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

Motoyuki Ishimori and Saneyuki Kawabata*

Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

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 combinatory 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* (*API*), B class includes *APETALA3* (*AP3*)

and *PISTILLATA* (*PI*), C class includes *AGAMOUS* (*AG*), D class includes *SEEDSTICK* (*STK*), and E class includes *SEPALLATA1-4* (*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* (Pařenicová 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^{*}-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,

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* Corresponding author (E-mail: ayuki@mail.ecc.u-tokyo.ac.jp).

such as the ABCDE MADS-box genes. To examine the involvement of MADS-box genes in flower development in *E. grandiflorum*, we identified MIKC^c-type MADS-box genes from the above transcriptome and characterized their floral organ-specific expressions. MIKC^c-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 MIKC^c-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) repositied 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

Table 1. Primers used in this study.

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
<i>EgPLE1</i>	tactgataccaccagcacaggat	tttctcagctgtctgattctt
<i>EgPLE2</i>	gtgagtactgataccaccagtatagg	cttatatgcttcccagctttt
<i>EgPLE3</i>	gcttctgctgataccacyaattcgg	cttcttagctgtgctgctgttg
<i>EgSTK</i>	gaccgttacaggaagcaactcag	gcttaaacctctcccatcatgatgc
<i>EgAPIa</i>	gaagacaatggcgcaacaac	gaggaaaggtgtcgaatttgc
<i>EgAPIb</i>	tgtcccttcaactgttctctca	ctacaggttggtatgcgtcact
<i>EgDEF1</i>	ggctctgatcttaccactttg	taaagagatgaggagacaagacc
<i>EgDEF2</i>	cctaaccgcccagttttat	agcaatgcgaaggtggaag
<i>EgDEF3</i>	agaaccgaaccaagacaagt	ttgaacagcccatttctctt
<i>EgTM6</i>	gtactcttctgcttgcagtct	taatctcaagcctgagggtct
<i>EgGLO1</i>	gaaaatgctagaggatcagcacaagcag	tcgaaatgcaaaaggcatatgggaatc
<i>EgGLO2</i>	gaaatgctagaggaggagcacaagcac	gaaatgcaaaaggcatatgggagtgtatg
<i>EgSEP1</i>	gaagcttaagctcgttatgaagmcctcc	ccgtgtagatctgatttgcctcaagaca
<i>EgSEP2</i>	ctcgttatgaagccctacaacggtcac	atctgatttgcctcagcgacgtatcg
<i>EgSEP3</i>	aagcccgatatgaagctctacaacgg	ccgctgtagaacggatttgccttaagtac
<i>EgSEP4</i>	aagcccgtatgaagctctacaagg	ccgggtagaacggatttgccttaagcagc
<i>EgSEP5</i>	atgctcttctggatgtagtcagcca	gtgataacgttggaaggacctgactct
<i>EgSEP6</i>	tttctctaccatggaagctc	tttcaagtgttctctgcatgct
<i>EgUbi</i>	aagtttgcctcaagcttttgc	ggaacaacagagatataggttcaca

using SuperScript III Reverse Transcriptase (Life Technologies, Gaithersburg, MD, USA) or ReverTra 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^c-type MADS-box genes

Through analysis of the assembled transcripts from *E. grandiflorum* flowers, 23 MIKC^c-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 (*API/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 (*API/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 *API/FUL* clade genes, *EgAPIa* and *EgAPIb*, showed 62% amino acid sequence identity and were close to *AtAPI* and *AtFUL*, 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).

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^c-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^c 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 I domain. Motif 13 preceded the

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

No.	Gene Name	Protein Length	Clade
1	<i>EgPLE1</i>	227	AG/PLE
2	<i>EgPLE2</i>	241	AG/PLE
3	<i>EgPLE3</i>	256	AG/PLE
4	<i>EgSTK</i>	218	STK
5	<i>EgAPIa</i>	245	API/FUL
6	<i>EgAPIb</i>	242	API/FUL
7	<i>EgDEF1</i>	226	AP3/DEF
8	<i>EgDEF2</i>	226	AP3/DEF
9	<i>EgDEF3</i>	230	AP3/DEF
10	<i>EgTM6</i>	222	AP3/DEF
11	<i>EgGLO1</i>	212	PI/GLO
12	<i>EgGLO2</i>	212	PI/GLO
13	<i>EgSEP1</i>	237	SEP
14	<i>EgSEP2</i>	238	SEP
15	<i>EgSEP3</i>	244	SEP
16	<i>EgSEP4</i>	244	SEP
17	<i>EgSEP5</i>	247	SEP
18	<i>EgSEP6</i>	187	SEP
19	<i>EgAGL6</i>	254	AGL6
20	<i>EgFLC</i>	202	FLC
21	<i>EgSVP</i>	—	SVP
22	<i>EgSOC1a</i>	—	SOC1
23	<i>EgSOC1b</i>	—	SOC1

—; unknown length.

