The expression of two DEFICIENS-like genes was reduced in the sepaloid tepals of viridiflora tulips

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According to the modified ABC model, class B genes are expressed in whorl 1 as well as in whorls 2 and 3, therefore the organs of whorls 1 and 2 have the same petaloid structure in many monocots. The viridiflora tulip, a well-known cultivar group, has flowers in which the tepals in whorls 1 and 2 have greenish stripes. This phenotype could be expected as class B mutant by the modified ABC model. In this study, we discovered that tepal phenotypes correlated with that of stamens. We isolated two class A genes (TGSQA and TGSQB) from wild type tulip. Northern hybridization of these two class A genes, and the class B genes (TGDEFA, TGDEFB and TGGLGLO), showed that the expression of TGDEFA and TGDEFB in the viridiflora tulips were weaker than those in wild type cultivars, whereas the expression patterns of the rest genes were almost identical. From our results, we suggest that reduced expression of the two DEF-like genes, TGDEFA and TGDEFB, is involved in the development of the viridiflora phenotype. This reduced expression could be caused by the amino acid difference.

Key Words: floral organ identity, MADS-box gene, class B gene, monocot, petaloid tepal, sepal, tulip.

Introduction

Flowers, the reproductive organs of plants, share principal points in common besides their various shapes and colors. In eudicots, they consist of four types of organs: sepals, petals, stamens and carpels. To explain floral organ identity, the ABC model was proposed for the model plants, Arabidopsis thaliana and Antirrhinum majus (Bowman et al. 1991, Coen and Meyerowitz 1991). According to this model, from the outside of a flower moving inwards, sepals are identified by A-function genes, petals are determined by A- and B-function genes, stamens by B- and C-function genes and carpels by C-function gene.

The genes that perform the functions of the ABC model (except for APETALA2 (AP2), one of the A-functional genes) belong to the MADS-box gene family. The function that divides petals from sepals and stamens from carpels, known as the B-function, consists of genes from two closely related clades, named after A. majus genes, DEFICIENS (DEF)-like and GLOBOSA (GLO)-like. In Arabidopsis, APETALA3 (AP3), a DEF-like gene and PISTILLATA (PI), a GLO-like gene, regulate each other’s expression and are involved in the B-function (Jack et al. 1994, Goto and Meyerowitz 1994).

In contrast, many monocots have flowers with petaloid organs instead of sepals in whorl 1. To explain the organ identity of a flower that has a doubled petaloid perianth (tepal), such as tulip or lily, the modified ABC model was proposed based on the morphological analysis of mutant tulip flowers (van Tunen et al 1993). They suggested that the petaloid character of the outer tepal is due to the expression of class B genes, not only in whorls 2 and 3, but also in whorl 1. In this model, a tulip designated as ‘viridiflora’ was predicted to be a class B mutant. It was described that this mutant had a flower with tepals transformed into sepaloid organs in whorls 1 and 2, and infused carpels containing ovules instead of stamens (van Tunen et al. 1993). This model was supported by Northern blot analysis of two types of class B MADS-box genes isolated from Tulipa gesneriana and designated as TGDEFA, TGDEFB and TGGLGLO (Kanno et al. 2003). The mRNAs of these genes were detected in whorls 1, 2 and 3 as predicted by the modified ABC model. In other monocots with two-layered petaloid organs, such as the lily (Theissen et al. 2000), Phalaenopsis equestris (Tsai et al. 2004, 2005), Dendrobium crumenatum (Xu et al. 2006) and Crocus sativus (Tsafarlis et al. 2006), similar expression patterns for class B genes were also reported. In situ hybridization analyses revealed the presence of mRNAs of class B genes in the primordia of the outer tepals in Agapanthus praecox (Nakamura et al 2005) and Alstroemeria ligu (Hirai et al. 2007). However, expression of these genes was not detected in the primordia of the outer tepals.
tepals in *Asparagus officinalis* (Park et al. 2003, 2004). In addition, there is another type of genes that is important for eudicots sepal and petal identities, class A *SQUAMOSA* (*SQ*)-like genes, named after *A. majus* gene. Only few studies have so far been made at *SQ*-like genes of monocots with two-layered petaloid organs. Thus, it is important to study about *SQ*-like genes of these monocots.

The viridiflora tulip is a well-known cultivar group of tulips. This cultivar group has flowers in which the tepals in whorls 1 and 2 are partially sepaloid structures. According to the modified ABC model, both class A and B genes should be expressed in whorls 1 and 2. Thus, in order to investigate the molecular mechanism of this viridiflora phenotype, we have cloned class A and class B genes from the tulip, and compared their amino acid sequences and expression patterns in wild type and viridiflora cultivars.

