The Artificial Promoter rMdAG2I Confers Flower-specific Activity in Malus

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Abstract: Genetic modifications of floral organs are important in the breeding of Malus species. Flower-specific promoters can be used to improve floral organs specifically, without affecting vegetative organs, and therefore developing such promoters is highly desirable. Here, we characterized two paralogs of the Arabidopsis thaliana gene AGAMOUS (AG) from Malus domestica (apple): MdAG1 and MdAG2. We then isolated the second-intron sequences for both genes, and created four artificial promoters by fusing each intron sequence to a minimal 35S promoter sequence in both the forward and reverse directions. When transferred into tobacco (Nicotiana benthamiana) by Agrobacterium tumefaciens-mediated stable transformation, one promoter, rMdAG2I, exhibited activity specifically in flowers, whereas the other three also showed detectable activity in vegetative organs. A test of the four promoters’ activities in the ornamental species Malus micromalus by Agrobacterium-mediated transient transformation showed that, as in tobacco, only rMdAG2I exhibited a flower-specific expression pattern. Through particle bombardment transformation, we demonstrated that rMdAG2I also had flower-specific activity in the apple cultivar ‘Golden Delicious’. The flower-specific promoter rMdAG2I, derived from M. domestica, thus has great potential for use in improving the floral characteristics of ornamental plants, especially the Malus species.

Keywords: flower-specific promoter; Malus; AGAMOUS; intron

1. Introduction

The genus Malus comprises more than 30 species with both ornamental and edible uses [1]. Flowering crabapples belonging to this genus are widely used in landscape gardening, due to their varied and attractive flower color, size and fragrance [2]. Malus plants are always self-sterile, requiring cross-pollination by insects, such as bees, which is important for fruit production in domesticated apples [3–7]. Flowers are key reproductive organs, because they play important roles in attracting pollinating insects. Therefore, Malus flowers are important both for the plants’ value as ornamentals and for pollination purposes [8–11]. Improvement of flower traits is a crucial target in breeding Malus species.
Transgenic technology has been used in the genetic improvement of a series of flower traits, including flower color, size and stress resistance [12–14]. However, constitutive expression of target genes may have undesirable effects on the vegetative organs. For instance, although cytokinins can be used to regulate flower size, constitutive regulation of cytokinins would also affect vegetative growth [15,16]. A flower-specific promoter could be of great utility by enabling the application of transgenic methods to improve flower traits, specifically without affecting vegetative growth.

In *Arabidopsis thaliana*, the gene AGAMOUS (AG), initially expressed in the third stage of flower development, is the key factor determining the identity of carpel and stamen [17,18]. The second intron of AGAMOUS contains cis-regulatory elements influencing the flower-specific expression of target genes [19,20]. In woody plants, Li et al. cloned the second intron of one of the poplar AG paralogs, PTAG2, and demonstrated that, when fused with the minimal 35S promoter, the reverse version of the PTAG2 second intron exhibits flower-specific activity [21]. However, a flower-specific promoter derived from the *Arabidopsis* AG second intron does not retain its specificity in tobacco plants [21]. Therefore, isolating the second intron from *Malus* paralogs of AG as a tool for the transgenic improvement of flowers in the *Malus* species is needed.

In *M. domestica*, two MADS-box genes, *MdMADS15* and *MdMADS22*, have been reported as paralogs of AGAMOUS [22]. Furthermore, RNA interference (RNAi) experiments indicate that RNA interference against two apple AG paralogs cause male and female sterility, suggesting these genes play important roles in both male and female floral structures [23], similar to the AG genes in *Arabidopsis* [24–26]. In this study, we cloned the second introns of two AG paralogs, and characterized the promoter activity by fusing them to a β-glucuronidase gene (*uidA, or GUS*) in both forward and reverse directions. We reported that the reverse version of the second intron of *MdMADS22* drove the flower-specific expression of target genes both in tobacco and in two *Malus* species, *Malus domestica* (apple) and the ornamental *Malus micromalus* (midget crabapple). This artificial promoter could thus serve as a tool for the genetic manipulation of *Malus* species and other flowering plants.

