Dissecting promoter of InMYB1 gene showing petal-specific expression

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Abstract We had previously reported that the InMYB1 promoter, the 1023 bp upstream region of InMYB1, works petal-specifically in various dicot plants by recognizing petal identity at a cellular level. To determine the petal-specific region in the InMYB1 promoter, Arabidopsis plants harboring InMYB1_1023b::GUS (β-glucuronidase), InMYB1_713b::GUS, InMYB1_506b::GUS, InMYB1_403b::GUS, InMYB1_332b::GUS, InMYB1_200b::GUS and InMYB1_140b::GUS were produced and confirmed a shortest region, which has the petal-specific promoter activity by using histochemical GUS assay. Petal-specific GUS staining was not observed in the Arabidopsis plants transformed with InMYB1_200b::GUS and InMYB1_140b::GUS, but observed in transgenic Arabidopsis plants harboring from InMYB1_1023b::GUS to InMYB1_332b::GUS. cDNA sequence of InMYB1 shows that 120 bp upstream region of InMYB1 is 5′ untranslated region, suggesting that the 332-121 bp upstream region of InMYB1 contains an important element for petal-specific gene expression. In the Arabidopsis harboring the InMYB1_332-121b×3_TATA_Ω::GUS, petal-specific GUS staining was observed and the staining was stronger than in the Arabidopsis harboring InMYB1_1023b::GUS. This result shows that the 332-121 bp region is enough and essential for the petal specificity and the InMYB1_332-121b×3_TATA_Ω could be used for the molecular breeding of floricultural crops.

Key words: cis-element, InMYB1 promoter, petal-specific gene expression.

Introduction

Genetic transformation has been used to produce novel crops with superior traits and to analyze gene function (Fukui et al. 2003; Katsumoto et al. 2007; Sasaki et al. 2014, 2016; Tanaka et al. 2013). For the genetic transformation, not only transgenes but also promoters, which control transgene expression in desired tissues and timings, are important (Oshima and Mitsuda 2016; Sasaki et al. 2016). Although cauliflower mosaic virus 35S (CaMV35S) promoter is used most frequently to drive constitutive transgene expression (Benfey and Chua 1990; Odell et al. 1985), tissue-specific promoters is also conventionally employed. For instance, 2A11 and E8 promoters were used as fruit-specific promoters in tomato (Deikman and Fischer 1988; Pear et al. 1989). Seed-specific promoters for soybean, broad bean, and carrot and endosperm-specific promoters for rice and corn were also reported (Abbadi et al. 2004; Karunanandaa et al. 2005; Peremarti et al. 2010; Raclaru et al. 2006; Zhu et al. 2008). We had previously reported that the InMYB1 promoter from Japanese morning glory (Ipomoea nil) functions petal-specifically in various dicot plants (Azuma et al. 2016a, b). The InMYB1 promoter (InMYB1_1023b) is an ideal tool for molecular breeding of floricultural crops, because it can restrictively modify phenotype in petals without inducing undesirable changes in other organs or tissues (Azuma et al. 2016a, b).

Promoter consists of three parts: core promoter, proximal region and distal enhancer (Dey et al. 2015). Core promoter usually contains TATA-box element which is bound by TATA-box binding protein and the protein complex containing RNA polymerase. Promoter...
can’t work by core promoter element alone, because binding of transcription factors (TFs) to cis-elements in proximal region is necessary to give spatio-temporal preference to the gene expression. Distal enhancer, which sometimes presents far upstream of proximal region, is also bound by TFs and modify the gene expression. Several databases, such as Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PLACE (https://sogo.dna.affrc.go.jp/cgi-bin/sogo.cgi?lang=en&pj=640&action=page&page=newplace), provide information about cis-elements in plants (Higo et al. 1999; Lescot et al. 2002).

In this study, we attempted to identify the petal-specific region of InMYB1 promoter by deleting the upstream region. Our results showed that the 332–121 bp upstream region of InMYB1 is an essential element for petal-specific promoter function. Furthermore, we investigated the promoter activity of a combination of three-tandem repeat of the 332-121 bp upstream region of InMYB1, the TATA-box element of CaMV35S promoter, and translational enhancer Ω and showed that the modified system can promote higher gene expression than the original InMYB1 promoter.