**Materials and Methods**

**Plant materials**

Plants of *T. gesneriana* were grown in the experimental garden of Tohoku University, Japan. We collected young tulip flower buds and dissected them into their four floral organs (outer tepals, inner tepals, stamens and carpels). The organs were frozen in liquid nitrogen and stored at −80°C. The *T. gesneriana* cultivars, ‘White Dream’ and ‘Spring Green’ were used for morphological observation and cDNA isolation. The three wild type tulips, ‘White Dream’, ‘Pink Diamond’ and ‘Sweety’, and four viridiflora tulips, ‘Spring Green’, ‘Green River’, ‘Artist’, and ‘Adriaant Dominique’ were used for northern blot analyses.

**Scanning electron microscopy**

Leaves and two-layered tepals from mature flowers were dissected and fixed in 0.3% (w/v) glutaraldehyde in acetone overnight at −80°C. The fixed samples were gradually warmed and transferred to 100% acetone. These samples were dried by means of a technique known as ‘critical point drying’, using liquid CO₂ in JCPD-3 (JEOL Ltd., Japan). Dehydrated samples were mounted onto stubs and gold-coated with a sputter coater (JFC-1100, JEOL). The epidermal cells of each organ were observed using a scanning electron microscope (SEM) (JSM-5800LV, JEOL).

**cDNA cloning of MADS-box genes from tulip**

Partial cDNAs of *TGSQA* and *TGSQB* were isolated using the 3' RACE method (Kanno et al. 2003). Total RNA was isolated from 4 cm flower buds of the *T. gesneriana* cultivar ‘White Dream’ using the method of Chomczynski and Sacchi (1987). DYNABEADS (DYNAL, Norway) were used for separating poly(A)⁺ RNA, and cDNA synthesis was performed using reverse transcriptase AMV (Roche). For the PCR reaction from ‘Spring Green’ cDNA, the 5' and 3' UTR sequence of ‘White Dream’ (5'-GAAACCCCTCACCCACCACT-3' and 5'-GCTACTCAACTGCAGAAAT-3') for *TGDEFA*; (5'-GAAACCCCTCACCCACCACT-3' and 5'-GCTACTCAACTGCAGAAAT-3') for *TGDEFB*; (5'-GACCTCCCCACCTCGCA-3' and 5'-CTAGCTGGTGAGCTCCAGA-3') for *TGGLO* were used as primers. PCR products were cloned and sequenced as described above.

**Phylogenetic analysis**

Predicted amino acid sequences for the MADS-box genes were obtained from the EMBL/DDBJ/Genebank DNA databases. These were aligned using Clustal W, and a phylogenetic tree was constructed using the neighbor-joining (NJ) method. The GenBank accession numbers of the amino acid sequences used for alignments are shown in Supplemental Table 1. The phylogenetic tree was drawn using Njplot (Perrière and Gouy 1996). Bootstrap values for this phylogenetic tree were derived from 100 replicate runs (Thompson et al. 1994).

**Northern blot analysis**

Total RNA was isolated from floral bud, stem, leaf, bulb, root, outer tepals, inner tepals, stamens and carpels using the method of Chomczynski and Sacchi (1987), and 10 μg of total RNA for each organ was separated by electrophoresis on 1% agarose gels containing 5% formaldehyde and 1× MOPS. The gels were blotted overnight onto positively charged nylon membranes (Roche Diagnostics, USA) using the standard technique of Sambrook and Russell (2001). In order to avoid cross-hybridization with other members of the MADS-box gene family, gene-specific hybridization probes for *TGSQA*, *TGSQB*, *TGDEFA*, *TGDEFB* and *TGGLO* were obtained from the C domains and 3' UTR regions of each gene and were labeled with the DIG Probe Synthesis Kit...
Primer sequences employed for preparation of gene specific probes were (5′-CAGGAGCATAAACATCCTGG-3′) and (5′-CAGAAGATTTAGATGTCGCCAT-3′) for TGSQA; (5′-CTTCTGGAGGAGCAGAAGTC-3′) and (5′-GCGATCTGAGAACGATAATCTG-3′) for TGSQB; (5′-TCTAGCATGTACGAGTTCCGC-3′) and (5′-GCCTACTCAACTGCGAAAATAG-3′) for two DEF-like genes of tulip; and (5′-GGATGAAAATATAAGGGACATTGG-3′) and (5′-CTAGCTGTTGGAGCTCCAGA-3′) for TGGLO. Blots were hybridized with anti-DIG-AP probes (Roche) and the chemiluminescence reaction was performed using the CDP-Star Reagent (New England Biolabs, Beverly, MA, USA). Expression signals were detected using X-ray film (Hyperfilm, Amersham Biosciences, Amersham, UK).

