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A Floral Meristem Identity Gene Influences Physiological and Ecological Aspect of Floral Organogenesis

Ho-Sung Yoon*
Department of Botany, University of Wisconsin, 430 Lincoln Drive, Madison WI 53706, USA

The architecture of a flower is tightly linked to the way a plant pollinates, making it one of the most physiologically and ecologically important traits of angiosperms. Floral organ development is proposed to be governed by the activity of three different classes of organ identity genes (the ABC model), and the expression of those genes are regulated by a number of meristem identity genes. Here we use a transgenetic strategy to elucidate the role of one floral meristem identity gene, *LEAFY* (*LFY*), in the evolution of floral organogenesis of a self pollinator *Idahoa scapigera* and a obligatory out-crosser *Leavenworthia crassa* in the mustard family, Brassicaceae. By introducing the *LFY* genes from these two types of pollination habit into the genetic model species *Arabidopsis thaliana*, we provide evidence that changes in *LFY* influenced flower architecture probably by controlling the downstream organ identity genes.

Keywords: floral organogenesis, meristem identity gene, *LEAFY*, pollination type, transgenetics

Flowers of Brassicaceae family display strongly conserved composition of organ types that are arranged spatially in four concentric whorls; starting from the outermost whorl - sepals, petals, stamens, and carpels. According to the ABC model, the organ types are determined by the combination of three different classes of organ identity genes (Weigel and Meyerowitz, 1994). The outermost whorl sepals are specified by the class A gene activity (*AP1* and *AP2*), the second whorl petals are specified by the combination of A (*AP1* and *AP2*) and B class gene (*AP3* and *PI*) activities, the third whorl stamens are specified by the combination of B (*AP3* and *PI*) and C class gene (*AG*) activities, and the fourth whorl carpels are specified by C class gene (*AG*) activity. The model also postulates that A, B and C activities are capable of functioning in all whorls of the flower, and A and C functions are suggested to repress each other, such that a mutation in either of these functions results in the expansion of the other function into all four floral whorls.

The five organ identity genes mentioned above have been cloned and shown to be transcriptional factors which regulate downstream specific floral organ developmental genes (Jack et al., 1994; Mandel et al., 1992; Yanofsky et al., 1990). Upstream of the regulatory circuit in floral development is a meristem identity gene *LEAFY* (*LFY*). *LFY* is one of the first genes to be activated during floral evocation, whereupon it activates (in some cases directly) other meristem identity genes and floral organ identity genes (Weigel et al., 1992). *LFY* encodes a 412-424 residue DNA-binding transcriptional regulator. There is evidence that *LFY* homologs can act non-cell-autonomously (Hantke et al. 1995; Session et al. 2000). *LFY* homologs have been reported from a wide range of flowering plants (Weigel and Nilsson 1995; Hofer et al. 1997; Kyozuka et al. 1998) and sequence conservation is high: for example, 70% amino acid identity between *Arabidopsis* and *Antirrhinum*. The high level of sequence conservation suggests conservation of *LFY* function, at least across eudicots.

There has been no direct evidence that *LFY* affects architecture of a particular organ type from the studies of *lfy* mutant lines. *Arabidopsis lfy* mutants fail to activate the floral organ genes resulting in the production of a shoot-like structure (Weigel et al., 1992) rather than affecting an individual organ type. *LFY* overexpression promotes early flowering without clearly affecting particular floral organogenesis (Weigel and Nilsson, 1995). However, since *LFY* is a regulator of all the organ identity genes, we hypothesized that changes in the *LFY* gene may have played some role in the formation of characteristic morphologies of different flowers of closely related species. This could be achieved by the evolutionary changes of the *LFY* gene in the way of interacting with each organ identity genes and so by affecting the activity of the downstream floral structural genes. Among the close relatives of *Arabidopsis* in Brassicaceae family, *Idahoa scapigera* (*I. scapigera*) and *Leavenworthia crassa* (*L. crassa*) display strikingly different floral mor-

*Corresponding author; fax +82-53-950-6750
e-mail: hyoon@knu.ac.kr

Figure 1. Comparison of the floral architecture of *Idahoa scapigera* (**A**), and *Leavenworthia crassa* (**B**). **A**, Different stages of floral development of *I. scapigera* are shown starting from the opening of the flower (left) to the maturation of the fruit (right). Self-pollinator *I. scapigera* produces minute size petals which are indicated by arrows. **B**, In contrast, obligate out-crosser *L. crassa* produces relatively large petals among *Brassicaceae* family to capture the attention of insect pollinators. Some petals are indicated by arrows.

