 2013). In this study, two bHLH transcription factors were selected to test the promoter activity of the *CfAOC* and *CfJMT* genes and identify their exact interaction sites.

To explore the promoter activity of the *CfAOC* and *CfJMT* genes from the MeJA biosynthesis pathway of *C. faberi*, these two promoters were cloned and their expression patterns were assessed in tobacco. The crucial interaction cis-elements with CfbHLH transcription factors in *CfAOC* and *CfJMT* promoters were studied and discussed in this study.

Materials and methods

Plant materials and growth conditions

The wild *C. faberi* was transplanted from Dangyang mountain (30°55′25′′N, 111°51′24″E), Hubei province, China in 2011, and preserved at the greenhouse of Wuhan University of Bioengineering. *Nicotiana tabacum* and *Nicotiana benthamiana* were kindly provided by associate professor Wenjun Huang (Wuhan Botanical Garden, Chinese Academy of Sciences). Transgenic and wild-type tobacco plants were grown in the greenhouse under a 16 h light/8 h dark cycle at 25 °C and 60% humidity.

Cloning and bioinformatic analysis of the promoter sequence of the *CfAOC* gene

The genomic DNA was extracted from young leaves of *Cymbidium faberi* using the CTAB protocol and a modified TAIL-PCR was applied to amplify the promoter of *CfAOC* as described previously (Xu et al. 2019). The universal primers used in the TAIL-PCR were the same as those reported by Xu et al., and the three nested specific primers PCfAOC-SP1, SP2 and SP3 are listed in Table S1. The TAIL-PCR products of the promoter sequence of the *CfAOC* gene were sequenced by Sangon Biotech (Shanghai, China) and then assembled using ContigExpress software. PLACE software (http://www.dna.affrc.go.jp/PLACE/signalscan.html) was used to predict the cis-acting elements and DNA-binding regions within the *CfAOC* promoter sequence. The promoter of the *CfJMT* gene was preserved in our lab from a previous study (Xu et al. 2019).

Vector construction and tobacco transformation

The vectors for the testing of the *CfAOC* and *CfJMT* promoters were constructed based on the pCambia1301-GUS backbone using the restriction endonucleases *Hind* III and *Bgl* II, or *Pst* I and *Bgl* II, respectively. The primers used to amplify the *CfAOC* and *CfJMT* promoter sequences are listed in Table S1. The resulting recombinant plasmids pC1301-PCfAOC and pC1301-PCfJMT were transformed into *Agrobacterium tumefaciens* GV3101, which was used to transform *Nicotiana tabacum* plants as described before (Huang et al. 2016).

Based on the bioinformatic analysis of the promoter sequences, a series of truncated fragments of the *CfAOC* and *CfJMT* promoters were obtained by PCR. The corresponding primers of the PCfAOC-F series, which were combined with PCfAOC-R, and the PCfJMT-F series, which were combine with PCfJMT-R, are also listed in Table S1. The resulting fragments starting at –81, –161, –217, –406, –445, –629, –862, –1038, –1197, and –1347 nt upstream of the translation start site of the *CfAOC* gene, as well as the fragments starting at –265, –302, –417, –593, –742, –887, –960, –1002, –1121, –1245, and –1377 nt upstream of the translation start site of the *CfJMT* gene, were integrated into the pGreen II 0800-Luc vector to identify the conserved cis-elements in the promoter sequences, which would influence their interactions with the corresponding transcription factors. The effector vectors pGreenII 62-SK-CfbHLH5 and

pGreenII 62-SK-CfbHLH36, as well as the reporter vectors pGreen II 0800-Luc-PCfAOC truncated series and pGreen II 0800-Luc-PCfJMT truncated series were individually transformed into *A. tumefaciens* GV3101 together with pSoup. Then, the *Agrobacteria* with effector vector of pGreenII 62-SK-CfbHLH5 and reporter vectors of pGreen II 0800-Luc-PCfAOC, pGreen II 62-SK-CfbHLH36 and pGreen II 0800-Luc-PCfJMT truncated series were transiently co-transformed into *N. benthamiana* plants as described previously (Xu et al. 2020a). The enzymes used to amplify the promoter fragments and construct the vectors were purchased from TaKaRa (Dalian, China) and other reagents from Sinopharm (Shanghai, China).

GUS staining

The seedlings were screened for positive transformants by PCR using the same primers employed in the recombinant vector construction. T1 and T2 generations were selected on Murashige and Skoog (MS) medium with 10 μg/mL hygromycin (neoBioFroxx GmbH, Germany). GUS staining was detected in T2 tobacco seedlings using a previously described protocol (Zhou et al. 2012). The images were recorded using a Scanner (Microtek, ScanMaker i800, Zhongjing, China).

Dual-luciferase detection of LUC activity

Following 3 days of infiltration with the effector and reporter vectors, the tobacco leaves were subjected the dual-luciferase detection as described before (Xu et al. 2020a). Each sample was analyzed in biological triplicates.

Statistical analysis

Data on the relative LUC activity as determined by dual-luciferase detection were presented as the means ± SE from $n = XY$ parallel experiments, and were subjected to analysis of variance using SigmaPlot 12.5 software.

Results

The promoter of the *CfAOC* gene was successfully cloned and bioinformatically analyzed

To identify the cis-elements in the promoter of the *CfAOC* gene, TAIL-PCR was applied to clone the promoter sequence. In the first round of TAIL-PCR, fragments with a length above 750 bp were purified for DNA sequencing (Figure S1a). The sequencing results revealed a fragment of about 320 bp adjacent to the ORF of the *CfAOC* gene upstream of the ATG start codon. The second round of TAIL-PCR using the PCfAOC-SP2 and AD4 primers yielded another fragment with a length of approximately 750 bp (Figure S1b). After three rounds of TAIL-PCR, the amplification products were sequenced and aligned, yielding the promoter region of the *CfAOC* gene spanning 1347 bp (Figure S1c). PlantCARE analysis predicted many cis-acting elements within the cloned sequence (Fig. 1 and Table 1), mainly including ERE, ABRE, CGTCA-motif, TGACG-motif, and GARE elements responsive to plant hormones, TCA-motif, STRE, and LTR responsive to stress, some G-box, BOX4 and circadian elements responsive to light, as well as some G-box, MYB, MBS, and MYC elements responsive to related transcription factors (marked background color in Fig. 1), suggesting that the expression of the *CfAOC* gene in *C. faberi* is influenced by many biotic and abiotic factors and controlled by a plethora of transcription factors, which is consistent with the pleiotropic effects of MeJA on plant physiology.