Results

Morphological description of the viridiflora cultivar

The flower of the wild type tulip has two whorls of petaloid tepals, six stamens and three carpels, while the viridiflora cultivars have greenish tepals in the outer two whorls and the stamens are often degenerated in whorl 3 (Fig. 1A and 1B). A typical flower of the viridiflora tulip has tepals whose centers are green, similar to a sepal or leaf, and whose edges are white, or variegated as for normal tepals, and has stamens with thin anthers (Fig. 1C). The tepal phenotypes vary between individuals, and appear to correlate with that of the stamens. The flower of the weak phenotype has slightly greenish tepals and stamens with mature anthers capable of releasing pollen (Fig. 1C: 1a, 1b), while the flower of the strong phenotype has sepaloid- or leaf-like tepals and stamens with highly degenerate anthers, which do not release pollen, or filamentous organs instead of anthers (Fig. 1C: 5a, 5b).

In addition, we examined the epidermal cells of each organ (leaf, inner tepal and outer tepal) in wild type and viridiflora tulips using a SEM. The surface structures of each of the three organs of the wild type and viridiflora tulips were very similar, having a line of long cells interspaced by many stomata, though the middle part of the outer tepal of viridiflora was a little waxier than that of the wild type (Fig. 2).
searches of public databases using predicted amino acid sequences indicated that two of these clones shared a high level of sequence identity with the proteins encoded by SQ-like genes and we named them TGSQA (AB472012) and TGSQB (AB472011). Each homolog was independently isolated at least three times independently and the 5' regions of the each gene were isolated using 5'RACE method.

TGSQA and TGSQB contained conserved paleoAP1
motif regions, as well as the MADS- and K-boxes. Phylogenetic analyses of the MADS- and K-box amino acid sequences supported the conclusion that TGSQA and TGSQB belonged to the class A genes, and this phylogenetic reconstruction confirmed that the two genes belonged to the monocot FRUITFUL (FUL)-like gene subfamily (Fig. 3).

Expression analyses of A- and B-class MADS-box genes from wild type and viridiflora tulips

Positive signals for the TGDEFA and TGDEFB mRNAs were previously detected in floral organs of the tulip, especially in whorls 1, 2 and 3 (outer and inner tepals and stamens), while TGGLO was shown to be strongly expressed in both types of tepals and stamens, and weakly expressed in carpels, leaves, stems and bracts (Kanno et al. 2003). In this paper, we have analyzed the expression of the class A genes TGSQA and TGSQB in the wild type tulip ‘White Dream’, and we found that TGSQA was strongly expressed in stem and leaf, and weakly expressed in floral bud and root, while mRNA for TGSQB was detected in all organs analyzed (Fig. 4).

Next, we investigated the expression patterns of A and B class genes in both the wild type and viridiflora tulips (Fig. 5). Total RNA was isolated from carpels, stamens, inner tepals and outer tepals of young floral bud from the wild type cultivar ‘White Dream’ and the viridiflora cultivar ‘Spring Green’ (In this stage, their phenotype strength were not distinguished). To avoid cross hybridization, 3’ cDNA fragments specific to TGSQA, TGSQB, TGDEFA, TGDEFB and TGGLO from ‘White Dream’ were used as probes. In the probe sequences, TGDEFA had 3 bp/331 bp (0.9%), TGDEFB had 8 bp/346 bp (2.3%) and TGGLO had 4 bp/372 bp (1.1%) differences between ‘White Dream’ and ‘Spring Green’. Because of their little differences, we judged that it would not affect the hybridization strength of each gene. The class A gene TGSQA was expressed very weakly in whorls 1, 2 and 3, but was stronger in whorl 4; while the expression of TGSQB was detected in all four whorls, and was especially strong in whorl 4. The expression patterns of TGSQA and TGSQB were similar in both cultivars. Both TGDEFA and TGDEFB were expressed in the outer tepals, inner tepals and stamens in ‘White Dream’ and in ‘Spring Green’, but their expression levels were weaker in ‘Spring Green’. TGGLO was strongly expressed in the outer tepals, inner tepals, stamens and carpels of both cultivars.

