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Full Length Article

Cloning and Characterization of *OfMYBR1* Gene in Response to Circadian Rhythm affecting Floral Fragrance of *Osmanthus fragrans*

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Abstract

Osmanthus fragrans is a famous fragrant woody plant whose fragrance is influenced by circadian rhythm. To explore the molecular mechanism of circadian rhythm affecting floral fragrance of *O. fragrans*, an MYB transcription factor named *OfMYBR1* was cloned from *O. fragrans* 'Liuye Jingui' in this study. The full-length ORF of *OfMYBR1* is 905bp, encoding 304 amino acids and containing a conserved MYB-like domain. The encoded protein has the closest relationship with *Fraxinus velutina Fv*MybR1, followed by soybean *Gm*MYB1R1, and is clustered into a group with R1-MYB transcription factors from other plants, which belong to CCA1-like II subclass. Spatio-temporal expression pattern analysis showed that *OfMYBR1* gene had the highest expression in petals, followed by young leaves. *OfMYBR1* showed continuous expression during the whole flowering process; expression level increased after blooming, but changed insignificantly from the initial to the late flowering stage. The expression of *OfMYBR1* gene increased from 0:00 to 6:00 and decreased from 12:00 to 18:00. Subcellular localization showed that *Of*MYBR1 protein played a role in the nucleus. Given the previous analysis of synthesis and emission of floral volatiles and metabolic pathway genes expression, it is possible to infer that *OfMYBR1* gene regulates the synthesis of floral fragrance, possibly in response to circadian rhythm that positively regulates the transcription of structural genes involved in floral fragrance synthesis.

Keywords: *Osmanthus fragrans*; Floral fragrance; Circadian rhythm; R1-MYB transcription factor

Introduction

Osmanthus fragrans is a well-known fragrant woody plant with a long history of cultivation in China. Its flesh flowers have extremely strong and unique aroma, containing more than 70 floral volatiles mainly including terpenes, aromatics, esters, etc. (Cao *et al.* 2009; Xin *et al.* 2013; Fu *et al.* 2019; Zou *et al.* 2019). It is found that there is an obvious circadian rhythm in the synthesis and release of floral volatiles from *O. fragrans*. Zheng *et al.* (2017) examined the circadian rhythm of the emission and accumulation of terpene compounds in *O. fragrans* flowers, and suggested that the expression of genes involved in the synthesis of these compounds is also affected by circadian rhythm. The expression of alcohol acyltransferase (AAT) gene involved in the synthesis of ester compounds also shows circadian rhythm in *O. fragrans* flowers (Liu *et al.* 2016). These results indicated that the release and synthesis of floral volatiles in *O. fragrans* are generally regulated by circadian rhythm, but the molecular mechanism of this phenomenon is still unclear.

Previous studies have shown that MYB, especially the CIRCADIA CLOCK ASSOCIATED 1 (CCA1) subclass of R1-MYB transcription factor, is an important transcription factor regulating circadian rhythm. R1-MYB is an MYB transcription factor containing one R conserved domain. There are 49 and 84 gene members of R1-MYB in *Arabidopsis thaliana* and rice respectively (Katiyar *et al.* 2012). Compared with R2R3-MYB transcription factor, little is known about the function of R1-MYB transcription factor. Baranowskij *et al.* (2010) firstly found that only one R conserved domain in R1-MYB from potato can also play the role of transcriptional activation, which is different from other MYB transcription factors in DNA binding activity. CCA1 from *A. thaliana* is also R1-MYB type transcription factors that could bind to light-responsive promoters and act as a special activator to transmit photosensitive pigmentation-related signals and regulate circadian rhythm (Wang *et al.* 1997). Constitutive expression of *CCA1* gene in plants results in elongation of cotyledon hypocotyl and

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lag of flowering time (Wang and Tobin 1998). Therefore, R1-MYB transcription factor plays an important role in regulating circadian rhythm, while no findings about R1-MYB transcription factor in *O. fragrans* have been reported.