The promoter of the *CfAOC* gene could induce the expression of the *gus* reporter gene in all the vegetative organs of transgenic tobacco, while the *CfJMT* promoter could not

To test the promoter activity and expression pattern of *CfAOC* and *CfJMT* genes, the T2 generation of tobacco transformants carrying the pCambia1301-GUS plasmid with the promoters of the *CfAOC* and *CfJMT* genes, respectively, were obtained by hygromycin screening. A total of 10 tobacco seedlings, respectively (named AOC# and JMT#) were subjected to GUS staining. The results showed that the AOC#7 and AOC#10 transformants were stained blue in all the vegetative organs, especially in the roots, while there was no staining in wild type (WT) (Fig. 2). However, none of the T2 generation of tobacco transformants with the promoter of the *CfJMT* gene could be stained blue (Fig. 2), just like the WT.

After the transformants grew up to the reproductive period, all the organs were collected for GUS staining. As shown in Fig. 3, the root, stem and leaf of AOC#7 were stained blue (Fig. 3A, B, C), but the reproductive organs were not stained (Fig. 3D, E, F). The staining in root is obvious compared to that in other organs, which is consistent with that in the seedling. The root of JMT#1 is not stained blue (Fig. 4A), but stem, leaf and petal were stained faint blue (Fig. 4B, C, D). However, the stamen and sepal were not stained blue (Fig. 4E, F). This result suggested that the *CfJMT* promoter has a weak activity in tobacco, because only very limited regions even a few cells in these tissues could be stained blue.

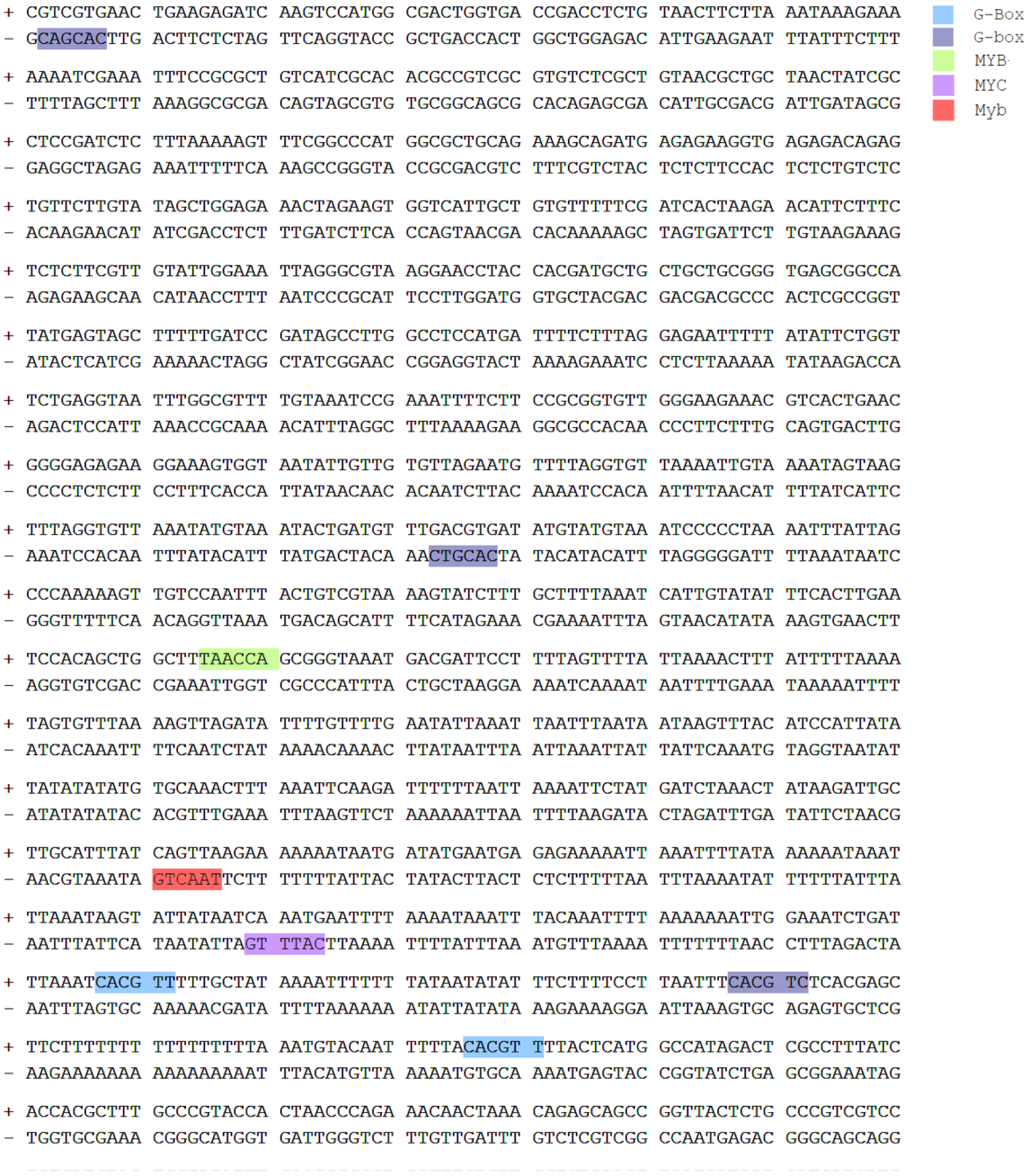

Fig. 1 Sequence of *CfAOC* promoter and partial cis-elements, including G-box (in pale blue and pearl violet), MYB (in pale green and brick red) and myc (in purple)

The transient co-transfection of the promoter of the *CfAOC* gene with CfbHLH5 transcription factor could increase its promoter activity in tobacco

To identify the exact interaction sites of the promoter of the *CfAOC* gene with the basic helix–loop–helix (bHLH) transcription factors, a series of truncated promoter sequences of *CfAOC* gene were inserted into the pGreen II 0800-Luc vector. The LUC activity in the dual-luciferase assay showed that shorter promoter truncations had weaker interactions with the transcription factor (Fig. 5). In the region from –629 nt upstream of the translation start codon, the promoter activity was significantly activated by CfbHLH5. However, when the fragment between –629 nt and –1038 nt was truncated, the LUC activity decreased to a certain extent, suggesting that some cis-elements in this region could be recognized and integrated with CfbHLH5. When compared with the activity of single transformation with