To confirm the reduced expression of the two class B DEF-like genes in the viridiflora cultivar, we investigated the expression patterns of the three class B genes in three wild type cultivars: ‘White Dream’, ‘Pink Diamond’ and ‘Sweetie’, and in four viridiflora cultivars: ‘Green River’, ‘Artist’ and ‘Adriaant Dominique’ (Fig. 6). The expression levels of the TGDEFA and TGDEFB genes were weaker in the outer and inner tepals and stamens of the viridiflora cultivars than in those of the wild type cultivars, while the TGGLO gene was strongly expressed in all four organs in both cultivars (Fig. 6).

cDNA cloning of DEF- and GLO-like MADS-box genes from the viridiflora tulip

In order to compare the amino acid sequences of the three class B genes (TGDEFA, TGDEFB and TGGLO) in the viridiflora tulip, we used PCR to isolate cDNA clones of these three genes from the ‘Spring Green’ cultivar. Comparison of the amino acid sequences from the wild type and viridiflora cultivars revealed no differences for the TGGLO gene. However, the two DEF-like genes, TGDEFA and TGDEFB, from ‘Spring Green’ had the following differences: 1195ser ->Asn in TGDEFA, and 12Glul->Gly and 74Met->Ala in TGDEFB. We did not observe any deletion or frame shift mutations in these genes (data not shown).

Discussion

Morphological investigations

Viridiflora tulips have three sepaloid, or slightly leaf-like tepals, in each of whorls 1 and 2, and six stamens, which are often degenerate, in whorl 3. Morphological analyses showed that the strength of the tepal phenotype appeared to correlate with that of the stamen phenotype (Fig. 1). This suggests that some genes involved in the formation of both tepals and stamens cause abnormalities seen in the viridiflora cultivar.

Expression analyses

The floral morphology of the tulip was explained by the modified ABC model, as previously described (van Tunen et al. 1993, Kanno et al. 2003). Since the class B genes (TGDEFA, TGDEFB and TGGLO) have already been isolated (Kanno et al. 2003), in this study we have isolated two class A genes (TGSQA and TGSQB) from the wild type tulip (Fig. 3). We found that TGSQA and TGSQB were expressed in all floral organs (Fig. 5), which does not fit the modified ABC model: In this model, identically with the original ABC model, class A genes should be expressed only in outer two whorls, and should not be expressed in whorls 3 and 4.

In model plants of monocots, rice has four SQ-like genes, OsMADS14, OsMADS15, OsMADS18 and OsMADS20 (Fornara et al. 2004). Among these genes, Preston and Kellogg (2007) discussed that OsMADS14 will concern identity of all floral organs and OsMADS15 have A-function because OsMADS14 is expressed in all floral organs except lodicules, and OsMADS15 is expressed only in the outer bracts and perianth (lodicules). On the other hand, Jeon et al. (2000) described over expression of OsMADS14 caused producing shoot, embryo and flower like structure from calli, but not mentioned change of floral organ identity. About OsMADS18, Fornara et al. (2004) reported that it is widely expressed all plant tissues and cannot complement A-function. In our opinion, it will be open question that OsMADS15 have A-function, because OsMADS15 lack the expression in rice lemma and palea, which are considered as rice sepal. Though it remains discussions about which they are sepal or leaf-like organs, they are certainly outer floral
Fig. 3. NJ trees of *SQ*-like genes. The numbers next to the nodes represent bootstrap values from 100 replicates. Arrows indicate the genes from tulip. In the figure, the following gene names were abbreviated; CAULIFLOWER (CAL), SEPALLATA (SEP), AGAMOUS-LIKE6 (AGL6).
organ of lodicule, which is considered as rice petal. Additionally, because rice has highly derived flower, it is difficult to compare the floral organ identity with eudicot or tulip-like monocot flowers.

Among monocots which have petaloid perianth, several SQ-like genes were isolated from orchids and lily. In *Phalaenopsis*, ORAP11 and ORAP13 expressed in primordia of all floral organs and also in vegetative organs (Chen et al. 2007). DOMADS2 from *Dendrobium* expressed in column (Yu and Goh 2000), and OMADS10 from *Oncidium* expressed in vegetative organs, lip, carpel, anther cap and stigmatic cavity of mature flower (Chang et al. 2009). In lily, three SQ-like genes (*LMADS5/6/7*) were isolated from *Lilium longiflorum*. All of these genes expressed in vegetative stem and inflorescence meristem. *LMADS5* and *LMADS6* also strongly expressed in vegetative leaf and carpel, while weakly expressed whors 1, 2 and 3. Although *LMADS7* expression was only detected in vegetative stem and inflorescence meristem (Chen et al. 2008). Since tulip SQ-like genes expressed in most vegetative organs and all floral organs, especially in whorl 4, SQ-like gene expression in vegetative organs and strong expression in carpel are common characters in monocots with petaloid perianth. These results indicate that monocot SQ-like genes are not likely to have simple A-function.