In this study, we cloned a R1-MYB transcription factor named *OfMYBR1* from *O. fragrans*. To gain an insight into the function of *OfMYBR1*, we applied sequence alignment, protein structure and gene expression pattern analysis, as well as subcellular localization to the *OfMYBR1* gene. The hypothesis to be tested was whether R1-MYB transcription factor could regulate the synthesis of the flower fragrance needs further study. The present work would be helpful for understanding of the molecular mechanism that regulates the synthesis of flower fragrance in *O. fragrans*.

Materials and Methods

Plant materials

In this experiment, all the samples were harvested from the adult tree of *O. fragrans* 'Liuye Jingui' (about 50 years old) in Huazhong Agricultural University (Wuhan, China). Drawing upon the studies by and Zeng *et al.* (2016), we separately collected the petals (also known as corolla lobes) at four stages: tight bud stage (S1), initial flowering stage (S2), full flowering stage (S3) and late flowering stage (S4). Flowers at the full flowering stage were divided into three parts: petals (P), stamens (S) and the remaining pedicels and pistils (PP). The young leaves (YL) of the current year's branches were collected in May. The sampling time for circadian rhythm analysis was 6:00–24:00 from the initial to the full flowering stage, once every six hours, and samples for other analysis were collected between 7:00 and 9:00.

Isolation of *OfMYBR1* gene and bioinformatics analysis

Total RNA was isolated using TRIzol reagent by the manufacturer's instructions (CoWin Biotech Co., Ltd., Beijing, China). The full-length of *OfMYBR1* gene sequence was obtained via the SMARTER^{TM} RACE method drawing upon the study by Zeng *et al.* (2015). The primers for 5'- and 3'- RACE-PCR (Table 1) were based on transcript-derived fragment from cDNA-AFLP (Zeng *et al.* 2019).

The DNAMAN 6.0 software (Lynnon Biosoft, USA) was used for sequence splicing and multiple sequence alignment. The *OfMYBR1* open reading frame (ORF) was predicted by the NCBI ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). The construction of the phylogenetic tree was based on the default parameters of neighbor-joining computational method by the MEGA 6.1 software. The protein structure and subcellular localization were performed according to Expash website (http://www.expasy.org/tools/) and WoLf PSORT software (http://www.genscript.com/psort/wolf_psort.html).

Real-time PCR analysis

The first-stranded cDNA was synthesized using RevertAid^{TM} First Strand cDNA Synthesis Kit, following the manufacturer's instructions (Fermentas, Thermo Fisher Scientific Inc., USA). Then, the qRT-PCR analysis was carried out according to the study by Zeng *et al.* (2015), on an Applied Biosystems 7500 Fast Real Time PCR platform (Applied Biosystems Life Technologies). The qRT-PCR primers based on the *OfMYBR1* gene full length cDNA sequence are listed in Table 1. Using β-actin as the endogenous control gene for data normalization, relative transcript levels were calculated by using the $2^{-\Delta\Delta Ct}$ method with three biological replicates and each reaction carried out in triplicate.

Subcellular localization of *OfMYBR1* gene

The upstream and downstream primers containing restriction sites of XbaI and PstI were used for *OfMYBR1* gene full-length cloning (see Table 1). The PCR product of *OfMYBR1* full-length was digested with XbaI and PstI. The restriction enzyme-generated inserts were cloned into the Super-1300::GFP binary vector with the XbaI-PstI restriction sites to create Super-1300::*Of*MYBR1:GFP via T4 DNA ligase (Fermentas, Thermo Fisher Scientific Inc., USA). The correct plasmid was transformed into *Agrobacterium tumefaciens* strain EHA105.