Table 1 Cis-acting elements in the *CfAOC* promoter predicted by PlantCARE

Site name	Organism	Position	Strand
AAGAA-motif	*Avena sative*	–1053	–
AAGAA-motif	*Avena sative*	–237	–
ABRE	*Arabidopsis thaliana*	–733	+
ABRE	*Arabidopsis thaliana*	–172	–
ABRE	*Arabidopsis thaliana*	–270	–
AC-II	*Phaseolus vulgaria*	–862	–
ACE	*Petroselinum crispum*	–174	–
AE-box	*Arabidopsis thaliana*	–110	+
as-1	*Arabidopsis thaliana*	–847	–
as-1	*Arabidopsis thaliana*	–597	+
as-1	*Arabidopsis thaliana*	–735	+
as-1	*Arabidopsis thaliana*	–48	+
ATCT-motif	*Arabidopaia thaliana*	–371	–
Box 4	*Petroselinum crispum*	–518	–
CAAT-box	*Nicotiana glutinosa*	–421	–
CAAT-box	*Nicotiana glutinosa*	–782	–
CAAT-box	*Nicotiana glutinosa*	–181	+
CAAT-box	*Pisum sativum*	–304	+
CAAT-box	*Nicotiana glutinosa*	–682	+
CAAT-box	*Pisum sativum*	–328	+
CAAT-box	*Arabidopsis thaliana*	–683	+
CAAT-box	*Arabidopsis thaliana*	–290	–
CAAT-box	*Nicotiana glutinosa*	–645	–
CGTCA-motif	*Hordeum vulgare*	–847	+
CGTCA-motif	*Hordeum vulgare*	–597	–
CGTCA-motif	*Hordeum vulgare*	–735	–
CGTCA-motif	*Hordeum vulgare*	–48	–
chs-CMA2a	*Petroselinum crispum*	–635	+
circadian	*Lycoperalcon esculentum*	–956	–
ERE	*Nicotiana glutinos*	–563	–
ERE	*Nicotiana glutinos*	–319	–
ERE	*Nicotiana glutinos*	–321	+
ERE	*Nicotiana glutinos*	–301	+
ESE	*Brassica oleracea*	–256	–
ESE	*Brassica oleracea*	–877	–
G71-motif	*Solanum tubercaue*	–628	+
GARE-motif	*Brasslca oleracea*	–254	+
G-box	*Pisum sativum*	–270	+
G-box	*Pisum sativum*	–172	+
G-box	*Zea mays*	–734	–
G-box	*Zea mays*	–270	+
GT1-motif	*Arabidopsis thaliana*	–613	–
IC-rich rereata	*Nicotiana tabacum*	–1102	–
IC-rich rereata	*Nicotiana tabacum*	–874	+
IC-rich rereata	*Nicotiana tabacum*	–937	+
LAMP-element	*Pisum sativum*	–144	+
LTR	*Hordeum vulgare*	–1167	–
LTR	*Hordeum vulgare*	–879	+
MBSII	*Petunia bybrida*	–548	+
MYB	*Arabidopsis thaliana*	–612	+
MYB	*Arabidopsis thaliana*	–406	–
MYC	*Arabidopsis thaliana*	–328	–
O2-site	*Zea mays*	–737	+
SKN-1 motif	*Oryza sativa*	–598	–
SKN-1 motif	*Oryza sativa*	–1085	+
STRE	*Arabidopsis thaliana*	–713	–
SUTR Py-rich stretch	*Lycopersicon esculentum*	–832	–
TATA	*Arabidopsis thaliana*	–364	–
TATA	*Arabidopsis thaliana*	–259	+
TATA-box	*Arabidopsis thaliana*	–241	–
TATA-box	*Arabidopsis thaliana*	–54	–
TATA-box	*Arabidopsis thaliana*	–334	–
TATA-box	*Arabidopsis thaliana*	–482	–
TATA-box	*Arabidopsis thaliana*	–427	–
TATA-box	*Pisum sativum*	–363	–
TATA-box	*Brassica napus*	–336	+
TATA-box	*Helianthus annuus*	–248	–
TATA-box	*Brassica napus*	–242	–
TCA	*Pisum sativum*	–11	+
TGACG-motif	*Hordeum vulgare*	–847	–
TGACG-motif	*Hordeum vulgare*	–597	+
TGACG-motif	*Hordeum vulgare*	–735	+
TGACG-motif	*Hordeum vulgare*	–48	+
TGACG-motif	*Hordeum vulgare*	–847	–
TGACG-motif	*Hordeum vulgare*	–735	+
TGACG-motif	*Hordeum vulgare*	–847	–
TGACG-motif	*Hordeum vulgare*	–597	+

the LUC vector carrying the promoter of the *CfAOC* gene without any transcription factor, the results showed that the CfbHLH5 transcription factor could play a positive role in the expression of the *CfAOC* gene in tobacco.

The transient co-transfection of the promoter of the *CfJMT* gene and the CfbHLH36 transcription factor decreased its promoter activity in tobacco

Similarly, a series of truncated promoter sequences of the *CfJMT* gene were inserted into pGreen II 0800-Luc vector to characterize the interaction sites of the *CfJMT* promoter. The LUC activity also showed that the core sequence of the promoter near the translation start site played a crucial role in the promoter activity (Fig. 6), the same as in the *CfAOC* promoter. In the region from –960 nt upstream of the translation start codon, the promoter activity was significantly activated by CfbHLH36. However, when the fragment between –960 nt and –1121 nt was truncated, the LUC activity was significantly decreased, suggesting that some cis-elements in this