A-function was found in *A. thaliana*, however, the generality of this role across angiosperms is questionable (Theissen et al. 2000). To date, SQ-like genes, that *A. thaliana* class A gene (*APETALA1*) belongs to, were isolated from many angiosperm species, such as Amborellaceae, Nymphaeaceae, monocots and magnolids. Many of these SQ-like genes were expressed not only in floral meristems but also in vegetative organs (Shan et al. 2007), like *TGSQA* and *TGSQB* in tulip. The broad expression patterns of SQ-like genes indicating that these gene products would have varied functions. Thus, it is unclear that these genes have A-function.

In tulip, if we consider any possibility about SQ-like genes, we might mention some hypotheses. First, tulip might not have A-function genes and only B- and C-function genes decide the floral organ identity. Second, because TGSQA and TGSQB are belong to FUL-like clade, which is basal position of both euAP1 and euFUL clade genes, tulip SQ-like genes might have dual function of euAP1 and euFUL genes, thus, they express all floral organs. Third, another genes might concern about A-function with SQ-like genes and both type of genes may necessary for A-function. Although further analyses are needed to verify these hypotheses.
Fig. 6. Expression of class B genes in three wild type and four viridiflora cultivars. Northern hybridization analysis of *TGDEFA* (i), *TGDEFB* (ii) and *TGGLO* (iii) in three wild type cultivars of ‘White Dream’ (A), ‘Pink Diamond’ (B) and ‘Sweety’ (C) and four viridiflora cultivars, ‘Spring Green’ (D), ‘Green River’ (E), ‘Artist’ (F) and ‘Adriaant Dominique’ (G). Lane 1: outer tepal, lane 2: inner tepal, lane 3: stamen and lane 4: carpel. Ethidium bromide stained gels of rRNA are shown under each blot.
We performed Northern blot analyses on floral homeotic genes to compare their expression patterns in wild type and viridiflora tulips. The expression patterns of *TGSO4*, *TGSOB* and *TGGLO* did not differ between the wild type and viridiflora tulips. While the transcripts of *TGDEFA* and *TGDEFB* were detected in whorls 1, 2 and 3 in the wild type and viridiflora cultivars, the expression levels of these genes in the viridiflora cultivar were lower than that of the wild type (Fig. 5). These results suggest that weaker expression of these two DEF-like genes correlates to morphological shifts in tepals and stamens in the viridiflora cultivar.

Since there was not much difference in the expression levels of the *TGDEFA* and *TGDEFB* genes between the wild type (‘White Dream’) and viridiflora (‘Spring Green’) cultivars, we compared the expression levels of the three class B genes in three wild type and four viridiflora cultivars. Although *TGGLO* was expressed in all four whorls, and its expression levels in wild type and viridiflora cultivars did not differ, we found that the expression levels for the two DEF-like genes from the all viridiflora cultivars were lower than those from the wild type cultivars. These results indicated that there might be one or more mutations in one or both of the DEF-like gene(s) in viridiflora tulips.

**Consideration of amino acid sequences**

Unlike the GLO-like genes in most angiosperms contain a PI-motif, the DEF-like genes have diverged in some lineages. Most of the higher eudicots have euAP3-lineage genes that contain the euAP3-motif, although some species have *TM6*-lineage genes that contain the paleoAP3-motif and whose function has not yet been elucidated. The lower eudicots, monocots and magnolid dicots, on the other hand, have only paleoAP3-lineage genes that contain the paleoAP3-motif (Kramer et al. 1998). Until now, concerning GLO-like genes except higher eudicots, there have also been reports using virus-induced gene silencing. For example, *AqPI* in *Aquilegia* (Kramer et al. 2007) and *PeMADS6* in *Phalaenopsis* (Lu et al. 2007), which showed that petaloid organs changed to sepaloid organs upon down-regulation of these class B genes. With regard to the DEF-like genes, it has been reported that in the *supergonwman* mutant of rice and the *silky1* mutant of maize, lodicules, which are considered to correspond to petals, change to palea or lemma-like organs and stamens change to carpels (Nagasawa et al. 2003, Ambrose et al. 2000). However, with respect to non-grass monocots with petaloid tepals, our study on the viridiflora cultivars of tulip is the first report, as far as we know, to suggest that the reduced expression of DEF-like genes may result in a homeotic change from petaloid organs to sepaloid organs, and the degeneration of stamens.