2. Results and Discussion

2.1. Isolation and Expression Analysis of *MdAG1* and *MdAG2*

To confirm the apple AGAMOUS (AG) paralogs, we performed a BLAST analysis of the protein sequence of *Arabidopsis* AG against the protein database of the *M. domestica* variety ‘Golden Delicious’. We identified two AG paralogs, which we named *MdAG1* and *MdAG2*. *MdAG1* and *MdAG2* were reported previously as *MdMADS15* and *MdMADS22*, respectively [22,23]. A phylogenetic tree constructed based on the protein sequences of various well-studied AG genes revealed that *MdAG1* and *MdAG2* were clustered closely, compared to other AG genes (Figure 1).

Next, we characterized the expression patterns of *MdAG1* and *MdAG2* in *M. domestica*. Both *MdAG1* and *MdAG2* exhibited similar floral-organ-predominant expression patterns (Figure 2). Both genes had relatively high expression in pistils and stamens, whereas in vegetative organs such as root, stem and leaf, their expression levels were rarely detectable. In many *Malus* species, fruits develop mainly from receptacles [27]. Interestingly, although both *MdAG1* and *MdAG2* were expressed in receptacles, we did not detect their expression in fruits, indicating that these genes may regulate fruit development in a dynamic fashion [28].
Figure 1. Phylogenetic analysis of AG protein sequences. The phylogenetic tree was constructed using the neighbor-joining method of MEGA 6.0 software based on the protein sequences of Phaseolus vulgaris AG (Phvul.006G169600), Medicago truncatula AG (XP_013462626.1), Arachis hypogaea AG (XP_025640343.1), Malus domestica AG2 (MDP0000250080), Malus domestica AG1 (MDP0000324166), Vitis vinifera AG (NP_001268097.1), Primula vulgaris AG (AZZ09252.1), Sesamum indicum AG (AIS82595.1), Nicotiana tabacum AG (NP_001312829.1), Populus trichocarpa AG1 (AAC06237.1), Theobroma cacao AG (XP_007025252.1), Citrus clementina AG (XP_006449577.1), Citrus sinensis AG (XP_024950555.1) and Arabidopsis thaliana AG (NP_001328877.1). Values at nodes indicate bootstrap percentages.

Figure 2. Relative expression levels of MdAG1 and MdAG2 in various tissues of Malus domestica ‘Golden Delicious’. Expression levels of MdActin were used as an internal reference. Data represent means of three biological replicates ± SE. Bars with different letters are significant different at p < 0.05 based on one-way analysis of variance (ANOVA).

2.2. Cloning and Analysis of the Second Introns of MdAG1 and MdAG2

We cloned the sequences of the second introns of MdAG1 and MdAG2 and named them as MdAG1I (MN403053) and MdAG2I (MN401764), respectively. The two sequences showed 87% identity and were 4,239 bp and 4,236 bp in length, respectively. We further analyzed the putative cis-regulatory elements of both intron sequences using PlantCARE [29]. The distributions of some known cis-regulatory elements in both intron sequences are shown in Figure 3. Both MdAG1I and MdAG2I have core cis-regulatory elements, such as TATA-box and CAAT-box elements. For instance, MdAG1I has 25 TATA boxes and 24 CAAT boxes, while MdAG2I has 30 TATA boxes and 28 CAAT boxes. In addition, we identified a series of hormone- and environmental-signal-related cis-regulatory elements, including three light-responsive elements (GT1 motif, AAAC motif, and G box), one circadian control element
(circadian), one drought-responsive element (MBS), one low-temperature-responsive element (LTR), three hormone-related cis-regulatory elements (GARE motif, TGA element, and TGACG motif), and one leafy (LFY)-binding site. The LFY-binding site is reported to bind with AG second-intron sequences, a process that plays important roles in maintaining AG expression in floral organs [30–32]. We identified one LFY-binding site in MdAG1I and two in MdAG2I, and they were also distributed differently within the two intron sequences. These differences may result in different expression patterns when the introns act as promoters.

**Figure 3.** Distributions of ten types of predicted cis-regulatory elements in the second introns of MdAG1 and MdAG2. The different distribution modes may cause different activities when these are used as promoters.