Fig. 1. Phylogenetic trees of MADS-box proteins constructed by neighbor-joining method. A: MIKC-type MADS-box proteins; B: AG/PLE clade proteins; C: SEP clade proteins. ♦: *E. grandiflorum* genes. Akt, *Akebia trifoliata*; Am, *Antirrhinum majus*; At, *Arabidopsis thaliana*; Bn, *Brassica napus*; Bp, *Betula pendula*; Ca, *Corylus avellana*; Can, *Capsicum annuum*; Cit, *Citrus unshiu*; Cm, *Chrysanthemum × morifolium*; Cs, *Cucumis sativus*; De, *Daucus carota*; Eg, *Eustoma grandiflorum*; Esca, *Eschscholzia californica*; Eucg, *Eucalyptus grandis*; Fa, *Fragaria × ananassa*; Gh, *Gerbera hybrida*; Goh, *Gossypium hirsutum*; Gt, *Gentiana triflora*; Ha, *Helianthus annuus*; In, *Ipomoea nil*; Ls, *Liquidambar styraciflua*; Md, *Malus × domestica*; Ns, *Nicotiana sylvestris*; Nt, *Nicotiana tabacum*; Pi, *Petunia integrifolia* subsp. *inflata*; Pb, *Populus balsamifera* subsp. *trichocarpa*; Pg, *Panax ginseng*; Ph, *Petunia hybrida*; Ps, *Pisum sativum*; Pt, *Populus tremuloides*; Rr, *Rosa rugosa*; Sa, *Sinapis alba*; Sl, *Solanum lycopersicum*; Sla, *Silene latifolia* and Vv, *Vitis vinifera*.

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 corre-

sponded 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.,

2004). In addition to the predicted motifs by MEME, EgAP1a and EgAP1b proteins contained euAP1 and paleoAP1 motifs (Shan et al., 2007), respectively.

Floral organ-specific expression of ABCDE class genes

The ABCDE model predicted that MADS-box genes

belonging to the six clades (*API/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

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 $\pm$ SE ($n = 3$).

RT-PCR (Fig. 3). All the *AG/PLE* clade genes were expressed in both stamens and carpels, in agreement with the C-function (Fig. 3A–C). *EgSTK* was expressed in the carpels (Fig. 3D). In the *API/FUL* clade, *EgAPIa* was mainly expressed in the sepals, while *EgAPIb* was also expressed in three other whorls (Fig. 3E, F). Of the three euAP3 sub-clade genes, *EgDEF1* and *EgDEF2* exhibited similar expression patterns, predominantly in the petals, followed by the sepals and stamens, and faintly in the carpels (Fig. 3G, H). *EgDEF3* expression was also detected in all four whorls (Fig. 3I). *EgTM6*, the paleoAP3 gene, was expressed strongly in the carpels and secondly in the stamens (Fig. 3J). Both *PI/GLO* clade genes, *EgGLO1* and *EgGLO2*, were expressed in the petals and stamens (Fig. 3K, L). Meanwhile, *EgSEP* genes showed various expression patterns. Both *EgSEP1* and *EgSEP2* were expressed in all four whorls, but their expression patterns were different. *EgSEP1* expression was the highest in the sepals, whereas *EgSEP2* expression was not different among the four whorls (Fig. 3M, N). On the other hand, *EgSEP3* and *EgSEP4* exhibited similar expression patterns, relatively higher in the petals and carpels (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^c-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^c-type MADS-box genes, respectively (Diaz-Riquelme et al., 2009; Hu and Liu, 2012; Pařenicová et al., 2003). In this study, we identified 23 MIKC^c-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, *EgAPIa* and *EgAPIb*, 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

euAPI1 motif, which was reported to be specific to the euAPI sub-clade, whereas *EgAPIb* contained paleoAPI1 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 *EgAPI* genes into euAPI and euFUL/*FUL*-like sub-clades.

AP3/DEF and PI/GLO clade genes

According to the ABC model, B class genes, corresponding to *AP3/DEF* and *PI/GLO* clades, function in petal and stamen identities (Krizek and Fletcher, 2005). In *E. grandiflorum*, two *PI/GLO* 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 slightly in the stamens, but they were also expressed in the sepals, and *EgDEF3* was expressed in all four whorls (Fig. 3G–I). Therefore, the expression patterns of *E. grandiflorum* euAP3 genes were not in agreement with the similar B-function of *Arabidopsis*. Among the three *EgDEF* genes, *EgDEF3* may not have a significant function in the determination of floral organ identity, since the expression level of *EgDEF3* was relatively low as compared with other *EgDEFs*. The expression level often correlates with the function. For instance, loss-of-function analysis of two redundant C class genes of *Petunia hybrida* indicated that one C class gene *FBP6* uniquely contributes to style and stigma development, while the other gene *PMADS3* exhibited weak expression and did not strongly influence floral organ identity in these organs (Heijmans et al., 2012; Kater et al., 1998).

Another explanation is that the expression patterns of the euAP3 genes (*EgDEFs*) do not influence their functions. In *Arabidopsis*, *AP3* is post-transcriptionally regulated (Jack et al., 1994), and the AP3 function needs PI as an interacting partner (McGonigle et al., 1996). If either *EgGLO1* or *EgGLO2* protein is necessary for *EgDEF* functions in *E. grandiflorum*, the functions of B class genes will be limited to the petals and stamens where *EgGLOs* are functional.

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 eu*AG* 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 eu*AG*-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 eu*AG* 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 eu*AG* 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 eu*AG* 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^c-type MADS-box genes during evolution in *E. grandiflorum*. We identified 23 MIKC^c-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 sepals at later stages (Mandel and Yanofsky, 1998). *AtAP3* were also expressed in mature ovules (Jack et al., 1992). In some species, *euAPI*-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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