phology in the size of petals (Fig. 1, compare A and B). *I. scapigera* produces self-pollinating flowers with four minute petals, while self-incompatible, insect-pollinated *L. crassa* produces very large flowers relative to *Arabidopsis*. Floral morphology is very tightly related to the pollination habit influencing the physiology and ecology of the species (Rollins 1994; Baskin et al. 1995). We used a transformation strategy to test our hypothesis. *LFY* homologs with their flanking DNA sequences (ca. 3-kb upstream and 1-kb downstream) were cloned from *I. scapigera* and *L. crassa* and introduced into *Arabidopsis lfy* mutant lines. A 2.3-kb DNA fragment upstream of the *Arabidopsis LFY* has been reported to contain all the necessary elements for proper expression (Blázquez et al., 1997; Blázquez et al., 1998). Transgenic lines in which *LFY* homologs from these two species had replaced the endogenous *Arabidopsis LFY* gene were examined in the T2 and T3 generations and compared to control lines containing an *Arabidopsis LFY* transgene. As another control, a distantly related plant in Brassicaceae, *Ionopsidium acaule* (*I. acaule*), was used for transformation. The transgenic lines containing *LFY* genes from *I. scapigera* or *L. crassa* produced flowers resembling the aspect of the donor flowers particularly in the petal size. Our results suggest that *LFY* influences the determination of floral morphology by affecting the expression of the downstream floral organ identity genes.

MATERIALS AND METHODS

Plant Materials

Wild type and *lfy* mutant *Arabidopsis* seeds were provided by D. Weigel. Seeds of *I. scapigera* were collected by D. Baum in Pullman, WA, USA (Voucher: Baum 365; GH). Seeds of *L. crassa* were provided by Reed Rollins (Cultivated voucher: Baum 379; WIS). The *I. acaule* lines were described previously (Shu et al., 2000).

Cloning, Transformation, and Phenotype Scoring

To clone the *LFY* homologues and the flanking DNA regions, we first cloned fragments within *LFY* coding regions of *I. scapigera*, *L. crassa*, and *I. acaule* by PCR with primers designed from *Arabidopsis LFY*. Sequences of the coding regions were then used to design primers for genome walking (Clontech) which allowed us to obtain flanking sequences. Primers were designed to amplify an approximately 7-kb DNA fragment of each gene containing ca. 3-kb upstream of the *LFY* open reading frame and ca. 1-kb downstream. 2.3-kb upstream sequences of *Arabidopsis LFY* has been shown to contain all the necessary regulatory elements for normal function (Blazquez et al., 1997). Using high-fidelity polymerase chain reaction (PCR using *Pfu* DNA polymerase, Stratagene) and UV-free gel extraction (Invitrogen), we successfully cloned *Idahoa LFY*, *Leavenworthia LFY*, and *Ionopsidium LFY* in the PCR-Blunt II-TOPO vector (Invitrogen). Six independent *LFY* clones from each donor species (three in each orientation) and four clones from *Arabidopsis* (two in each orientation) were then moved into the binary vector pCAMBIA3300 (Cambia) which includes the BAR selectable marker conferring BASTA resistance.

Arabidopsis thaliana Landsberg *erecta* heterozygous *lfy-6/LFY* plants were transformed with the 22 clones using the floral dip method (Clough et al. 1998). An empty vector pCAMBIA3300 (Cambia) was used as an additional control. T1 seeds were selected by spraying 0.2 mg/ml BASTA. Resistant plants were genotyped at the endogenous *LFY* locus using CAPS markers (Blazquez et al., 1997). A modified CAPS marker method was used to genotype the endogenous *LFY* locus for *Arabidopsis LFY* transgenic lines, because endogenous and exogenous *Arabidopsis LFY* copies are indistinguishable with regular CAPS markers. We selected resistant T1 plants that were determined to be *lfy-6/lfy-6* at the endogenous locus and used them for analysis in the T2 generation. We generally characterized 2 such

"replacement" lines per construct and grew up 20-30 T2 plants in long-day conditions (16 hours light: 8 hours dark). However, due to reduced fertility of some lines this was not always possible. The segregation ratio of $BASTA^R$:$BASTA^S$ in T2 and T3 plants was used to estimate the number of transformed T-DNA loci.

