Conservation and Diversification of Floral Homeotic MADS-box Genes in 
*Eustoma grandiflorum*

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The MADS-box gene family is one of the largest transcription factor gene families in plants and is necessary at various developmental stages. Many studies on flower development show that especially MIKC c-type MADS-box genes are essential for proper floral organ development. We identified and characterized MIKC c-type MADS-box genes expressed in *Eustoma grandiflorum* flowers. Twenty-three genes were identified and grouped into 10 clades, which were characterized by conserved specific motifs. Phylogenetic analysis indicated the diversification of *AG*/PLE, *AP3*/DEF, PI/GLO, and SEP clades and the occurrence of recent gene duplication events. The floral organ-specific expression patterns were partly diversified within the gene members of AP3/DEF and SEP clades, while they were conserved in AG/PLE and PI/GLO clades. These results suggest that genes with conserved expression as well as those with diversified expression contribute to specifying floral organ identity in *E. grandiflorum*.

Key Words: ABC model, floral organ identity, gene duplication, lisianthus, transcription factor family.

**Introduction**

For several decades, many plant scientists have tried to elucidate the molecular mechanisms of flower development. One of the most fruitful results from this research was the discovery of MADS-box transcription factor family genes that play important roles in floral organ development (Sommer et al., 1990; Yanofsky et al., 1990). This finding led to the floral “ABC” model, which has been modified as the “ABCE” or “ABCDE” model (Coen and Meyerowitz, 1991; Krizek and Fletcher, 2005). Most of the gene members of the “ABCDE” model belong to the MADS-box family, except for the *APETALA2* (AP2) gene (Goto and Meyerowitz, 1994; Jack et al., 1992; Jofuku et al., 1994; Mandel et al., 1992; Pelaz et al., 2000; Pinyopich et al., 2003; Yanofsky et al., 1990). This model explains the determination of floral organ identity by combinatorial functions of MADS-box proteins classified into different classes (A, B, C, D, and E). The combinations of A+E, A+B+E, B+C+E, C+E, and D+E specify sepal, petal, stamen, carpel, and ovule identity, respectively (Immink et al., 2010). The A class gene includes *APETALA1* (AP1), B class includes *APETALA3* (AP3) and *PISTILLATA* (PI), C class includes *AGAMOUS* (AG), D class includes *SEEDSTICK* (STK), and E class includes *SEPPALLATA1-4* (SEP) in *Arabidopsis* (Krizek and Fletcher, 2005).

The MADS-box genes comprise a large gene family in plants. For example, 107 MADS-box genes were identified in *Arabidopsis* (Pařeniová et al., 2003). Both plant and animal MADS-box genes can be classified into type I and type II (Alvarez-Buylla et al., 2000). Plant type II MADS-box genes belong to “MIKC-type”, named after their four specific domains, a MADS-box (M), intervening (I), keratin-like (K), and C-terminal (C) domains (Kaufmann et al., 2005). Of the four domains, the MADS-box domain is especially important for DNA binding and dimerization, and is highly conserved throughout eukaryotes. MIKC-type MADS-box genes are further divided into MIKC* c-type and MIKC* c-type genes, but many seed plant type II and all ABCDE MADS-box genes belong to the former group.

*Eustoma grandiflorum* is one of the most popular species in the cut flower market and many cultivars with different flower shapes and colors, and different growth characteristics have been developed. In spite of its economic value, genomic information about this species is still limited. We previously analyzed the transcriptome of *E. grandiflorum* during flower development (Kawabata et al., 2012). The transcriptome was expected to include a number of genes involved in floral organ development,
such as the ABCDE MADS-box genes. To examine the involvement of MADS-box genes in flower development in *E. grandiflorum*, we identified MIKCc-type MADS-box genes from the above transcriptome and characterized their floral organ-specific expressions. MIKCc-type MADS-box genes included not only ABCDE genes but also other genes involved in the regulation of flowering time.