### 2.3. Floral-Specific Expression of rMdAG2I::GUS in Tobacco

To characterize the promoter activity of the two intron sequences, we fused each sequence with the β-glucuronidase (GUS) gene. It has been reported that the directions of intron sequences may direct different expression patterns [21]. Therefore, we created four different constructs by fusing the two intron sequences with GUS in both forward and reverse directions, creating the constructs fMdAG1I::GUS, rMdAG1I::GUS, fMdAG2I::GUS and rMdAG2I::GUS (Figure 4).

**Figure 4.** Gene constructs used for tobacco and Malus transformation. 35S, cauliflower mosaic virus 35S gene promoter sequence; GUS, coding sequence of the β-glucuronidase gene; tNos, nopaline synthase terminator; fMdAG1I, forward orientation of the second intron of Malus AGAMOUS1 (MdAG1); rMdAG1I, reverse orientation of the second intron of MdAG1; fMdAG2I, forward orientation of the second intron of MdAG2; rMdAG2I, reverse orientation of the second intron of MdAG2; Min35S, 60 bases of the 35S gene promoter sequence.

We first tested these constructs in tobacco. An Agrobacterium-mediated stable transformation of tobacco with the four constructs produced 28, 23, 31 and 20 lines for fMdAG1I::GUS, rMdAG1I::GUS, fMdAG2I::GUS, and rMdAG2I::GUS, respectively. We also produced eight 35S::GUS transgenic tobacco
lines. Next, we assayed the leaf, stem and flower tissues of the transgenic tobacco or non-transgenic control by histochemical staining for GUS activity. All the tested tissues exhibited GUS activity in the eight 35S::GUS lines (Figure 5A–C and Table 1), whereas none of these tissues had GUS activity in the non-transformed control (Figure 5D).

Figure 5. GUS staining in tobacco transformed with the four artificial promoters and 35S::GUS control. (A–C) GUS expression using 35S::GUS as a positive control. All organs are stained. (D) GUS expression in non-transformed flowers. None of the plant was stained. (E–G) GUS expression of tobacco tissues transformed with fMdAG1I::GUS (E), rMdAG1I::GUS (F), and fMdAG2I::GUS (G), respectively. Flowers, leaves and stems all show different degrees of blue color. (H–I) Two lines of rMdAG2I::GUS transgenic tobacco. Only flowers are stained.

For the MdAG1 intron sequence, both the forward and reverse promoters exhibited GUS expression patterns similar to those of the 35S::GUS plants, with GUS activity in all tested tissues. However, fMdAG1I::GUS caused stronger GUS activity than rMdAG1I::GUS, indicating that the direction of the intron sequence did affect the activity of the artificial promoter (Figure 5E,F). Of the two promoters derived from the MdAG2 intron sequence, the forward promoter had GUS activity in both vegetative and floral organs (Figure 5G), whereas the reverse promoter had GUS activity only in floral organs, and not in vegetative organs (Figure 5H,I), suggesting a strong likelihood that rMdAG2I::GUS governs flower-specific expression of target genes in Malus. The difference in expression patterns between the forward and reverse directions of the two intron-sequences demonstrated that their activities are largely dependent on their orientation, which is consistent with our previous findings for the poplar AG gene [21]. The direction difference presumably leads to differences in the distances between the cis-regulatory sequences and transcription start sites, thus resulting in different expression patterns. In support of this, MdAG2I has one more LFY-binding site than MdAG1I at the 5' end (Figure 3). Also, we identified only AAAC motif and TGA elements in MdAG1I, suggesting that MdAG1I may be more easily affected by environment stimuli.
**Table 1.** Characterization of floral specificity of fMdAG1I::GUS, rMdAG1I::GUS, fMdAG2I::GUS, and rMdAG2I::GUS transgenic tobacco.

| Vectors          | No. of Transgenic Lines | Plant Tissues | Floral Specificity |
|------------------|-------------------------|---------------|--------------------|
|                  |                         | Stem | Leaf | Sepal | Petal | Stamen | Carpel |           |
| 35S::GUS         | 8                       | +    | +    | +     | +     | +      | +      | No        |
| fMdAG1I::GUS     | 28                      | +    | +    | +     | +     | +      | +      | No        |
| rMdAG1I::GUS     | 23                      | +    | +    | +     | +     | +      | +      |            |
| fMdAG2I::GUS     | 31                      | +    | +    | +     | +     | +      | +      |            |
| rMdAG2I::GUS     | Type I                  | 11   | −     | −     | +     | +      | +      | Yes       |
|                  | Type II                 | 9    | −     | −     | −     | +      | +      |            |