In this study, we also isolated the three class B genes from ‘Spring Green’ and compared their sequences with those from ‘White Dream’, and found that the two DEF-like genes, *TGDEFA* and *TGDEFB* of ‘Spring Green’, had the following amino acid differences: residue 119 (Ser->Asn) in *TGDEFA*, residue 12 (Glu->Gly) and residue 74 (Met->Ala) in *TGDEFB*. In contrast, the amino acid sequences of *TGGLO* in ‘White Dream’ and ‘Spring Green’ are identical. Since amino acid 74 is located in the I-region, a non-conserved region of the MADS-box genes, and since the SILKY gene in maize also has an Ala residue at this position (Ambrose et al. 2000), the amino acid change at 74 (Met->Ala) in *TGDEFB* would not affect protein-protein interactions. In addition, since the amino acid residue at position 119 of *TGDEFB* in both ‘White Dream’ and ‘Spring Green’ is Asn, the amino acid change at 119 (Ser->Asn) in *TGDEFA* would not affect protein-protein interactions, although residue 119 is located in the K-box, which is a conserved region among MADS-box genes. On the other hand, the amino acid residue at position 12 (Glu->Gly) is located in the MADS-box, which is a highly conserved region among many DEF-like genes from various plants. Furthermore, Glu and Gly have different characteristics; glutamic acid is acidic and glycine is nonpolar. This indicated that the amino acid change at position 12 (Glu->Gly) in TGDEFA might affect protein-protein interactions and/or DNA binding to the CArG box, at which MADS-box proteins bind.

**Speculations about the mechanism that reduce expression of the two DEF-like genes**

What is the mechanism underlying the reduction in expression levels of the two DEF-like genes? One possibility is that the amino acid differences found in the viridiflora *TGDEFA* gene may affect the gene expression of the two DEF-like genes. In higher eudicots, DEF- and GLO-like proteins form heterodimers that are required for the B-function in eudicots. DEF/GLO heterodimers bind to the CArG-box present in the promoter region of these genes, and the expression of these genes is thus autoregulated (Tröbner et al. 1992). In tulip, *TGDEFA* and *TGGLO*, and *TGDEFB* and *TGGLO*, have been shown to heterodimerize (Kanno et al. 2003). If the amino acid difference at position 12 (Glu->Gly), observed in *TGDEFA*, affects protein-protein interactions between *TGDEFA* and *TGGLO*, and/or DNA binding to CArG-boxes, then expression of both the *TGDEFA* and *TGGLO* genes might be reduced. However, the expression level of *TGGLO* was not found to be different between wild type and viridiflora cultivars as shown in Fig.5 and Fig.6. Since *TGGLO* can also bind to a CArG-box as a homodimer (Kanno et al. 2003), it is possible that *TGGLO* expression might be regulated by a TGGLO homodimer as well as by *TGDEFA*/TGGLO and *TGDEFB*/TGGLO heterodimers, and thus the expression of the *TGGLO* gene might not be affected. Other possible explanations have not been excluded, including the promoter regions of *TGDEFA* and/or *TGDEFB*, and or that an upstream gene, for example, *UNUSUAL FLORAL ORGANS (UFO)* in Arabidopsis, which induces gene expression of both *TGDEFA* and *TGDEFB* in viridiflora cultivars, might be mutated.

Although the detailed molecular mechanism underlying the viridiflora phenotype remains unclear, reduced expression of two DEF-like genes may produce the phenotype
observed in this cultivar. Further studies are needed to investigate the protein-protein interactions and genomic structures of these DEF-like genes.

Comparative expression analyses of class A and class B genes between wild type and viridiflora cultivars of tulip showed that the expression of the two DEF-like genes, TGDEFA and TGDEFB, in the viridiflora cultivars was weaker than that of their wild type cultivars, whereas the expression patterns of the class A genes TGSQA and TGSQB and the class B gene TGGLO were almost identical in both the wild type and viridiflora tulips. Our results suggest a possible mechanism for the viridiflora phenotype in the tulip.

Acknowledgment

We thank Prof. Masumi Yamagishi (Hokkaido University, Japan) for providing help and advice. We are also grateful to all the members of our lab.

This study was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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