The transient genetic transformation was applied as described in the study by Zeng *et al*. (2015). About 35-day-old greenhouse-grown *Nicotiana benthamiana* seedlings were infiltrated with the *A. tumefaciens* strain EHA105, harboring the Super-1300::*Of*MYBR1:GFP and pCAMBIA 2300::p19 (1:1 pair-wise matching). *N. benthamiana* leaves infiltrated with the Super-1300::GFP and pCAMBIA 2300::p19 *Agrobacterium* cultures mixed in a 1:1 ratio were used as control. The processed leaves were cultured for 48–54 h in greenhouse, and then the location of fluorescence was detected by laser confocal microscopy.

Statistical analysis

Three biological replications of each sample were performed. The differentiation of gene expression level at different flowering period and in different tissues was performed with one-way ANOVA followed by comparison of means with LSD test ($P < 0.05$), using SPSS 19.0 software.

Results

Sequence characterization of *OfMYBR1* gene in *O. fragrans*

The *OfMYBR1* ORF sequence was 915 bp, encoding 304 amino acids (Fig. 1). The molecular formula of its encoded

Table 1: Primers used for gene cloning and expression analysis

Name of primers	Sequence of primers (5'–3')
RACE PCR	
OfMYBR1-3'-1	AAGAACACCACGATCCCTACACC
OfMYBR1-3'-2	ACACGTACACCCACACAGGTTGCAA
OfMYBR1-5'-1	TTGGGTGGTATGATTTTTCTTGATGC
OfMYBR1-5'-2	ATTTTTGAAGATGGGGGAGGTGGAA
Cloning the full-length ORF	
OfMYBR1-FL-F	GGCCTCTAAACCTTATATGCGCC
OfMYBR1-FL-R	TTATTCCCATCAAGAAACACTAACC
Real-time PCR	
OfMYBR1-F	CAAGAACACCACGATCCCTACA
OfMYBR1-R	TAACCATGCTATCTCCACTACCG
Actin-F	ATTATTTCCTTGCTCATACGGTCAG
Actin-R	ATTAGTCCTCTTCCAGCCTTCTTTG
Constructing the subcellular localization vector	
OfMYBR1-Y-F	GCTCTAGAATGCGCCAAAACTCCATTAATT
OfMYBR1-Y-R	AACTGCAGGAAACACTAACCATGCTATCTCCAC