+, detectable GUS activity; −, no detectable GUS activity.
Interestingly, rMdAG2I::GUS lines exhibited two different patterns of GUS expression in flower organs (Table 1). Eleven of the 20 lines (Type I) showed GUS activity in all floral organs (sepals, petals, stamens and carpels), while the remaining nine lines showed GUS activity only in stamens and carpels (Type II). Those two patterns were probably due to variable copy numbers or different transgene insertion sites among different lines. Both expression patterns would be useful in practice for breeding purposes. For instance, to produce flowerless plants without any floral organs by fusing a promoter with a toxin gene, we would need a flower-specific promoter with activity in all floral organs as Type I [21]. However, if we planned simply to produce sterile plants with normal petal development for ornamental purposes, the Type II expression pattern would be needed [33–35].

2.4. Optimization of Agrobacterium-Mediated Transient Transformation Conditions for Malus Flowers

We further tested the four constructs in two Malus species. Because there is no well-established stable transformation protocol for most Malus species, we employed Agrobacterium-based transient transformation to test all four constructs. The transient transformation method has the advantages of being simpler and faster than the stable transformation [36–39] and is regularly used in both Arabidopsis and tobacco to characterize the subcellular localization of functional genes [40–42]. Additionally, it has been reported that combining the clustered regularly interspersed short palindromic repeat (CRISPR)/Cas9 system and Agrobacterium-mediated transient transformation makes it possible to produce a target mutant with no integration of transgenes, which can be very important to accelerate the application of CRISPR technology in asexually-propagated woody plants [43–46]. In Malus species, especially M. domestica, Agrobacterium-mediated transient transformation has been widely used in studying fruit traits because it allows quick testing of the functions of genes associated with fruit traits, avoiding the lengthy periods associated with stable transformation methods because of the long juvenile phase of these species [47]. However, although well established for Malus fruit, Agrobacterium-mediated transient transformation is not equally successful in different sample types in Malus species. Therefore, we first optimized the Agrobacterium-mediated transient transformation system for flower tissues.

First, because the efficiency of T-DNA delivery into the host cell is one of the most important factors affecting Agrobacterium-mediated transient transformation, we included a vacuum infiltration step in our transient transformation system. Vacuum infiltration is reported to enhance Agrobacterium infection in some species, such as Arabidopsis, cotton and wheat [48], by improving the penetration efficiency of Agrobacterium cells into target plant tissues. We optimized the vacuum infiltration procedure using a 35S::GUS construct in one representative ornamental Malus species, Malus micromalus (midget crabapple). Different plant tissues may have different tolerances of vacuum pressure due to the major variations in their cell walls and intercellular spaces. Moreover, short vacuum times may not achieve ideal results, while long vacuum times may damage the tissues [49]. Therefore, to achieve an appropriate vacuum time for flower tissues, we tested a gradient of vacuum times: 2.0 min, 3.0 min and 4.0 min, corresponding to vacuum levels of -70 kPa, -80 kPa and -85 kPa, respectively. We also observed that the length of the vacuum application affected the transformation efficiency. Therefore, we tested the results of maintaining the samples at each pressure for four different time periods: 1.0 min, 1.5 min, 2.0 min and 3.0 min. The flowers sustained more serious damage at increased vacuum levels (Table 2); in particular, the -85 kPa vacuum level was unacceptable because it caused relatively serious damage. Maintaining samples under relatively low vacuum pressure (-70 kPa) for a long time improved the staining efficiency. At -80 kPa, a large increase of the treatment time also caused tissue damages. Ultimately, we decided that a -80 kPa vacuum maintained for 1.5 min was ideal, and we used this for later experiments. By employing a modified setting, we were also able to detect GUS activity in stem materials with the 35S::GUS construct, suggesting that, besides the floral organs, the optimized protocol is also available for stem tissues.
Table 2. Optimization of conditions for Agrobacterium-mediated transient transformation of Malus flowers using 35S::GUS construct.