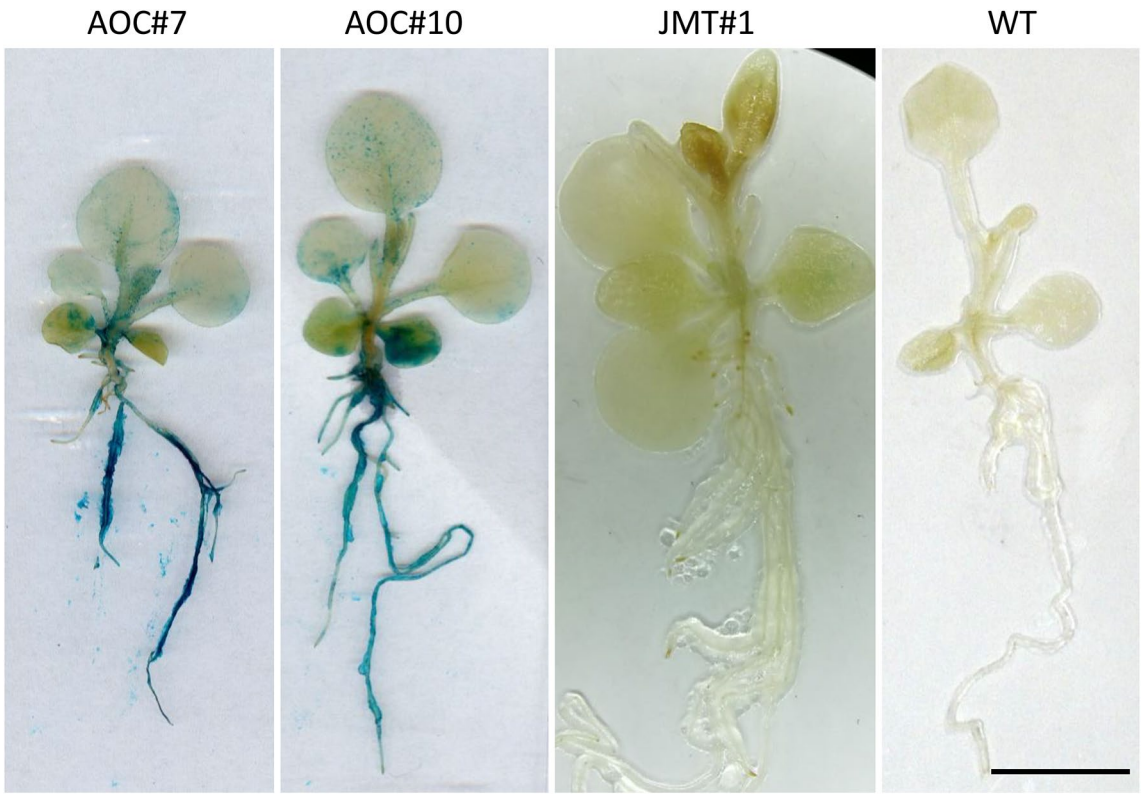

Fig. 2 GUS staining of transgenic tobacco seedlings transformed with pC1301-PCfAOC-GUS. bar = 1 cm. AOC#7, AOC#10 and JMT#1, transgenic tobaccos; WT, wild type

region could be recognized and integrated with CfbHLH36. When compared with the activity of single transformation with a LUC vector carrying the promoter of the *CfJMT* gene without any transcription factor, the results showed that the CfbHLH36 transcription factor had a negative effect on *CfJMT* promoter activity in tobacco.

Discussion

Orchids are famous for their fragrance, color, and charm, and account for a great proportion of sales in the flower market. However, their production is mostly limited to traditional propagation and breeding methods. In recent years, along with the rapid development of molecular and transgenic technology, extensive studies have focused on flower induction and development in orchids (Wang et al. 2017, 2019). Due to the complicated components of the flower fragrance of orchids, it is difficult to study fragrance production at the molecular level. In *Cymbidium faberi*, the main component of the flower fragrance is methyl jasmonate (MeJA) (Omata et al. 1990; Zhou et al. 2018). The genes encoding the crucial enzymes in the MeJA biosynthesis pathway, *CfAOC* and *CfJMT*, have been cloned from *C. faberi* and ectopically expressed in tomato. However, they were found to participate in the stress response but rather than influencing the volatile emissions of the plant (Zhou et al. 2018; Xu et al. 2019).

The promoter of a gene plays a great role in its expression and regulation at the transcriptional level. Many studies have focused on promoter modification to improve target traits in plant biotechnology (Hernandez-Garcia and Finer 2014). A chimeric promoter derived from potato and Arabidopsis can induce guard cell-specific expression of genes of interest under water deficit conditions (Na and Metzger 2014). In rice, bacterial transcription activator-like (TAL) effectors can bind to effector-binding elements (EBEs) in SWEET gene promoters. EBE variants that could not be recognized by TAL effectors abrogated this induction, causing resistance (Eom et al. 2019), Therefore, a corresponding diagnostic kit enables the analysis of the bacterial blight in the field and identification of suitable resistant lines. Many specific promoters were cloned and could induce genes of interest expressed in specific tissues or during specific developmental periods (Chen et al. 2014; Luan et al. 2019), which can provide great convenience in the genetic engineering of novel cultivars (Kaur et al. 2020); Ren et al. 2019; Tzean et al. 2020).

To intensively explore the expression patterns of the *CfAOC* and *CfJMT* genes, we cloned their promoter