**Materials and Methods**

*Plant materials*

*E. grandiflorum* ‘Azuma no Murasaki’ (Sakata Seed, Kanagawa, Japan) was grown in a growth chamber controlled at 20–22°C with a 16h photoperiod. After two months, they were transplanted to pots in March 2013 and grown in a greenhouse under natural light conditions until flowering. Flowers at the 12 mm bud stage were collected in June and dissected into sepals, petals, stamens, and carpels. The samples were frozen in liquid nitrogen immediately and kept at −60°C until RNA extraction.

**Gene identification**

Previously sequenced reads of the floral transcriptome (Kawabata et al., 2012) were re-assembled using a GS De Novo Assembler (Roche Applied Science, Mannheim, Germany). Contigs containing the MIKCc-type MADS domain were screened by the hidden Markov model (HMM) approach using the HMM-FRAME program (Zhang and Sun, 2011). The HMM profile of the MADS domain was constructed with the HMMER program (Eddy, 2009), using the multiple alignment file of the conserved MADS domain (Entry name: MADS_BOX_1, accession number; PS00350) repositioned at PROSITE (http://prosite.expasy.org/). The identified MADS-box genes were aligned by Clustal X version 2.0 (Larkin et al., 2007), and the redundant sequences were removed manually. The original sequence reads that corresponded to each identified MADS-box sequence were collected by conducting a blast search. These reads were mapped to the contigs to manually correct sequence errors and extend contig length. The ORFs were predicted using FrameDP (Gouzy et al., 2009) with a complete set of *Arabidopsis* proteins (TAIR_10_pep_20101214_updated, ftp://ftp.arabidopsis.org/home/tair/Sequences/blast_datasets/TAIR10_blastsets/) as the reference database.

**Phylogenetic analysis**

Phylogenetic trees for the identified MADS-box genes were constructed along with related genes of other species retrieved from the GenBank database using MEGA v5.2 (Tamura et al., 2011). Clustal W (Larkin et al., 2007) was used for aligning the full-length amino acid sequences.

**Motif prediction**

MEME (Bailey et al., 2006) was used for detecting motifs in *E. grandiflorum* MADS-box proteins. InterProscan (Quevillon et al., 2005) was employed to examine whether the predicted motifs were known domains. Schematic diagrams of protein domain structures were drawn using DOG 2.0 (Ren et al., 2009).

**Expression analysis**

Total RNA was extracted from the tissue using a Spectrum Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA). The cDNA was reverse-transcribed...
using SuperScript III Reverse Transcriptase (Life Technologies, Gaithersburg, MD, USA) or RevertAq Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). For real-time PCR analysis, SYBR Green Realtime PCR Master Mix-Plus-(TOYOBO) and the Eco Real-Time PCR System (Illumina, San Diego, CA, USA) were used. The real-time PCR program consisted of 95°C for 1 min followed by 45 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The primer list is shown in Table 1. Real-time PCR analysis was conducted in three biological replicates, each of which was calculated as the average of three technical replicates.

Results

Identification of Eustoma MIKC-type MADS-box genes

Through analysis of the assembled transcripts from E. grandiflorum flowers, 23 MIKC-type MADS-box genes were identified (Table 2). Based on protein sequence similarity, they were grouped into 10 clades, including 4 clades related to flowering time (FLC, SVP, SOC1, and AGL6) and 6 clades related to floral organ identity (AP1/FUL, AP3/DEF, PI/GLO, AG/PLE, STK, and SEP). Full-length protein sequences could not be obtained for EgSOC1a, EgSOC1b, and EgSVP, so they were not used for phylogenetic and motif analyses. Floral homeotic genes included two A class genes (AP1/FUL clade), six B class genes (AP3/DEF and PI/GLO clades), three C class genes (AG/PLE clade), one D class gene (STK clade), and six E class genes (SEP clade) (Fig. 1A).