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1    ACATGGGGCCTCTAAACCTTATATGCGCCAAAACTCCATTAATTTGCACAATCTCCAGCATTCGTTAATA
                                M  R  Q  N  S  I  N  L  H  N  L  Q  H  S  L  I
71   TACCTTTTAATGGAGGCGGCGGAGGCGGCCGGTGGAAACAACGATCAGAATTCGCCGGAGGTCAGAGGT
      Y  L  L  M  E  A  A  E  A  A  G  G  N  N  D  Q  N  S  P  E  V  R  G
140  GGCGGCGGCAAAGGGTTCATGCTGTTTGGTGTAAGAGTCATGGAAGGATCGTTTAGGAAGAGTGCTAGT
      G  G  G  K  G  F  M  L  F  G  V  R  V  M  E  G  S  F  R  K  S  A  S
209  TTGAATAACCTGGCTCAGTATGAGCAACCCCATGAGTCCAACAATGATGTGGCCGCCGGCTATGCTTCT
      L  N  N  L  A  Q  Y  E  Q  P  H  E  S  N  N  D  V  A  A  G  Y  A  S
278  GACGATATTGTCCACCCTTCTGGCCGGAGTCATGATCGGAAAAGAGGAGTGCCATGGACTGAGGAGGAA
      D  D  I  V  H  P  S  G  R  S  H  D  R  K  R  G  V  P  W  T  E  E  E
347  CACAGGTTATTTCTAATAGGGTTGCAGAAAGTAGGGAAAGGAGATTGGAGAGGGATTTCAAGAAACTTT
      H  R  L  F  L  I  G  L  Q  K  V  G  K  G  D  W  R  G  I  S  R  N  F
416  GTGAAGACACGTACACCCACACAGGTTGCAAGCCATGCTCAAAAGTACTTTCTTCGCCGGAATAACCAT
      V  K  T  R  T  P  T  Q  V  A  S  H  A  Q  K  Y  F  L  R  R  N  N  H
485  AGCCGCCGGCGCCGGAGATCTAGTCTCTTTGATATCACCACTGATACGGTTTTGGGTTCGAAAATTGGA
      S  R  R  R  R  R  S  S  L  F  D  I  T  T  D  T  V  L  G  S  K  I  G
554  GACCAAAGGCATCAAGAAAAATCATACCACCCAACAACGGTAAGCAAAAATAATGAAAAATTCCCCGTG
      D  Q  R  H  Q  E  K  S  Y  H  P  T  T  V  S  K  N  N  E  K  F  P  V
623  TCAGCTTTTCTTGTACCAATGACGATAGAAAATTCAACAGAGAATCTCACTCTAGGAATGAAACATTCA
      S  A  F  L  V  P  M  T  I  E  N  S  T  E  N  L  T  L  G  M  K  H  S
692  ACCAATCTCATCCCTCCAATTCCAAATCTTCCACCTCCCCCATCTTCAAAAATGGCCAATTTAGATCTG
      T  N  L  I  P  P  I  P  N  L  P  P  P  P  S  S  K  M  A  N  L  D  L
761  AACAAGAACACCACGATCCCTACACCTGAGCCTCTCCCTTTAACACTTAAGCTGTCCATATCACCAACT
      N  K  N  T  T  I  P  T  P  E  P  L  P  L  T  L  K  L  S  I  S  P  T
830  CCGCCACCAGACAATTATCCATCTCCGGCGAGACACGTGTCGGGTTTCCAGACAATGCAAGCTAGCTTT
      P  P  P  D  N  Y  P  S  P  A  R  H  V  S  G  F  Q  T  M  Q  A  S  F
899  AGTAGCGGTAGTGGAGATAGCATGGTTAGTGTTTCTTGATGGGAATAATGTTATTGATATCTGTGTTGT
      S  S  G  S  G  D  S  M  V  S  V  S  -
968  AAAGTTGGGAAAAAAAAATAGACTAAAGTTGGATGATTAATTACTTAGGTTTGGATTATAATTTAATAT
1037 TTAGATTATTGTTGATTAGGTGGGTAGGTGATGATGATATCTTATTATAAAGGATAGAAAAGATTGTGA
1106 AATTTGGTGGCAAAAAAAAAAAAAAAAAAAAAA
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Fig. 1: Nucleotide and amino acid sequence of *OfMYBR1*

protein was $C_{1466}H_{2322}N_{440}O_{451}S_9$, with molecular weight 33.70 kDa and theoretical isoelectric point (pI) 9.98. There were 26 negative charge amino acid residues (Asp + Glu) and 35 positive ones (Arg + Lys) in the *Of*MYBR1 protein. Protein multi-alignment (Fig. 2) of *Of*MYBR1 with R1-MYB from other plants revealed that *Of*MYBR1 contained a conserved MYB-like domain. Phylogenetic analysis (Fig. 3) of the predicted amino acid sequence compared with R1-MYB in other species showed that *Of*MYBR1 had the closest relationship with *Fraxinus velutina Fv*MybR1 (AGK29591.1), followed by soybean *Gm*MYB1R1 (NP_001304346.2). It was grouped together with potato *St*MYB1R1 (ABB86258.1), rose *Rh*MYB (ABU53684.1), soybean *Gm*MYB176 (ABH02865.1), *At*1g19000 (BAH19529.1) and *At*lg74840 (BAH56970.1) of *A. thaliana* belonging to CCA1-like II subclass. Therefore, it is possible