SEM

Tissue was fixed in FAA (50% ethanol, 5% acetic acid, 3.7% formaldehyde) and dehydrated in a graded ethanol series to 100%. The samples were then critical point dried and sputter coated with palladium and viewed on an ISI 30 at 10 kV voltage.

RESULTS AND DISCUSSION

Floral Phenotype of the *Idahoa LFY* Transgenic Plants

Brassicaceae family including wild-type *Arabidopsis* plant produce flowers with 4 sepals, 4 petals, 6 stamens, and 1 gynoecium with 2 fused carpels. As a control, we cloned *Arabidopsis LFY* from *Arabidopsis* genome with the same procedure that was used to clone the *LFY* homologues from *I. scapigera* and *L. crassa* (see Materials and Methods). When *Arabidopsis LFY* was used to transform *Arabidopsis lfy* mutant, the transgenic plants produced flowers that are indistinguishable to wild-type flowers (Fig. 2, A and B). This result indicates that the cloned *Arabidopsis LFY* gene and the flanking sequences (ca. 3-kb upstream of the *LFY* open reading frame and ca. 1-kb downstream) functions normally in transgenic plants.

Idahoa LFY gene shows 84.3% amino acid identity with *Arabidopsis LFY* (for detailed sequence comparisons and phylogenetic analysis, refer to Yoon et al., 2004). Transformation experiments were conducted and 10 *Idahoa LFY* transgenic lines that replace the endogenous *LFY* gene in *Arabidopsis* were examined (Table 1). All the transgenic lines produced flowers with fewer or no petals. There were differences in severity of the phenotype but we observed a specific effect on petals number in transgenic plants (Fig. 2, C and D). Other organs appeared in mostly normal numbers (Table 3 and Fig. 3), but there was a slight reduction in the size of all floral organs. The donor species *I. scapigera* pro-

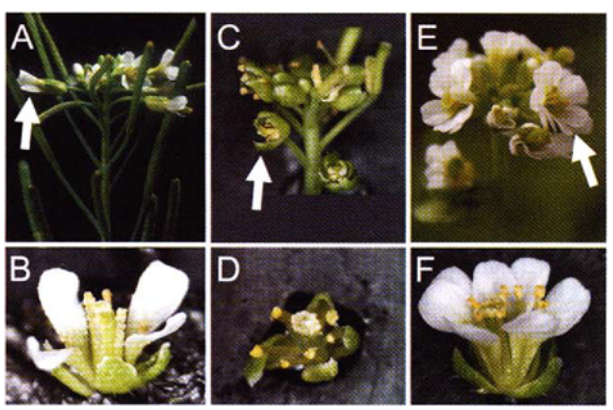

Figure 2. Phenotypes of the transgenic *Arabidopsis* plants containing *LFY* transgenes instead of the intrinsic *Arabidopsis LFY* gene. **A,** Transformed *Arabidopsis LFY* replaces endogenous *Arabidopsis LFY* function resulting in a wild-type floral architecture. One of the flowers is indicated by an arrow. The whole inflorescence (**A**) or an individual flower (**B,** indistinguishable from a wild-type flower) is shown. **C,** An inflorescence of a transgenic *Arabidopsis* plant containing *Idahoa LFY*. Most of the flowers are missing petals (arrow). A close up photograph of a flower is shown in **D**. All the other organs (sepals, stamens, and carpels) are formed normally. **E,** An inflorescence of a transgenic *Arabidopsis lfy* mutant containing *Leavenworthia LFY*. Most of the flowers produced extra petals (arrow). **F,** An example of flowers with extra petals. 7 petals instead of normal 4 petals are produced in this flower.

Table 1. Phenotype of the transgenic lines of *Arabidopsis* in that *LFY* gene was replaced with *Idahoa LFY*.

Lines	Number of plants	Floral phenotype[a]	
		WT	Fewer petals
1-5	12	0	12
1-19	16	1	15
6-13	12	0	12
7-30	19	0	19
7-31	6	5	1
11-9	16	0	16
11-22	21	0	21
15-8	13	0	13
15-17	21	0	21
23-2	16	0	16

[a]Number represents the number of plants that contain overall such floral morphology.