The two AP1/FUL clade genes, EgAP1a and EgAP1b, showed 62% amino acid sequence identity and were close to ATAP1 and AIFUL, respectively. Arabidopsis and Antirrhinum are known to have two different B class genes, which represent two sub-clades, the AP3/DEF and PI/GLO clades. Of the six B class genes identified, four belonged to the AP3/DEF clade and two belonged to the PI/GLO clade (Fig. 1A). The AP3/DEF clade could be further divided into the euAP3 and TM6 sub-clades, although TM6-like genes were absent from Arabidopsis and Antirrhinum (Kramer et al., 1998). In E. grandiflorum, EgDEF1, 2, and 3 were included in the euAP3 sub-clade, while EgTM6 was included in the TM6 sub-clade (Fig. 1A). Amino acid sequences of EgDEF1 and EgDEF2 were 91% identical and were the same length, but were only 63% and 64% identical to EgDEF3, respectively. The amino acid sequence of the two PI/GLO clade genes (EgGLO1 and EgGLO2) was almost identical (92% identity).

Three AG/PLE clade genes (EgPLE1, EgPLE2, and EgPLE3) and one STK clade gene were identified. The AG/PLE clade can be further divided into the euAG and PLE sub-clades (Kramer et al., 2004). The three EgPLE genes belonged to the PLE sub-clade (Fig. 1B). EgPLE1 and EgPLE2 had 89% identical amino acid sequences, while EgPLE3 showed lower similarity to EgPLE1 and 2 (66% and 71% identical, respectively). EgPLE3 protein was closely related to the gentian MADS-box gene, GtMADS3 (77% identity). EgSTK was the only candidate gene for the D class gene and its sequence was the same as EgMADS1 reported by Tzeng et al. (2002).

The six SEP genes (Fig. 1A) were grouped into three pairs (EgSEP1 and EgSEP2, EgSEP3 and EgSEP4, and EgSEP5 and EgSEP6). Each gene of the three pairs showed close sequence similarity (96% identical for EgSEP1–2 and EgSEP3–4, and 77% identical for the aligned region of EgSEP5–6). The two pairs (EgSEP1–4) belonged to the SEP3 sub-clade, while the other pair (EgSEP5 and EgSEP6) belonged to the SEP4 sub-clade (Fig. 1C).

Table 2. List of MIKC-type MADS-box genes identified from Eustoma floral ESTs.

<table>
<thead>
<tr>
<th>No.</th>
<th>Gene Name</th>
<th>Protein Length</th>
<th>Clade</th>
</tr>
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<td>1</td>
<td>EgPLE1</td>
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<td>AG/PLE</td>
</tr>
<tr>
<td>2</td>
<td>EgPLE2</td>
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<td>AG/PLE</td>
</tr>
<tr>
<td>3</td>
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<td>256</td>
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<td>4</td>
<td>EgSTK</td>
<td>218</td>
<td>STK</td>
</tr>
<tr>
<td>5</td>
<td>EgAP1a</td>
<td>245</td>
<td>AP1/FUL</td>
</tr>
<tr>
<td>6</td>
<td>EgAP1b</td>
<td>242</td>
<td>AP1/FUL</td>
</tr>
<tr>
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<td>PI/GLO</td>
</tr>
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</tr>
<tr>
<td>23</td>
<td>EgSOC1b</td>
<td>—</td>
<td>SOC1</td>
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—; unknown length.

Clade-specific motifs conserved in Eustoma MADS-box proteins

MEME motif search identified 16 conserved motifs from E. grandiflorum MADS-box proteins (Fig. 2). Of the four domains in the MIKC-type MADS-box protein, M (MADS-box) and K (K-box) domains were generally highly conserved, while the other two domains were variable among different MADS-box genes across plant species (Kaufmann et al., 2005). M and K domains were also conserved in E. grandiflorum MIKC+ MADS-box genes. The MADS-box domain corresponded to motif 1 and part of motif 3, whereas K domain corresponded to motif 2, 5, 7, 11, 12, and 15. Motif 2 was found in all the MADS-box proteins, except for EgFLC (Fig. 2). Motif 4 coincided with the 1 domain. Motif 13 preceded the
MADS domain, which is specific to AG/PLE-like proteins, such as AG, SHP1 (AGL1), and SHP2 (AGL5) in Arabidopsis (Ma et al., 1991). The other motifs in the C-terminal (motifs 6, 8, 9, 10, 14, and 16) were included in the C domain. Except for motif 10, these motifs corresponded to clade-specific motifs. Motif 6 and 14 included SEP I and II motifs, respectively (Zahn et al., 2005). Motif 8 partially matched PI and PI-derived motifs, and motif 16 was part of the euAP3 motif (Kramer et al., 1998). Motif 9 included AG motif I and II (Kramer et al., 1998).
belonging to the six clades (AP1/FUL, AP3/DEF, PI/GLO, AG/PLE, STK, and SEP) are essential for the proper development of floral organs (Krizek and Fletcher, 2005).