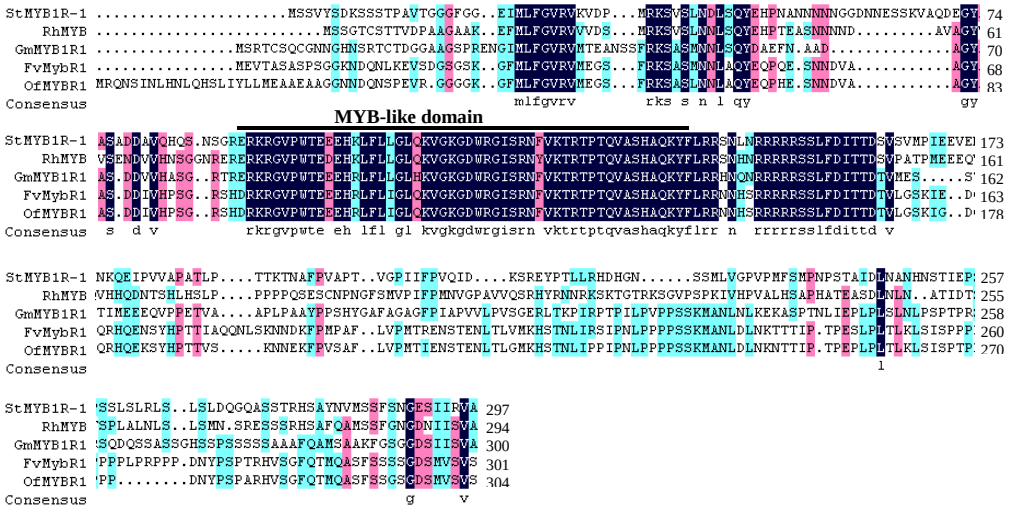

Fig. 2: Protein multi-alignment of *Of*MYBR1 with R1-MYB from other plants

to infer that the *OfMYBR1* gene in *O. fragrans* has similar function to those in the CCA1-like II subclass.

Temporal and spatial expression analysis of *OfMYBR1* gene in *O. fragrans*

The expression levels of *OfMYBR1* gene at different flowering stages detected by real-time PCR showed that this gene was continuously expressed during the whole flowering process from tight bud to late flowering stage (Fig. 4). Its expression level was low at the tight bud stage and had no significant change from the initial to late flowering stage. In analyzing the expression levels of the *OfMYBR1* gene in different tissues (Fig. 5), the highest expression level was found in petals, followed by young leaves, pedicels and pistils, and the lowest expression level was found in stamens. The detection of *OfMYBR1* gene expression levels for three consecutive days and nights showed that the gene expression presented a significant circadian rhythm, showing a gradual increase from 0:00 to 6:00 and a gradual decrease from 12:00 to 18:00 (Fig. 6).

Subcellular location of *OfMYBR1* gene

The subcellular localization of *OfMYBR1* was predicted by WoLf PSORT software. The result showed that *Of*MYBR1 protein might be located in the nucleus. We constructed Super-1300::*Of*MYBR1:GFP fusion vector and carried out transient genetic transformation in *N. benthamiana* leaves. 48 h after injection, the laser confocal fluorescence microscopy detected that the blank vector could find the fluorescence signal in the whole cell, while fluorescence signal could be found only in the nuclear region by the vector containing *OfMYBR1* gene (Fig. 7). These results indicated that *OfMYBR1* gene actually plays a role in the nucleus.