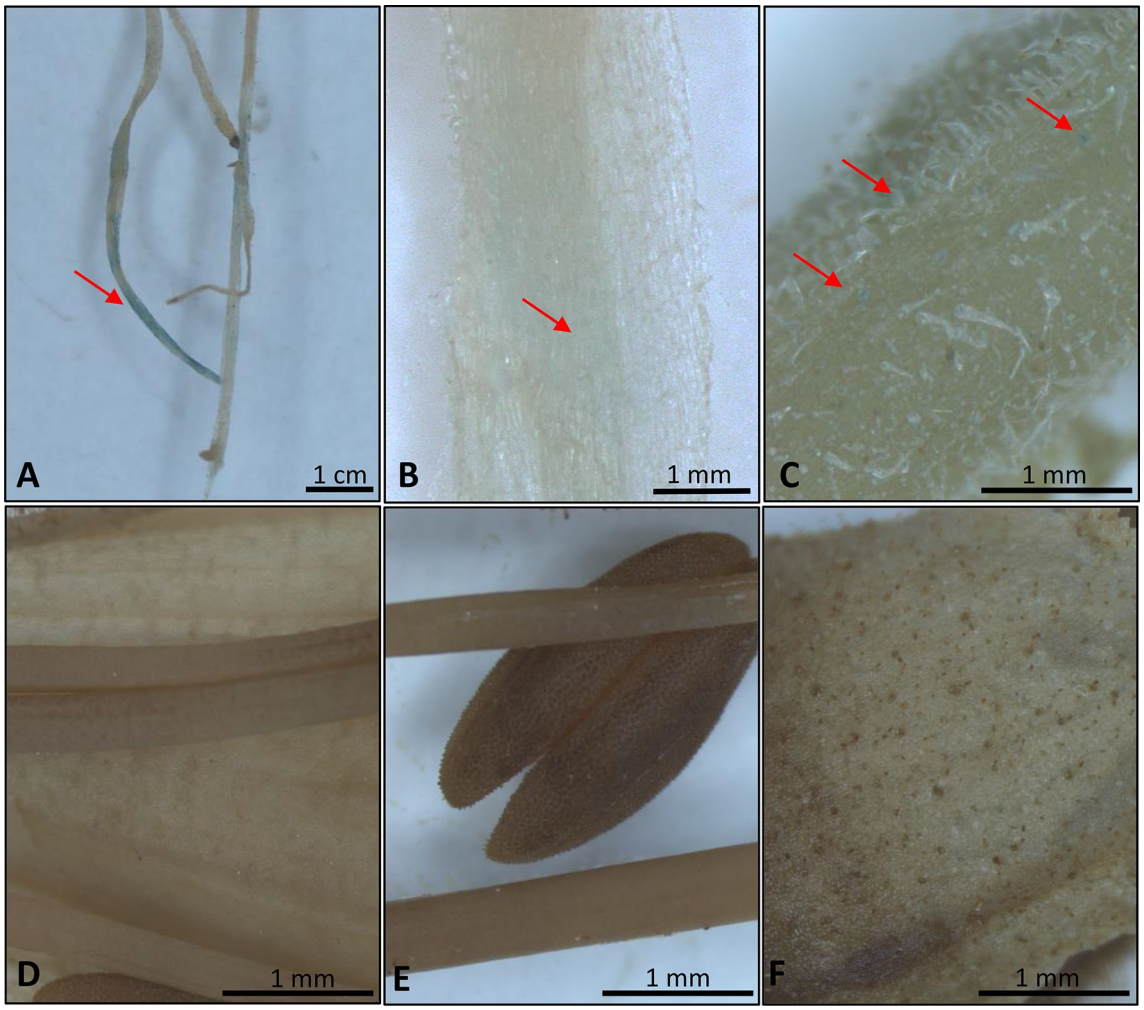

Fig. 3 GUS staining of transgenic tobacco plants AOC#7. **A** root; **B** stem; **C** leaf; **D** petal; **E** anther and filament; **F** sepal

sequences. Many conserved cis-elements were found in the promoters of *CfAOC* and *CfJMT*, mainly responsive to hormones, biotic and abiotic stresses, indicating the important functions of MeJA in *C. faberi* and its interconnection with other hormones, as reported in the mango (Winterhagen et al. 2019), grapevine (Ahmad et al. 2019), and tea (Zhang et al. 2019). In the promoter sequences of both *CfAOC* and *CfJMT*, the truncation of core elements such as the TATA and CAAT boxes proximal to the ATG was found to significantly reduce the promoter activity, as was observed in other organisms (Figs. 5 and 6) (Lubliner et al. 2015; Lin et al. 2008). The *CfAOC* gene was highly expressed in flowers at the blooming stage, but significantly lower in vegetative tissues. And the lowest expression of *CfAOC* gene was in flowers at the withered stage (Zhou et al. 2018). The expression level of *CfJMT* gene was the highest in flowers, especially at the blooming stage, but was almost undetectable in vegetative tissues (Xu et al. 2019). Combined with the spatiotemporal expression pattern of *CfAOC* and *CfJMT* genes in *C. faberi*, these results suggested that the *CfAOC* promoter is comparatively constitutive while the *CfJMT* promoter is flower-specific, since the former could express the *gus* gene in the seedlings of transgenic tobacco, and the latter could not. In the mature plant, *CfAOC* and *CfJMT* promoters both unlikely activated the expression of *gus* gene strongly except that *CfAOC* promoter had an activity in the root at any stages. Considering the differences between monocots and dicots, *CfAOC* and *CfJMT* promoters derived from *C. faberi* might have different regulatory ways in tobacco. For example, lack of corresponding regulatory proteins in tobacco could result in weak activities of *CfAOC* and *CfJMT* promoters.

In eukaryotes, gene expression is frequently mediated by multi-protein complexes (Zimmermann et al. 2004). Transcription factors are closely associated with a variety of plant physiological processes, and the basic helix–loop–helix transcription factor MYC2 is involved in the regulation of the MeJA pathway (Lorenzo et al. 2004; Song et al. 2014; Shin