Table 2. Phenotype of the transgenic lines of *Arabidopsis* in that *LFY* gene was replaced with *Leavenworthia LFY*.

Lines	Number of plants	Floral phenotype[a]	
		WT	Extra petals
L2-6	6	6	0
L3-13	27	8	19
L3-15	18	0	18
L4-2	14	14	0
L4-5	9	5	4
L5-7	9	9	0

[a]Number represents the number of plants that contain overall such floral morphology.

Table 3. Floral organ numbers of transgenic *Arabidopsis* lines containing introduced *Arabidopsis LFY*, *Idahoa LFY*, or *Leavenworthia LFY* in place of the intrinsic *LFY* gene.

	Sepals	Petals	Stamens	Gynoecia	
Arabidopsis LFY	4 +/− 0	4.04 +/− 0.20	5.92 +/− 0.34	1	N = 50
Idahoa LFY	3.98 +/− 0.13	0.88 +/− 0.90[a]	5.62 +/− 0.87[b]	1	N = 93
Leavenworthia LFY	4.06 +/− 0.35	6.65 +/− 1.27	6.19 +/− 0.56	1[c]	N = 93

[a]Petal number of *Idahoa LFY* transformants is a sum of petals (0.24), stamenoid petals (0.28), and filamentous organs (0.37).
[b]Stamen number of *Idahoa LFY* transformants is a sum of stamens (4.86), stamenoid sepals (0.03), stamenoid petals (0.28), stamenoid carpels (0.09), and filamentous organs (0.37).
[c]Occassional fused multiple carpels were found in *Leavenworthia LFY* transformants.

duces self-pollinating flowers with four, minute petals (Fig. 1A) raising the possibility that the observed effect is not simply the result of a failure to fully rescue the *lfy* mutation, but could indicate that changes at the *Idahoa LFY* locus contributed to the evolutionary reduction in flower size in *I. scapigera*.

Floral Phenotype of the *Leavenworthia LFY* Transgenic Plants

Leavenworthia LFY shares 92.6% amino acid identity with *Arabidopsis LFY*. Three of six independent *Leavenworthia LFY* transgenic lines manifest a highly modified floral architecture (Table 2). The flowers produced by those *Leavenworthia LFY* lines had more petals than wild-type plants and tended to form larger-than-normal gynoecia (Fig. 2, E and F). Similar to the result of the *Idahoa LFY* transgenic plants, the morphology and numbers of other floral organs appeared to be normal (Table 3 and Fig. 3). These results are noteworthy given that the self-incompatible, insect-pollinated *L. crassa* produces very large flowers relative to *Arabidopsis* (Fig. 1B). The floral organ specific effects of *Idahoa LFY* and *Leavenworthia LFY*, and their correlation with donor flower petal size, make it tempting to speculate that *LFY* plays a role in the proliferation of floral meristems but that in an *Arabidopsis* genetic background this effect is manifest primarily as changes in petal number rather than petal size: *Arabidopsis* may not be equipped with genetic machineries to produce bigger size petals thus producing more numbers of petals with the extra cells in the floral meristem.