Discussion

Circadian rhythms based on an endogenous transcriptional clock are observable biological oscillations that occur with a 24 h periodicity (McClung 2006). Circadian rhythms affect many important physiological processes of plants, such as hypocotyl elongation, leaf movement, stomatal switch and flowering (Greenham and McClung 2015; Han *et al.* 2016). The synthesis and release of flower fragrance are also influenced by circadian rhythm, which is often expressed in diurnal or nocturnal release patterns (Lerdau and Gray 2003; Martin *et al.* 2003; van Doorn and Woltering 2008). Our previous studies found out that there are also obvious circadian rhythms in the synthesis and release of floral volatiles in *O. fragrans* (Liu *et al.* 2016; Zheng *et al.* 2017). However, the molecular mechanism of these rhythmic synthesis and release controlled by circadian rhythm remains unclear. In this study, an MYB transcription factor encoding 304 amino acids was cloned from *O. fragrans*. There was only one conserved MYB-like domain in this predicted protein, which has the typical characteristics of R1-MYB transcription factors, named *Of*MYBR1. Phylogenetic tree analysis showed that the protein encoded by this gene was clustered into a group of R1-MYB transcription factors from soybean, potato, rose and other plants, and belonged to CCA1-like II subclass. Yan *et al.* (2011) reveal that R1-MYB transcription factor in rose is highly expressed in aromatic wild-type petals, and its expression changes with the amount of flower fragrance release. These results suggest that *OfMYBR1* obtained in this study may play a similar role to those of R1-MYB transcription factors in other plants that participate in the regulation of flower fragrance in response to circadian rhythm in *O. fragrans*.

Further analysis of the spatial and temporal expression pattern of the *OfMYBR1* gene showed that this gene had the highest expression level in petals and continuous high

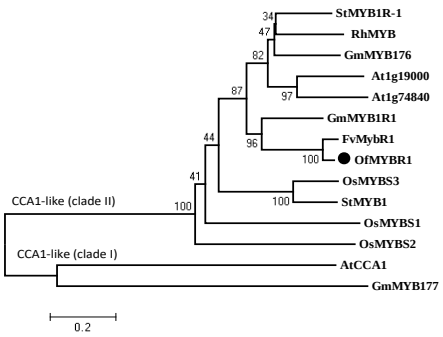

Fig. 3: Homology tree and phylogenetic tree of *Of*MYBR1and R1-MYB from other plants. StMYB1R-1: *Solanum tuberosum* ABB86258.1; RhMYB: *Rosa hybrid* ABU53684.1; GmMYB176: *Glycine max* ABH02865.1; At1g19000: *Arabidopsis thaliana* BAH19529.1; At1g74840: *A. thaliana* BAH56970.1; GmMYB1R1: *G. max* NP_001304346.2; FvMybR1: *F. velutina* AGK29591.1; OsMYBS3: *Oryza sativa* AAN63154.1; StMYB1: *S. tuberosum* AAB32591.2; OsMYBS1: *O. sativa* AAN63152.1; OsMYBS2: *O. sativa* AAN63153.1; AtCCA1: *A. thaliana* AAB40525.1; GmMYB177: *G. max* ABH02866.1

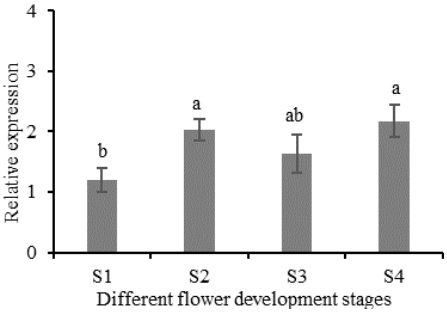

Fig. 4: Relative expression of *OfMYBR1* gene at different flowering periods. S1, Tight bud stage; S2, initial flowering stage; S3, full flowering stage; S4, late flowering stage. Identical superscript letters indicate that the difference is not significant, whereas different superscript letters imply a significant difference P<0.05

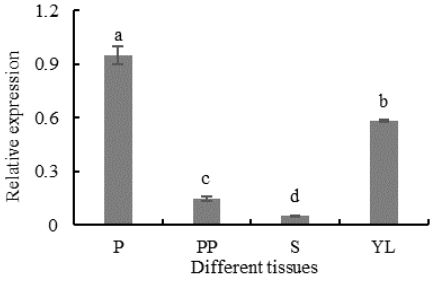

Fig. 5: Relative expression of *OfMYBR1* gene in different tissues. P, Petal; PP, Peduncle and pistil; S, Stamen; YL, Young leaf. Identical superscript letters indicate that the difference is not significant, whereas different superscript letters imply a significant difference. P<0.05