| Vacuum Time (min) | Soaking Time (min) | Condition of Treated Flowers |
|-------------------|--------------------|------------------------------|
| 2.0               | 1.0                | No damage, No staining       |
| 2.0               | 1.5                | No damage, Blue staining     |
| 2.0               | 2.0                | No damage, Blue staining     |
| 2.0               | 3.0                | No damage, Blue staining     |
| 3.0               | 1.0                | No damage, No staining       |
| 3.0               | 1.5                | No damage, Blue staining     |
| 3.0               | 2.0                | Slightly damaged             |
| 3.0               | 3.0                | Damaged                      |
| 4.0               | 1.0                | Damaged                      |
| 4.0               | 1.5                | Damaged                      |
| 4.0               | 2.0                | Damaged                      |
| 4.0               | 3.0                | Damaged                      |

2.5. rMdAG2I::GUS Confers Flower-Specific Expression in Malus

Next, we used the optimized transformation conditions for further experiments with the four promoter constructs. We were able to detect GUS activity in all tested organs for 35S::GUS-transformed flowers (Figure 6A), while, none GUS activity was detected for the non-transformed flowers (Figure 6B). In the fMdAG1I::GUS flowers, we detected GUS activity in both vegetative (stem) and floral organs (sepal, petal, stamen, pistil), which was consistent with the results in tobacco (Figure 6C, Table 3). In rMdAG1I::GUS flowers, we detected GUS activity in stem and stamen, but not in sepal, petal, or pistil (Figure 6D, Table 3). Notably, in tobacco, fMdAG1I::GUS produced higher activity than rMdAG1I::GUS in all instances; therefore, the undetectable activity of rMdAG1I::GUS in some flower organs in M. micromalus may be due to its global low activity. In fMdAG2I::GUS flowers, we detected activity in stems and flower organs (sepal, petal, stamen, pistil) (Figure 6E, Table 3). The reason that the rMdAG1I::GUS flowers exhibited no GUS activity in receptacles may be its global low activity. Based on the gene expression data in Figure 2, the expression pattern of both AG genes in fruits may undergo dynamic changes. Our results suggest that the second introns of Malus AG genes may play some role in regulating AG expression during fruit development. For rMdAG2I::GUS, we did not detect GUS activity in stems, but we did detect GUS activity in all tested floral organs (sepal, petal, stamen and pistil) (Figure 6F, Table 3). Therefore, rMdAG2I::GUS has strong potential as a flower-specific promoter in Malus.

Table 3. Characterization of floral specificity of fMdAG1I::GUS, rMdAG1I::GUS, fMdAG2I::GUS and rMdAG2I::GUS in Malus micromalus with Agrobacterium-mediated transient transformation.

| Vectors     | Organs | Stem | Sepal | Petal | Stamen | Pistil | Receptacle |
|-------------|--------|------|-------|-------|--------|--------|------------|
| 35S::GUS    | +      | +    | +     | +     | +      | +      | +          |
| fMdAG1I::GUS| +      | +    | +     | +     | +      | +      | +          |
| rMdAG1I::GUS| +      | −    | −     | +     | −      | −      | −          |
| fMdAG2I::GUS| +      | +    | +     | +     | +      | +      | +          |
| rMdAG2I::GUS| −      | +    | +     | +     | +      | +      | +          |

+, detectable GUS activity; −, no detectable GUS activity.
Figure 6. GUS staining of Malus micromalus with Agrobacterium-mediated transient transformation. (A) 35S::GUS-transformed flowers (positive control). Stem and flower show blue staining. (B) Non-transformed flowers (negative control) have no blue staining. (C–F) fMdAG1I::GUS- (C) rMdAG1I::GUS- (D) fMdAG2I::GUS- (E) and rMdAG2I::GUS- (F) transformed flowers show blue staining.