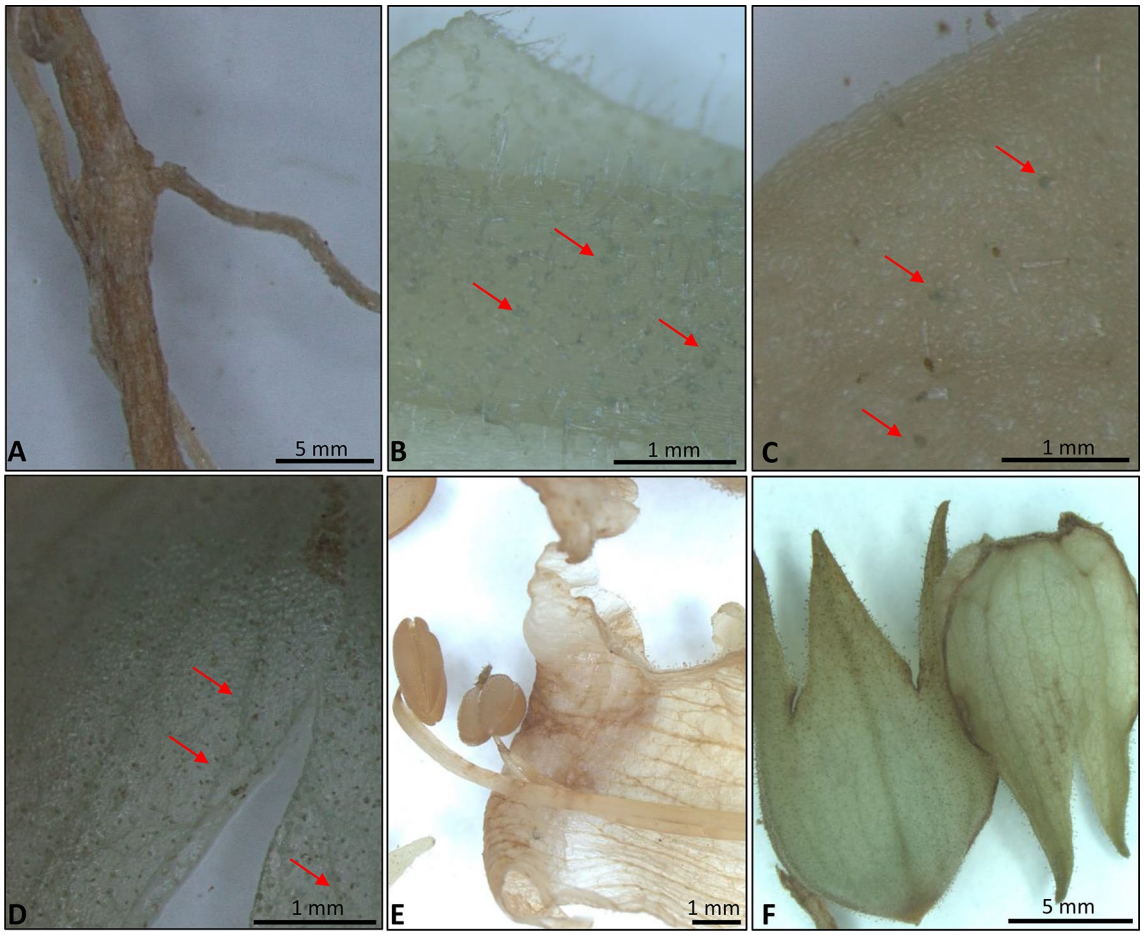

Fig. 4 GUS staining of transgenic tobacco plants JMT#1. **A**: root; **B** stem; **C** leaf; **D** petal; **E** anther and filament; **F** sepal

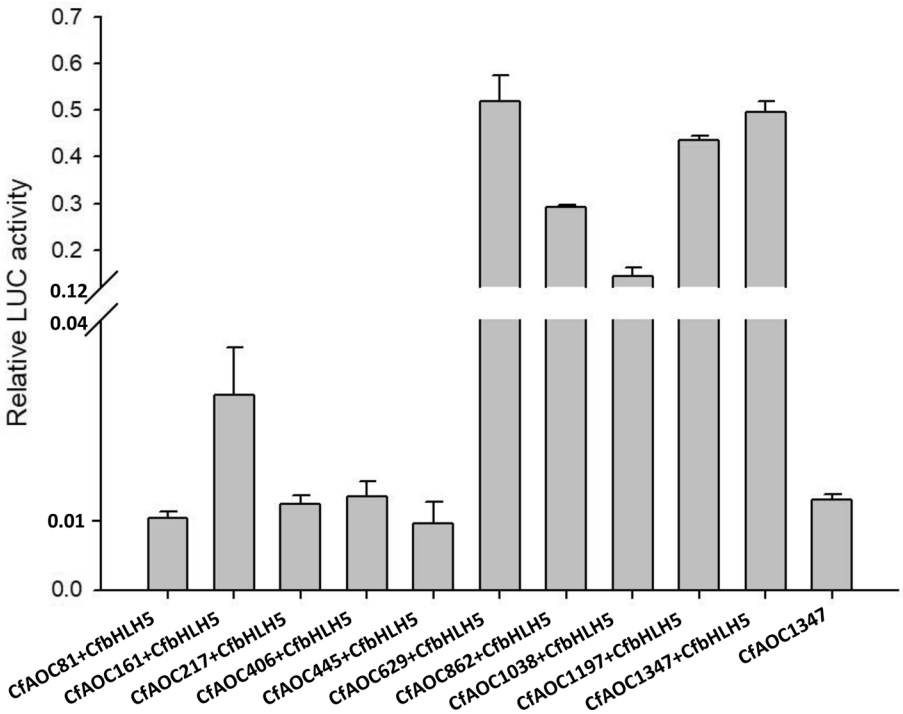

Fig. 5 Dual-luciferase assay of the relative LUC activity of the promoter sequence of the *CfAOC* gene when co-transformed with CfbHLH5. The LUC/REN ratio of the full length *CfAOC* promoter vector was used as control. The data represent the means and standard deviations from three biological replicates

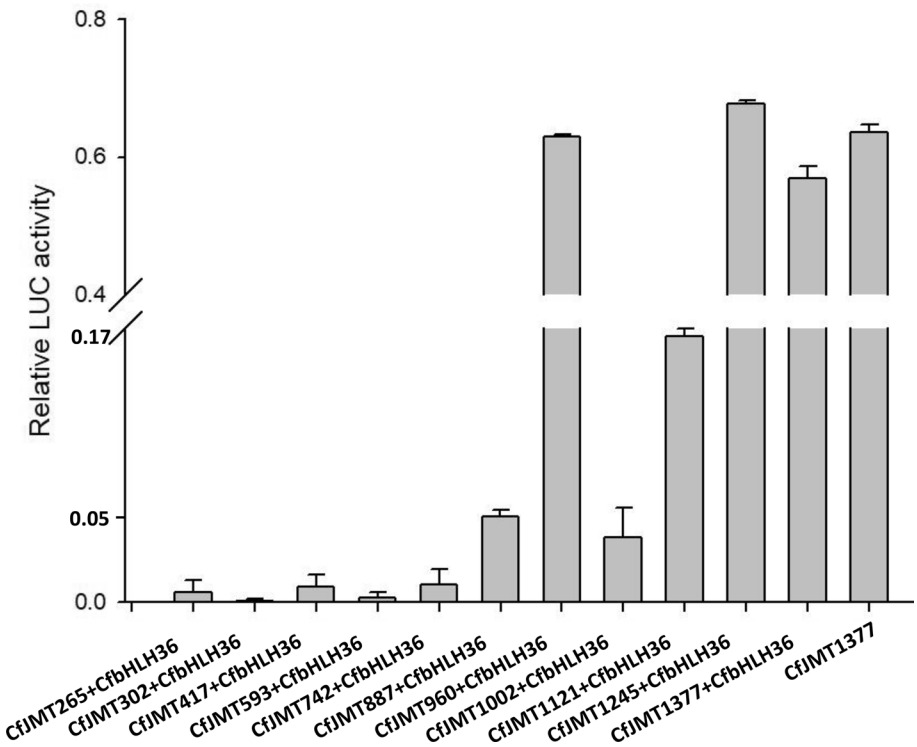

Fig. 6 Dual-luciferase assay of the relative LUC activity of the promoter sequence of *CfJMT* gene when co-transfected with CfbHLH36. The LUC/REN ratio of the full length *CfJMT* promoter vector was used as control. The data represent the means and standard deviations from three biological replicates