### Materials and methods

#### Construction of vectors

To produce the vector harboring InMYB1_1023b::GUS (β-glucuronidase), InMYB1_713b::GUS, InMYB1_506b::GUS, InMYB1_403b::GUS, InMYB1_332b::GUS, InMYB1_200b::GUS or InMYB1_140b::GUS, 1023 bp, 713 bp, 506 bp, 403 bp, 332 bp, 200 bp or 140 bp upstream region of InMYB1 was amplified by PCR from a genomic clone (DDBJ Accession number: AB232773; Morita et al. 2006) using the primers described in Table 1 and then replaced the CaMV35S promoter in pBI121 (Figure 1). To produce the vector harboring InMYB1_332-121b×3_TATA_Ω::GUS, 332-121 bp upstream region of InMYB1 was amplified by PCR using the primer described in Table 1 and then these three fragments were concatenated by In-fusion HF cloning Kit (TaKaRa, Shiga, Japan). The obtained three-tandem repeat fragment was fused to TATA element and Ω enhancer of pTATA-LUC-HSP vector (Yoshida et al. 2013). After that, the InMYB1_332-121b×3_TATA_Ω replaced the CaMV35S promoter in pBI121 (Figure 4).

**Plant transformation**

InMYB1_1023b::GUS, InMYB1_713b::GUS, InMYB1_

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**Table 1. List of primers used in this study.**

<table>
<thead>
<tr>
<th>Construction</th>
<th>Forward primer (5′-3′)</th>
<th>Reverse Primer (5′-3′)</th>
</tr>
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<tbody>
<tr>
<td>InMYB1_1023b::GUS</td>
<td>5′-CGAAGCTTCAATCAATTAATCAGTGGG-3′</td>
<td>5′-CAAGGATCCCGGCGCTGAAATT-3′</td>
</tr>
<tr>
<td>InMYB1_713b::GUS</td>
<td>5′-CGAAGCTTGGAGCTTGATGATAATTTGTTG-3′</td>
<td>5′-CAAGGATCCCGGCGCTGAAATT-3′</td>
</tr>
<tr>
<td>InMYB1_506b::GUS</td>
<td>5′-CGAAGCTTATGATGATGATAATTTTGTCC-3′</td>
<td>5′-CAAGGATCCCGGCGCTGAAATT-3′</td>
</tr>
<tr>
<td>InMYB1_403b::GUS</td>
<td>5′-CGAAGCTTCTTCATATATCATCTCTGCCC-3′</td>
<td>5′-CAAGGATCCCGGCGCTGAAATT-3′</td>
</tr>
<tr>
<td>InMYB1_332b::GUS</td>
<td>5′-CGAAGCTTCCCACTAATCTTCTGCCC-3′</td>
<td>5′-CAAGGATCCCGGCGCTGAAATT-3′</td>
</tr>
<tr>
<td>InMYB1_200b::GUS</td>
<td>5′-CGAAGCTTCTTCATATATCATCTCTGCCC-3′</td>
<td>5′-CAAGGATCCCGGCGCTGAAATT-3′</td>
</tr>
<tr>
<td>InMYB1_140b::GUS</td>
<td>5′-CGAAGCTTACACTGTTCATGTGGAG-3′</td>
<td>5′-CAAGGATCCCGGCGCTGAAATT-3′</td>
</tr>
<tr>
<td>InMYB1_332-121 b (1)</td>
<td>5′-ACTGGGCGCCGGTACCTGCACGACTTGGATGTTAATG-3′</td>
<td>5′-AACATCGGCGGAATCGACACTGACCACCCAAATT-3′</td>
</tr>
<tr>
<td>InMYB1_332-121 b (2)</td>
<td>5′-CAGGACTTTGATGGATGATAAT-3′</td>
<td>5′-AACATCGGCGGAATCGACACTGACCACCCAAATT-3′</td>
</tr>
<tr>
<td>InMYB1_332-121 b (3)</td>
<td>5′-CAGGACTTTGATGGATGATAAT-3′</td>
<td>5′-AACATCGGCGGAATCGACACTGACCACCCAAATT-3′</td>
</tr>
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**Gene expression**