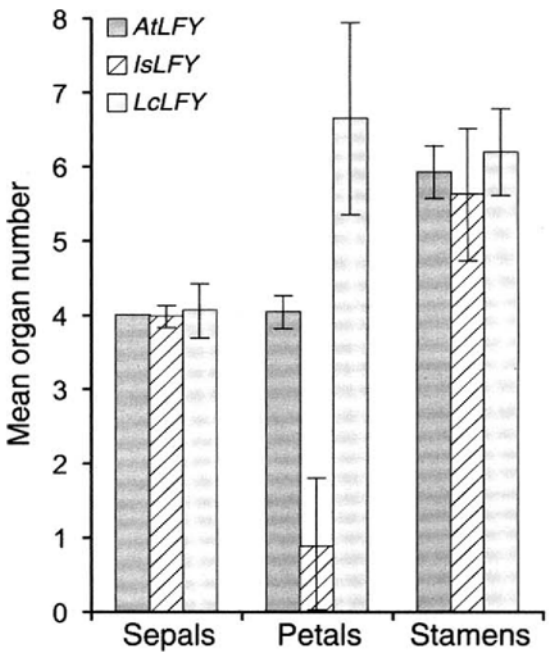

Figure 3. Floral organ numbers in transgenic *Arabidopsis* plants. Eight to ten flowers were counted from 5 *Arabidopsis LFY*, 10 *Idahoa LFY1*, 11 *Leavenworthia LFY* T2 transgenic plants (each from a single representative line) and the mean and standard deviations are indicated. Petal number of *Idahoa LFY1* transformants is a sum of petals (mean= 0.24/flower), stamenoid petals (mean= 0.28/flower), and filamentous organs (mean= 0.37/flower). Stamen number of *Idahoa LFY1* transformants is a sum of stamens (mean= 4.86), stamenoid sepals (mean= 0.03), stamenoid petals (mean= 0.28), stamenoid carpels (mean= 0.09), and filamentous organs (mean= 0.37).

LFY Affects Floral Morphology through the ABC genes

Ionopsidium acaule also belongs to Brassicaceae family but is not as closely related to *Arabidopsis* as *I. scapigera* or *L. crassa* (Galloway et al., 1998; Koch et al., 2001, also for detailed sequence comparisons and phylogenetic analysis, refer to Yoon et al., 2004). We transformed *Arabidopsis* plants with *Ionopsidium LFY* to examine the effect on floral morphogenesis. Ten independent *Arabidopsis* lines transformed with *Ionopsidium LFY* were examined and found to produce incomplete and abnormal flowers resembling *Arabidopsis ap2* mutation (Fig. 4; Jofuku et al., 1994). The flowers have carpeloid sepals, no petals, and reduced numbers of stamens. The *ap2*-like phenotypes could be due to a failure to properly activate A-function or a failure to

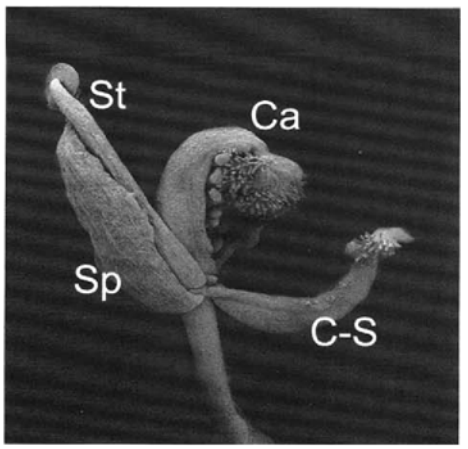

Figure 4. Electron micrograph (SEM) of an *Ionopsidium LFY* transgenic flower resembling *Arabidopsis ap2* mutation. One representative flower is shown. Only one sepal (Sp) is formed but the rest three sepals are missing. 5 stamens (St) are not produced and carpel (Ca) is not fully developed and unfused. A carpeloid sepal (C-S) displays characteristics of both organs.

exclude C-function from the outer whorls (e.g., through failure to activate *AP2* or ectopic activation of *AG*). Ectopic expression of *AG* driven by a viral promoter 35S has shown that this gene is sufficient to repress A function in the outer two floral whorls (Mandel et al., 1992, Mizukamu and Ma, 1992). Flowers of these plants contain carpeloid first whorl organs and stamenoid or missing second whorl organs; flowers which closely resemble *ap2* mutants. The phenotype obtained shows that there has been evolutionary divergence of function between *Ionopsidium LFY* and *Arabidopsis LFY* in the activation of A- or C-function organ identity genes. The fact that *Ionopsidium LFY* resulted in floral defects that resemble specific homeotic gene mutation implies the specific interaction between the introduced *LFY* and the intrinsic *Arabidopsis* ABC genes. Thus, functionally significant sequence change has occurred at the *LFY* locus subsequent to the splitting of the lineages leading to *Arabidopsis* and the donor species. It seems equally plausible that genetic chages in the cis-regulatory region of the *LFY* genes altered the temporal and spatial expression pattern of *LFY* thus causing altered regulatory effects on downstream genes of floral organogenesis.

Taking these data as a whole, we concluded that changes in the *LFY* gene had played a role in the evolution of floral organogenesis in close relatives of Brassicaceae, and this could be achieved by alteration of the way to interact with downstream organ identity genes of ABC class. Also *LFY* appears to have an important role in physiological and ecological aspects of plants being involved in the characteristic development of floral organs of particular pollination habit.

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