We also tested our optimized transformation protocol with one M. domestica cultivar, ‘Golden Delicious’; unfortunately, the results were poor, suggesting that Agrobacterium-mediated transient transformation is species or material dependent. Thus, we may need to modify this transformation protocol for each new species or cultivar. Given this result, we next turned our attention to an alternative transient transformation method, particle bombardment. Although costly, this method has some important advantages, such as the absence of limitations on species or sample types [50–52]. Therefore, we used particle bombardment to test the flower specificity of rMdAG2I::GUS in ‘Golden Delicious’, using 35S::GUS as control. With 35S::GUS, we detected GUS activity in both vegetative organs (leaf and stem) and floral organs (sepal, petal, stamen, pistil and receptacle) (Table 4). Meanwhile, with rMdAG2I::GUS, we did not detect any GUS activity in vegetative organs, but we did detect GUS activity in all floral organs tested (Figure 7 and Table 4). These results further confirm the floral specificity of rMdAG2I::GUS in Malus specie.
Table 4. Characterization of the activity of rMdAG2I in ‘Golden Delicious’ through particle bombardment.

| Organ        | Stem | Leaf | Sepal | Petal | Stamen | Pistil | Receptacle |
|--------------|------|------|-------|-------|--------|--------|------------|
| 35S::GUS     | +    | +    | +     | +     | +      | +      | +          |
| rMdAG2I::GUS | -    | -    | +     | +     | +      | +      | +          |

+, detectable GUS activity; −, no detectable GUS activity.

Figure 7. GUS staining of ‘Golden Delicious’ transformed with rMdAG2I::GUS through particle bombardment. (A) Flower organs with blue staining. RE, receptacle; SE, sepal; PE, petal; ST, stamen; PI, pistil. (B) Stem without blue staining.

In summary, we have successfully identified two AG paralogs (MdAG1 and MdAG2) from Malus. By fusing the second-intron sequences of these two genes to the minimal 35S promoter in both forward and reverse directions, we build four promoter constructs. Next, we demonstrated the flower specificity of the rMdAG2I promoter through the stable transformation of tobacco plants. We further verified its flower specificity in the Malus species by Agrobacterium-mediated transient transformation and particle bombardment. This newly designed rMdAG2I promoter thus has great potential as a means for the genetic modification of flower traits in the Malus species.

3. Materials and Methods

3.1. Phylogenetic Analysis

Phylogenetic analysis was conducted using MEGA 6.0 software [53]. The tree was compiled using the neighbor-joining method. Statistical reliabilities of the internal branches were assessed using 1000 bootstrap replicates. Values at nodes indicate bootstrap percentages [54].

3.2. RNA Extraction and qRT-PCR

The vegetative organs (roots, stems and leaves) and reproductive organs (receptacles, sepals, petals, stamens, pistils and fruits) of ‘Golden Delicious’ apples were prepared for RNA extraction. Total RNA of each sample was extracted using the cetyl trimethyl-ammonium bromide (CTAB) method. RNA quality and concentration were determined using a NANODROP 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The cDNA was synthesized using MLV reverse transcriptase (TakaRa Bio Inc., Dalian, China). We performed quantitative real-time PCR (qRT-PCR) analysis using the SYBR premix Ex Taq Kit (TakaRa Bio Inc., Dalian, China) in a 7500 real-time PCR system (Applied Biosystems Foster City, CA, USA).
The primer pair AG1-F (5′-GAGAGGTACAAGAAGGCAAGTGC-3′) with AG1-R (5′-CTCTTCAGTTCCAGACATAC-3′) was used for the analysis of MdAG1 transcripts, and the pair AG2-F (5′-ATGCCTATCAAGGACCTGAAGAG-3′) with AG2-R (5′-TAGTTCCGAGAGTCGTATGGCT-3′) was used for analysis of MdAG2 transcripts. The primer pair Actin-F (5′-TGACCGAATGAGCAAGTGAATCC-3′) with Actin-R (5′-TACTCAGCTTTGGCAATCCACATC-3′) was used to amplify the internal reference gene, MdActin [47]. Data were analyzed using the $2^{-\Delta\Delta C_T}$ method [55]. The gene expression in each sample was calculated using the expression level of MdActin in the same sample.

Each qRT-PCR reaction was performed with three biological replicates, and each sample was analyzed in triplicate (technical replicates).