To see whether the above genes in *E. grandiflorum* also follow the ABCDE model, their expression patterns in floral organs were evaluated by quantitative real-time

Floral organ-specific expression of ABCDE class genes

The ABCDE model predicted that MADS-box genes

**Motifs in MIKC domains**

M domain: 1, 3
I domain: 4
K domain: 2, 5, 7, 11, 12, 15
C domain: 6, 8, 9, 10, 14, 16

Other motif: 13

**Known clade-specific motifs**

SEP I motif: 6
SEP II motif: 14
PI or PI-derived motifs: 8
EuAP3 motif: 16
AG I and II motifs: 9
euAP1 motif: E
paleoAP1 motif: P

**Fig. 2.** Identification of conserved motifs in *E. grandiflorum* MADS-box proteins. The motifs were numbered by MEME outputs.

**Fig. 3.** Quantitative RT-PCR of *E. grandiflorum* MADS-box genes. EgPLE1 (A), EgPLE2 (B), EgPLE3 (C), EgSTK (D), EgAP1a (E), EgAP1b (F), EgDEF1 (G), EgDEF2 (H), EgDEF3 (I), EgTM6 (J), EgGLO1 (K), EgGLO2 (L), EgSEP1 (M), EgSEP2 (N), EgSEP3 (O), EgSEP4 (P), EgSEP5 (Q), EgSEP6 (R). Se: sepal, Pe: petal, St: stamen, Ca: carpel. Expression levels were calculated by relative values to ubiquitin (EgUbi). Each value is the mean ± SE (n = 3).
In this study, we identified 23 MIKC c-type MADS-AP1/FUL clade genes were orthologues of floral homeotic genes (Fig. 1A). Phylogenetic analysis suggested that 18 of these genes was mainly expressed in the sepals, while expressed in both stamens and carpels, in agreement with the C-function (Fig. 3A–C). 

The two A class genes, EgAP1a and EgAP1b, were grouped into the AP1/FUL clade. Litt and Irish (2003) reported that AP1/FUL clade genes could be divided into euAP1, euFUL and FUL-like sub-clades. While many of the euAP1 genes were expressed in specific organs, euFUL and FUL-like genes were found to be expressed in various organs in many species (Shan et al., 2007). In E. grandiflorum, the euAP1 sub-clade EgAP1a exhibited sepal-specific expression, while the euFUL/FUL-like sub-clade EgAP1b exhibited broad expression patterns (Fig. 3E, F). In addition, EgAP1a protein contained euAP1 motif, which was reported to be specific to the euAP1 sub-clade, whereas EgAP1b contained paleoAP1 motif, which was shared in the euFUL sub-clade and SEP genes (Litt and Irish, 2003; Shan et al., 2007). The expression pattern and motif conservation supported the diversification of EgAP1 genes into euAP1 and euFUL/FUL-like sub-clades.

AP3/DEF and PUGLO clade genes

According to the ABC model, B class genes, corresponding to AP3/DEF and PUGLO clades, function in petal and stamen identities (Krizek and Fletcher, 2005). In E. grandiflorum, two PUGLO clade genes, EgGLO1 and EgGLO2, were expressed in the petals and stamens, as expected by their putative B-function (Fig. 3K, L). However, the expressions of the three AP3/DEF genes (EgDEF1, 2, and 3) were not limited to the petals and stamens. EgDEF1 and 2 were expressed mainly in the petals and stamens, while EgDEF3 was expressed in all four whorls (Fig. 3M, N). On the other hand, EgSEP3 and EgSEP4 exhibited similar expression patterns, relatively higher in the petals and stamens (Fig. 3O, P). EgSEP5 was expressed predominantly in the sepals (Fig. 3Q). This expression pattern was quite different from that of EgSEP6, which was expressed strongly in the petals and carpels (Fig. 3R).