et al. 2014; Zhuo et al. 2020). To reveal the target sites of CfbHLH transcription factors in the promoters of the *CfAOC* and *CfJMT* genes, the transient co-transfection experiments were conducted in this study. The relative LUC activity in the dual-luciferase assay showed that the fragment between –629 nt and –1038 nt of the *CfAOC* promoter, as well as the fragment between –960 nt and –1121 nt of the *CfJMT* promoter were very important for the recognition by CfbHLH transcription factors. Coincidentally, there are two G-box motifs, –734 nt upstream of the ATG codon in the *CfAOC* promoter and –1088 nt upstream of the ATG codon in the *CfJMT* promoter, which are likely recognized by CfbHLH transcription factors and induce the expression of the *CfAOC* and *CfJMT* genes in *C. faberi*. This interaction will be further studied by EMSA in the future. The CfbHLH5 transcription factor could increase the activity of the *CfAOC* promoter but the CfbHLH36 transcription factor decreased the activity of the *CfJMT* promoter, suggesting that CfbHLH transcription factor may play both positive and negative roles in the regulation of MeJA biosynthesis, which was consistent with other studies in *Arabidopsis* (Nakata et al. 2013; Shin et al. 2014).

Conclusions

The flower fragrance is an important economical and appreciative trait of *C. faberi*. To reveal the regulation mechanism of flower fragrance in this oriental orchid, the promoter sequences of the *CfAOC* and *CfJMT* genes from MeJA pathway were cloned and analyzed. The expression pattern of *CfAOC* and *CfJMT* promoters was assessed in transgenic tobacco plants. Additionally, the target sites of CfbHLH transcription factors in the promoters were identified in tobacco in *vivo*, and will be further verified by EMSA in the future. This study provides further theoretic guidance for the genetic modification of the flower fragrance trait in non-scented orchids.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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All authors in the manuscript declare a consensus with publishing in Plant Biotechnology Reports.

References

Ahmad B, Zhang S, Yao J, Rahman MU, Hanif M, Zhu Y, Wang X (2019) Genomic organization of the B3-domain transcription factor family in grapevine (Vitis vinifera L) and expression during seed development in seedless and seeded cultivars. Int J Mol Sci 20:4553

Chen L, Jiang B, Wu C, Sun S, Hou W, Han T (2014) *GmPRP2* promoter drives root-preferential expression in transgenic Arabidopsis and soybean hairy roots. BMC Plant Biol 14:245

Choi H, Lyu JI, Lee HO, Kim JB, Kim SH (2020) Complete chloroplast genome sequence of an orchid hybrid *Cymbidium sinense* (♀) × *C. goeringii* (♂). Mitochondrial DNA B Resour 5:3784–3785

Eom JS, Luo D, Atienza-Grande G, Yang J, Ji C, Thi Luu V, Huguet-Tapia JC, Char SN, Liu B, Nguyen H, Schmidt SM, Szurek B, Vera Cruz C, White FF, Oliva R, Yang B, Frommer WB (2019) Diagnostic kit for rice blight resistance. Nat Biotechnol 37:1372–1379

Guan L, Denkert N, Eisa A, Lehmann M, Sjuts I, Weiberg A, Soll J, Meinecke M, Schwenkert S (2019) JASSY, a chloroplast outer membrane protein required for jasmonate biosynthesis. Proc Natl Acad Sci USA 116:10568–10575

Hernandez-Garcia CM, Finer JJ (2014) Identification and validation of promoters and cis-acting regulatory elements. Plant Sci 217–218:109–119

Hossain MM, Sharma M, Teixeira da Silva JA, Pathak P (2010) Seed germination and tissue culture of *Cymbidium giganteum* Wall. ex Lindl. Sci Hortic 123:479–487

Hossain MM, Kant R, Van PT, Winarto B, Zeng S, da Silva AT (2013) The application of biotechnology to Orchids. Crit Rev Plant Sci 32:69–139

Huang W, Khaldun ABM, Chen J, Zhang C, Lv H, Yuan L, Wang Y (2016) A R2R3-MYB transcription factor regulates the flavonol biosynthetic pathway in a traditional Chinese medicinal plant Epimedium sagittatum. Front Plant Sci 7:1089

Kaur A, Neelam K, Kitazumi A, Kaur K, Sharma P, Mangat GS, de los Reyes BG, Brar DS, Singh K, (2020) Novel *cis-acting* regulatory elements in wild *Oryza* species impart improved rice bran quality by lowering the expression of phospholipase D alpha1 enzyme (OsPLDα1). Mol Biol Rep 47:401–422

Ku YS, Sintaha M, Cheung MY, Lam HM (2018) Plant hormone signaling crosstalks between biotic and abiotic stress responses. Int J Mol Sci 19:3206

Lin X, Chen Y, Yi Y, Yan J, Zhang Z (2008) Promoter analysis of *Bombyx mori* nucleopolyhedrovirus *ubiquitin* gene. J Microbiol 46:429–435

Lorenzo O, Chico JM, Sánchez-Serrano JJ, Solano R (2004) *JASMONATE-INSENSITIVE 1* encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in *Arabidopsis*. Plant Cell 16:1938–1950

Luan A, He Y, Xie T, Chen C, Mao Q, Wang X, Li C, Ding Y, Lin W, Liu C, Xia J, He J (2019) Identification of an embryonic cell-specific region within the pineapple SERK1 promoter. Genes 10:883

Lubliner S, Regev I, Lotan-Pompan M, Edelheit S, Weinberger A, Segal E (2015) Core promoter sequence in yeast is a major determinant of expression level. Genome Res 25:1008–1017

Na JK, Metzger JD (2014) Chimeric promoter mediates guard cell-specific gene expression in tobacco under water deficit. Biotechnol Lett 36:1893–1899

Nakata M, Mitsuda N, Herbe M, Koo AJK, Moreno JE, Suzuki K, Howe GA, Ohme-Takagi M (2013) A bHLH-type transcription factor, ABA-inducible BHLH-type transcription factor/JA-associated MYC2-LIKE1, acts as a repressor to negatively regulate jasmonate signaling in *Arabidopsis*. Plant Cell 25:1641–1656