### 3.3. Cloning of MdAG1 and MdAG2 Second-Intron Sequences

Genomic DNA was extracted from the leaves of ‘Golden Delicious’ using a modified CTAB method [56]. Approximately 200 ng genomic DNA was used as templates for PCR amplification of the second-intron fragments from two MdAG genes. PCR amplification of the MdAG1 second intron was performed using the primer pair AG1I-F (5′-CGCCTCTATGAGTATGCCAAC-3′) and AG1I-R (5′-CTTTCTGTCCTTAATTGTTCCT-3′) under the following conditions: 5 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 58 °C, and 2 min at 72 °C, with a final extension of 7 min at 72 °C. PCR amplification of the MdAG2 second intron was performed with the primer pair AG2I-F (5′-GAGTATGCGCACACAGGTAAT-3′) and AG2I-R (5′-TGCTTTAACAAGCTCGACATATG-3′) under the following conditions: 5 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 58 °C, and 2 min at 72 °C, with a final extension of 7 min at 72 °C. The amplified fragments were inserted into the pEASY-Blunt Cloning Kit, and 10–12 clones were sequenced for verification of their sequence identity.

### 3.4. Plasmid Construction

Four chimeric promoters were created by fusing the intron sequence of either MdAG1 or MdAG2 to a 60-bp minimal 35S promoter in either forward or reverse direction. Those four artificial promoters were inserted upstream of the GUS coding sequence to create four plasmids: fMdAG1::GUS, rMdAG1::GUS, fMdAG2::GUS, and rMdAG2::GUS. A control vector was also created containing the GUS coding sequence driven by a constitutively active 35S promoter (35S::GUS).

### 3.5. Stable Transformation of Tobacco

Each of the four plasmids was introduced into Agrobacterium tumefaciens EHA105, which was subsequently used for transformation of tobacco (Nicotiana benthamiana) [57]. Leaf discs of tobacco (0.5 cm × 0.5 cm) were incubated for 20 min with A. tumefaciens (OD600 = 0.6) and then transferred to the Murashige and Skoog (MS) medium without hormone and antibiotics for two days of co-cultivation. After that, leaf explants were moved to a regeneration medium containing 3 mg/L 6-benzylaminopurine (6-BA), 0.2 mg/L 1-naphthaleneacetic acid (NAA), 300 mg/L cefotaxime, and 50 mg/L kanamycin. Putative transgenic shoots were moved to rooting medium containing 300 mg/L cefotaxime and 20 mg/L kanamycin. The rooting shoots were transferred to soil and grown in a greenhouse.

### 3.6. Vacuum Infiltration of Malus Micromalus Flowers

Agrobacterium tumefaciens EHA105 harboring each of four created constructs was prepared. Flowers of M. micromalus were immersed in the Agrobacterium suspension (OD600 = 1.0) and placed in a vacuum chamber. A total of 12 treatments with three vacuum treatment times (2 min, 3 min, 4 min) and four soaking times (1 min, 1.5 min, 2 min, 3 min) were used for orthogonal tests. After treatments, the samples were cultured on MS medium for two days and then subjected to GUS staining.
3.7. Particle Bombardment of ‘Golden Delicious’ Apple Flowers

To prepare the gold suspension, a mixture of gold powder and ethanol (60 mg:1 mL) was shaken for 1 min and centrifuged at 10,000 rpm for 10 s. The pellets were then resuspended in sterilized water. 50 µL of gold suspension, 20 µL 0.1 M spermidine, 50 µL 2.5 M CaCl₂, and 2.5 µg DNA were used for preparing the gold/DNA mixture. After having been shaken for 3 min, the mixture was centrifuged at 10,000 rpm for 20 s. The pellets were rinsed by ethanol for two times and then re-suspended by 60 µL ethanol, and 10 µL was used for each particle bombardment. The bombardment was repeated six times for each sample. The treated samples were cultured on MS medium for two days before GUS staining.

3.8. GUS Staining Assays

GUS assays were performed according to a previously described protocol with modifications [21]. Samples were incubated in X-Gluc solution (100 mM potassium phosphate buffer, 10 mM Na₂EDTA, 0.5 mM K₄Fe(CN)₆, 0.1% Triton X-100, and 1 g/L X-Gluc, pH 7.0) at 37 °C. After 24 h of incubation, the samples were decolorized with alcohol three to five times before being photographed.

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