Discussion

Identification of MIKC-type MADS-box genes in E. grandiflorum

Recent advances in whole-genome sequencing have enabled genome-wide analyses of the MADS-box gene family in economically important crops, such as in grapevine (Diaz-Riquelme et al., 2009), maize, sorghum (Zhao et al., 2011), cucumber (Hu and Liu, 2012), and soybean (Shu et al., 2013). The Arabidopsis, grapevine, and cucumber genomes contained 39, 38, and 30 MIKC-type MADS-box genes, respectively (Diaz-Riquelme et al., 2009; Hu and Liu, 2012; Pafenicová et al., 2003). In this study, we identified 23 MIKC-type MADS-box genes expressed in the Eustoma flower (Table 2). Phylogenetic analysis suggested that 18 of these genes were orthologues of floral homeotic genes (Fig. 1A).

API/FUL clade genes

The two A class genes, EgAP1a and EgAP1b, were grouped into the API/FUL clade. Litt and Irish (2003) reported that API/FUL clade genes could be divided into euAPI, euFUL and FUL-like sub-clades. While many of the euAPI genes were expressed in specific organs, euFUL and FUL-like genes were found to be expressed in various organs in many species (Shan et al., 2007). In E. grandiflorum, the euAPI sub-clade EgAPIa exhibited sepal-specific expression, while the euFUL/FUL-like sub-clade EgAPIb exhibited broad expression patterns (Fig. 3E, F). In addition, EgAPIa protein contained AG/PLE and STK clade genes

Genes of the AG/PLE clade, which comprise C class genes, were reported to be expressed in reproductive organs with a few exceptions (Zahn et al., 2006). This clade included three EgPLE genes (Fig. 1B) that not only shared similar DNA and protein sequences, but also similar expression patterns specific to the stamens and carpels (Fig. 3A–C). These results suggest that EgPLE1–3 genes function as C-class genes redundantly. AG/PLE and STK (AGL11) clades were considered to have an identical origin, but diverged into two sub-clades and were neo-functionalized after gene duplica-
tion (Kramer et al., 2004; Zahn et al., 2006). Further gene duplication in the AG/PLE clade resulted in the euAG and PLE sub-clades. In Arabidopsis and Antirrhinum, C-functions were attributed to AtAG and AmPLE, respectively (Bradley et al., 1993; Yanofsky et al., 1990). Although it is also known that Arabidopsis has two PLE-like genes, SHATTERPROOF1 and 2 (SHP1 and 2), and Antirrhinum has an euAG-like gene, FARINELLI (FAR), these genes do not mainly contribute to their C-functions (Davies et al., 1999; Liljegren et al., 2000). In contrast, in Petunia hybrida and Nicotiana benthamiana, both euAG and PLE sub-clade genes seem to retain C-functions (Fourquin and Ferrándiz, 2012; Heijmans et al., 2012). In E. grandiflorum, all of the putative C-class genes were grouped into the PLE sub-clade and no euAG sub-clade genes could be identified, suggesting that only PLE sub-clade genes specify the C-function.

EgSTK, a putative D class gene, was expressed in the carpels (Fig. 3D). The expression pattern was the same as in Arabidopsis, where the STK expression was limited to the ovules (Pinyopich et al., 2003; Tzeng et al., 2002). The results suggested that EgSTK can specify the ovule identity.