expression throughout the flowering process. The *OfMYBR1* gene expression levels within a day showed circadian rhythm, increasing from 0:00 to 6:00 and decreasing from 12:00 to 18:00. Flower petals are the main tissues for the synthesis and release of floral volatiles in plants (Dudareva *et al.* 2013). The synthesis and release of floral volatiles in *O. fragrans* increase significantly from the initial flowering stage (Zeng *et al.* 2015). Zheng *et al.* (2017) have analyzed the circadian rhythm of flower fragrance in *O. fragrans* and concluded that the volatile and free forms of the main aroma components, such as linalool, ocimene and ionone, increase from 0:00 to 6:00, decrease from 12:00 to 18:00, reach a low from 18:00 to 0:00 and peak from 6:00 to 12:00. The glycosidic form of linalool increases from 6:00 to 12:00 and decreases from 18:00 to 0:00. The structural genes involved in the biosynthetic pathway of these floral volatiles increase from 6:00 to 18:00 in the daytime and decrease from 18:00 to 6:00 in the night. It can be seen that the expression pattern of *OfMYBR1* was basically consistent with that of structural genes involved in floral volatiles synthesis and the regulation of floral volatiles synthesis and release. The expression time of *OfMYBR1* was earlier than that of structural genes involved in floral volatiles synthesis. Subcellular localization results showed that *Of*MYBR1 protein played a role in the nucleus. Thus, we hold that the *OfMYBR1* gene responding to the circadian rhythm might positively regulate the transcription of structural genes involved in floral volatiles synthesis, and affect flower fragrance synthesis and release during the day.

Conclusion

A R1-MYB transcription factor named *OfMYBR1* that may be involved in the regulation of flower fragrance in response to circadian rhythm has been obtained in *O. fragrans* for the first time. The protein structure, homology comparison, expression pattern and protein subcellular localization of the *OfMYBR1* gene have been preliminarily completed, laying a foundation for the further study of the molecular mechanism of circadian rhythm regulating the synthesis and release of flower fragrance in *O. fragrans*.

Acknowledgments

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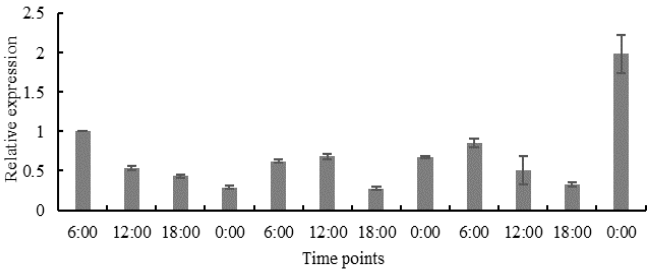

Fig. 6: Circadian change of *OfMYBR1* transcript level at different time points of three days

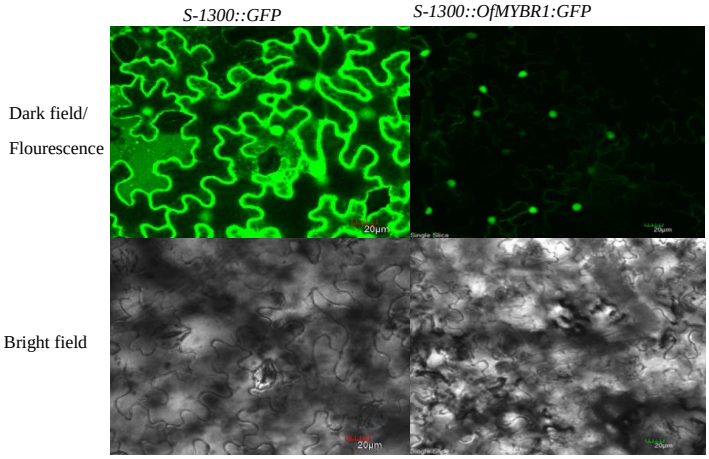

Fig. 7: Fluorescence detection of *OfMYBR1* subcellular location (Bar=20 μm)

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