<table>
<thead>
<tr>
<th>Forward primer (5′-3′)</th>
<th>Reverse Primer (5′-3′)</th>
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</thead>
<tbody>
<tr>
<td>AtEF1α</td>
<td>5′-CTGAGGTTTGAGGCTGATGATGATGATAAT-3′</td>
</tr>
<tr>
<td>GUS</td>
<td>5′-GGCTCACACCGCTACACGATC-3′</td>
</tr>
</tbody>
</table>
506b::GUS, InMYB1_403b::GUS, InMYB1_332b::GUS, InMYB1_200b::GUS, InMYB1_140b::GUS or InMYB1_332-121b×3_TATA_Ω::GUS vectors were transformed into Agrobacterium tumefaciens GV3101. Arabidopsis thaliana (Col-0) was transformed using the Agrobacterium harboring each vector by floral dip method (Clough and Bent 1998). The T1 generation of 8–22 independent transgenic lines for each construct were analyzed by histochemical GUS assay.

**Histochemical GUS assay**

We analyzed the promoter activity of InMYB1 upstream regions by histochemical GUS assay following the method of Jefferson (1987) with partial modification. Samples were soaked in X-Gluc buffer [12 mM potassium ferricyanide, 12 mM potassium ferrocyanide, 0.3% (v/v) Triton X-100 and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronide] and the buffer was infiltrated into the samples under vacuum. Following staining for 2 days at 37°C, the samples were washed in 70% Ethanol.
Petalspecific element of *InMYB1* promoter

(v/v) ethanol and then photographed using a stereomicroscope (VB-G25; Keyence, Osaka, Japan) and a CCD camera (VB-7100; Keyence).

**Measurement of promoter activity**

Level of GUS mRNA was detected by real-time RT-PCR using the primers in Table 1. *Elongation factor-1 α* (AtEF1) was used as an internal control. Total RNA was isolated from stage-3 flower buds (Azuma et al. 2016b) using Trizol (Thermo Fisher Scientific, MA, USA) and reverse-transcribed using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Shiga, Japan). Real-time PCR was carried out using obtained cDNA and SYBR Premix EX Taq (TaKaRa, Shiga, Japan). Real-time PCR was carried out using obtained cDNA and SYBR Premix EX Taq (TaKaRa, Shiga, Japan) with StepOnePlus real-time PCR system (Thermo Fisher Scientific, MA, USA). The PCR cycle was 95°C for 5 s, 58°C for 20 s. This cycle was repeated 40 times.

**Results and discussion**

**Identification of the petal-specific region by deleting the upstream region of InMYB1**

To identify the petal-specific region of *InMYB1* promoter, *InMYB1*~1023b::GUS, *InMYB1*~713b::GUS, *InMYB1*~506b::GUS, *InMYB1*~403b::GUS, *InMYB1*~332b::GUS, *InMYB1*~200b::GUS or *InMYB1*~140b::GUS transgenic Arabidopsis plants were produced and analyzed by histochemical GUS assay. In all organs of *InMYB1*~200b::GUS and *InMYB1*~140b::GUS transgenic plants, no GUS staining was observed (Figure 2). On the other hand, petal-specific GUS staining was observed in *InMYB1*~1023b::GUS, *InMYB1*~713b::GUS, *InMYB1*~506b::GUS, *InMYB1*~403b::GUS and *InMYB1*~332b::GUS transgenic plants (Figure 2). To know the extent of promoter activity, ratio of GUS-staining flowers was determined. In the *InMYB1*~1023b::GUS transgenic plants, 97% of the flowers were stained and more than 85% of the flowers were stained in the *InMYB1*~713b::GUS and *InMYB1*~506b::GUS transgenic plants (Figure 3). In the *InMYB1*~403b::GUS or *InMYB1*~332b::GUS transgenic plants, it was decreased to 50% or 30%, respectively, and no GUS staining was observed in the *InMYB1*~200b::GUS and *InMYB1*~140b::GUS transgenic plants (Figure 3). We previously reported that some kinds of *cis*-element such as CAAT-box, which increases promoter activity, exist in the 1023-332 bp upstream region of *InMYB1* (Azuma et al. 2016b). Although the petal-specific promoter activity became weaker and unstable by deletion of the several *cis*-elements, the 332 bp upstream region of *InMYB1* still functioned as a petal-specific promoter.