**SEP clade genes**

In addition to the ABCD class genes, E class genes are essential for specifying floral organ identities by forming heterodimers as predicted by the quartet model. Arabidopsis plants have four redundant SEP genes, since single, double, and triple mutants of SEP genes do not show any or full defects in the floral phenotype, but the quadruple sep1sep2sep3sep4 mutant develops leaf-like organs (Ditta et al., 2004; Pelaz et al., 2000). However, functional diversification of SEP-like genes was reported in other plants (Malcomber and Kellogg, 2005). In tomato, the SEP-like gene, RIN, is necessary for fruit ripening, although it might be also involved in floral organ development (Ito et al., 2008). In Gerbera hybrida, two SEP homologues, GRCD1 and GRCD2, were predicted to specify stamen and carpel identities, respectively, while GRCD4 and GRCD5 were proposed to act as general E-function regulators displaying hub-like positions in the interaction network of MADS-box proteins (Ruokolainen et al., 2010). These reports suggest that some SEP genes are functionally specialized to specific whorls, while others conserve the general E-functions. In E. grandiflorum, the transcripts of EgSEP1–4 were detected in all four whorls, indicating their functions are not specific to certain floral organs (Fig. 3M–P). By contrast, EgSEP5 was preferentially expressed in the sepals and EgSEP6 in the petals and carpels (Fig. 3Q, R), suggesting that these SEP4-like genes have specialized functions in specific floral organs.

**Gene duplication in BCE class genes**

The basic mechanism of floral organ specification and patterning regulated by MADS-box genes was found to be conserved in angiosperms (Krizek and Fletcher, 2005). This suggested that diversified flower forms might have evolved from ancient flowering plants along with the diversification of MADS-box genes. Evolutional changes in MADS-box genes include gene duplication and neo-functionalization of the duplicated genes, as well as the changes in protein-protein interactions that may contribute to the formation of MADS-box protein heterodimers (Immink et al., 2010).

From E. grandiflorum, as many as 18 MADS-box genes for ABCDE class gene clades were identified, whereas some of the orthologues for Arabidopsis floral MADS-box genes could not be identified. The phylogenetic tree suggested the presence of three AP3 orthologues, two PI orthologues and four SEP3 orthologues, while no euAG clade genes (Fig. 1).

Among the 23 identified MADS-box genes identified, multiple genes showed close sequence similarity. SEP genes, in particular, could be grouped into three pairs (Fig. 1C). EgSEP3 and 4 genes shared the same protein lengths, presence of specific motifs and similar expression patterns (Table 2; Figs. 2 and 3). In addition, EgPLE1–2, EgDEF1–2, and EgGLO1–2 showed very close sequence similarities and similar expression patterns. The presence of closely related MADS-box genes indicates the recent occurrence of gene duplication events. Duplicated genes would redundantly function to prevent unusual specification of floral organ identity under the loss of one of the duplicated genes, but the subsequent diversification of these genes would contribute to the evolution of floral architecture, as suggested for SEP clade genes by Malcomber and Kellogg (2005).

**Conclusion**

Our results showed the conservation and diversification of MIKC*-type MADS-box genes during evolution in E. grandiflorum. We identified 23 MIKC*-type MADS-box genes from Eustoma floral transcriptome. These genes were classified as floral homeotic genes, including the ABCDE genes, and flowering time genes. Phylogenetic analysis suggested that B, C, and E class genes have experienced recent gene duplication events. The identified genes contained some clade-specific motifs. The expression patterns of PI/GLO and PLE clade genes were conserved as predicted by the ABC model. However, diversified expressions were observed in AP3/DEF and SEP clade genes. Transcripts of some genes were observed not only in the whorls predicted from the ABCDE model, but also in other whorls. Such discordance suggested the functional diversification of these genes, but may also result from the flower developmental stages at which the gene expression was analyzed in this study. At later developmental stages, the expression domains of floral homeotic genes can sometimes extend more broadly. In Arabidopsis, for example, AtSEP3 is not expressed in sepal primordia, but is
on the adaxial side of sepal at later stages (Mandel and Yanofsky, 1998). *AtAP3* were also expressed in mature ovules (Jack et al., 1992). In some species, euAP1-like genes were also expressed in the carpels (Shan et al., 2007). To confirm the significant function of the MADS-box genes identified in this study, it is necessary to determine their spatial and temporal expression patterns in floral organ primordia.

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