Omata A, Nakamura S, Yomogida K, Moriai K, Ichikawa Y, Watanabe I (1990) Volatile components of TO-YO-RAN flowers (*Cymbidium faberi* and *Cymbidium virescens*). Agric Biol Chem 54:1029–1033

Qi T, Huang H, Wu D, Yan J, Qi Y, Song S, Xie D (2014) *Arabidopsis* DELLA and JAZ proteins bind the WD-repeat/bHLH/MYB complex to modulate gibberellin and jasmonate signaling synergy. Plant Cell 26:1118–1133

Ramya M, Park PH, Chuang YC, Kwon OK, An HR, Park PM, Baek YS, Kang BC, Tsai WC, Chen HH (2019) RNA sequencing analysis of *Cymbidium goeringii* identifies floral scent biosynthesis related genes. BMC Plant Biol 19:337

Ren Q, Zhong Z, Wang Y, You Q, Li Q, Yuan M, He Y, Qi C, Tang X, Zheng X, Zhang T, Qi Y, Zhang Y (2019) Bidirectional promoter-based CRISPR-Cas9 systems for plant genome editing. Front Plant Sci 10:1173

Riemann M, Haga K, Shimizu T, Okada K, Ando S, Mochizuki S, Nishizawa Y, Yamanouchi U, Nick P, Yano M, Minami E, Takano M, Yamane H, Lino M (2013) Identification of rice *Allene Oxide Cyclase* mutants and the function of jasmonate for defence against *Magnaporthe oryzae*. Plant J 74:226–238

Seo HS, Song JT, Cheong JJ, Lee YH, Lee YW, Hwang I, Lee JS, Choi YD (2001) Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. Proc Natl Acad Sci USA 98(8):4788–4793

Shin S, Lv J, Fazio G, Mazzola M, Zhu Y (2014) Transcriptional regulation of ethylene and jasmonate mediated defense respons e in apple (*Malus domestica*) root during *Pythium ultimum* infection. Hortic Res 1:14053

Song S, Huang H, Gao H, Wang J, Wu D, Liu X, Yang S, Zhai Q, Li C, Qi T, Xie D (2014) Interaction between MYC2 and ethylene insensitive3 modulates antagonism between jasmonate and ethylene signaling in *Arabidopsis*. Plant Cell 26:263–279

Tzean Y, Chang H, Tu T, Hou B, Chen H, Chiu Y, Chou W, Chang L, Yeh H (2020) Engineering plant resistance to *Tomato yellow leaf curl Thailand virus* using phloem-specific promoter expressing hairpin RNA. Mol Plant Microbe Interact 33:87–97

Wang H, Tong C, Jang S (2017) Current progress in orchid flowering/flower development research. Plant Signal Behav 12:e1322245

Wang SL, Viswanath KK, Tong CG, An HR, Jang S, Chen FC (2019) Floral induction and flower development of orchids. Front Plant Sci 10:1258

Wei Y, Jin J, Yao X, Lu C, Zhu G, Yang F (2020) Transcriptome analysis reveals clues into leaf-like flower mutant in Chinese orchid *Cymbidium ensifolium*. Plant Divers 42:92–101

Winterhagen P, Hagemann MH, Wünsche JN (2019) Different regulatory modules of two mango *ERS1*promoters modulate specific gene expression in response to phytohormones in transgenic model plants. Plant Sci 289:110269

Xu Q, Wang S, Hong H, Zhou Y (2019) Transcriptomic profiling of the flower scent biosynthesis pathway of *Cymbidium faberi* Rolfe and functional characterization of its jasmonic acid carboxyl methyltransferase gene. BMC Genomics 20:125

Xu Y, Zhou J, Lu S, Wang S, Zhou Y (2020a) Cloning and molecular characterization of CfMYBs associated with the regulation of methyl jasmonate biosynthesis in *Cymbidium faberi*. Hortic J 89:593–601

Xu Y, Zhou J, Liu Q, Li K, Zhou Y (2020b) Construction and characterization of a high-quality cDNA library of *Cymbidium faberi* suitable for yeast one- and two-hybrid assays. BMC Biotechnol 20:4

Yang J, Duan GH, Li CQ, Liu L, Han GY, Zhang YL, Wang CM (2019) The crosstalks between jasmonic acid and other plant hormone signaling highlight the involvement of jasmonic acid as a core component in plant response to biotic and abiotic stresses. Front Plant Sci 10:1349

Zhang X, Zhu Z, An F, Hao D, Li P, Song J, Yi C, Guo H (2014) Jasmonate-activated MYC2 represses ethylene insensitive3 activity to antagonize ethylene-promoted apical hook formation in *Arabidopsis*. Plant Cell 26:1105–1117

Zhang S, Yang Y, Li J, Qin J, Zhang W, Huang W, Hu H (2018) Physiological diversity of orchids. Plant Divers 40:196–208

Zhang X, He Y, He W, Su H, Wang Y, Hong G, Xu P (2019) Structural and functional insights into the LBD family involved in abiotic stress and flavonoid synthases in *Camellia sinensis*. Sci Rep 9:15651

Zhao ML, Wang JN, Shan W, Fan JG, Kuang JF, Wu KQ, Li XP, Chen WX, He FY, Chen JY, Lu WJ (2013) Induction of jasmonate signalling regulators MaMYC2s and their physical interactions with MaICE1 in methyl jasmonate-induced chilling tolerance in banana fruit. Plant Cell Environ 36:30–51

Zhou Y, Yau Y, Ow DW, Wang Y (2012) Site-specific deletions in the tomato genome by the CinH-RS2 and ParA-MRS recombination systems. Plant Biotechnol Rep 6(3):225–232

Zhou Y, Chen L, Xu Y, Wang Y, Wang S, Ge X (2018) The *CfAOS* and *CfAOC* genes related to flower fragrance biosynthesis in *Cymbidium faberi* could confer drought tolerance to transgenic tomatoes. Int J Agric Biol 20:883–892

Zhuo M, Sakuraba Y, Yanagisawa S (2020) A jasmonate-activated MYC2-Dof2.1-MYC2 transcriptional loop promotes leaf senescence in Arabidopsis. Plant Cell 32:242–262

Zimmermann IM, Heim MA, Weisshaar B, Uhrig JF (2004) Comprehensive identification of *Arabidopsis thaliana* MYB transcription factors interacting with R/B-like BHLH proteins. Plant J 40:22–34