**Determination of 5′UTR region of InMYB1 promoter**

To determine 5′ untranslated region (UTR), which shouldn’t contain *cis*-elements, cDNA sequence of *InMYB1* was searched in the Gene Index database of *Ipomoea nil* ([http://compbio.dfci.harvard.edu/cgi-bin/tgi/Blast/index.cgi](http://compbio.dfci.harvard.edu/cgi-bin/tgi/Blast/index.cgi)). The obtained cDNA sequence of *InMYB1* showed that the 120 bp upstream region of *InMYB1* is 5′UTR. Together with the data obtained by promoter-deletion analysis, we hypothesized that the 332-121 bp upstream region of *InMYB1* is essential for the petal-specificity.

**Improvement of InMYB1 promoter for molecular breeding**

It has been reported previously that the promoter activity including three-tandem repeat of *cis*-element was stronger than that of the native promoter (Espley et al. 2009). We thought that the three-tandem repeat of the 332-121 bp upstream region of *InMYB1* is possibly more stable and stronger than a single of 332-121 bp upstream region of *InMYB1*. Therefore, to demonstrate that the 332-121 bp upstream region is enough for the petal-specific expression, we investigated the promoter activity and petal-specificity of *InMYB1*~332-121b×3_TATA_Ω, a combination of three-tandem repeat of the 332-121 bp upstream region of the *InMYB1* gene, the TATA-box element of CaMV35S promoter, and translational enhancer Ω (Figure 4). We examined 8 independent lines of *InMYB1*~332-121b×3_TATA_Ω::GUS transgenic Arabidopsis plants and strong GUS staining in petals was observed in all the lines, indicating that the 332-121 bp upstream region is enough and essential for the petal-specific expression (Figure 4).

Furthermore, to compare the promoter activity of *InMYB1*~332-121b×3_TATA_Ω with the original

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Figure 3. The ratio of GUS-staining flowers of Arabidopsis harboring *InMYB1*~1023b::GUS, *InMYB1*~713b::GUS, *InMYB1*~506b::GUS, *InMYB1*~403b::GUS, *InMYB1*~332b::GUS, *InMYB1*~200b::GUS and *InMYB1*~140b::GUS. Light gray bars show the proportion of weak GUS-staining flowers while dark gray bars show the proportion of strong GUS-staining flowers. Each value represents mean of 11–22 independent transgenic lines.
In the **InMYB1** promoter, we investigated **GUS** mRNA level of each transgenic Arabidopsis plant by real-time RT-PCR. **GUS** mRNA level in **InMYB1** 332-121b×3_TATA_Ω::**GUS** transgenic Arabidopsis was almost double of that in **InMYB1** 1023b::**GUS** transgenic Arabidopsis (Figure 5).

In previous studies, several flower-specific promoters had been reported (Gustafson-Brown et al. 1994; Imai et al. 2013; Liu et al. 2011; Mandel et al. 1992; van der Meer et al. 1990). However, these promoters suffer from some drawbacks such as low petal specificity or restricted activity during the flowering stage. In addition, petal-specific promoter activity of these promoters was not tested in a wide range of plant species. We previously reported that the **InMYB1** promoter functions petal-specifically in various dicots plants by recognizing petal identity at a cellular level (Azuma et al. 2016a, b). In this study, we showed the promoter activity of **InMYB1** 332-121b×3_TATA_Ω is higher than that of the original **InMYB1** promoter, suggesting that the **InMYB1** 332-121b×3_TATA_Ω is a useful tool for molecular breeding of floricultural crops.

**Conclusion**

In this study, we revealed that the 332-121 bp upstream region of **InMYB1** is enough and essential element for the petal-specific promoter function. Furthermore, we showed that the modified artificial promoter, i.e. **InMYB1** 332-121b×3_TATA_Ω, can promote higher gene expression than the original **InMYB1** promoter in petal and therefore the artificial promoter could be a useful tool for molecular breeding of floricultural crops.

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References


Deikman J, Fischer RL (1988) Interaction of a DNA binding factor with the 5′-flanking region of an ethylene-responsive fruit ripening gene from tomato. EMBO J 7: 3315–3320


