Floral Patterning in *Lotus japonicus*[1][w]

Zhi-cheng Dong, Zhong Zhao, Cheng-wu Liu, Jiang-hong Luo, Jun Yang, Wei-hua Huang, Xiao-he Hu, Trevor L. Wang, and Da Luo*

National Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences (Z.D., C.L., J.L., J.Y., W.H., X.H., D.L.), and Graduate School of the Chinese Academy of Sciences (Z.D., C.L., J.L.), Chinese Academy of Sciences, Shanghai 200032, China; School of Life Science and Biotechnology, Shanghai Jiao Tong University, Shanghai 200030, China (D.L.); Department of Biochemistry, School of Life Sciences, Fudan University, Shanghai 200433, China (Z.Z.); and Department of Metabolic Biology, John Innes Centre, Colney, Norwich NR4 7UH, United Kingdom (T.L.W.)

Floral patterning in Papilionoideae plants, such as pea (*Pisum sativum*) and *Medicago truncatula*, is unique in terms of floral organ number, arrangement, and initiation timing as compared to other well-studied eudicots. To investigate the molecular mechanisms involved in the floral patterning in legumes, we have analyzed two mutants, *proliferating floral meristem* and *proliferating floral organ-2* (*pfo-2*), obtained by ethyl methanesulfonate mutagenesis of *Lotus japonicus*. These two mutants showed similar phenotypes, with indeterminate floral structures and altered floral organ identities. We have demonstrated that loss of function of *LjLFY* and *LjUFO/Pfo* is likely to be responsible for these mutant phenotypes, respectively. To dissect the regulatory network controlling the floral patterning, we cloned homologs of the ABC function genes, which control floral organ identity in Arabidopsis (*Arabidopsis thaliana*). We found that some of the B and C function genes were duplicated. RNA in situ hybridization showed that the C function genes were expressed transiently in the carpel, continuously in stamens, and showed complementarity with the A function genes in the heterogeneous whorl. In *proliferating floral meristem* and *pfo-2* mutants, all B function genes were down-regulated and the expression patterns of the A and C function genes were drastically altered. We conclude that *LjLFY* and *LjUFO/Pfo* are required for the activation of B function genes and function together in the recruitment and determination of petals and stamens. Our findings suggest that gene duplication, change in expression pattern, gain or loss of functional domains, and alteration of key gene functions all contribute to the divergence of floral patterning in *L. japonicus*.

Members of the legume family, one of the largest monophyletic families (approximately 700 genera and 18,000 species), display a range of flower structures. Studies on the ontogeny of flowers in two model plants, pea (*Pisum sativum*) and *Medicago truncatula* (Tucker, 1989; Ferrandiz et al., 1999; Benlloch et al., 2003), showed that there were substantial morphological differences between the flowers of Papilionoideae, the largest of the three subfamilies in the legume family, and many other eudicot plants like Arabidopsis (*Arabidopsis thaliana*) and Antirrhinum (*Antirrhinum majus*). Most flowers in Papilionoideae possess pentamerous whorls of sepals and petals, two whorls of stamens, and a single carpel (Tucker, 2003). Furthermore, in the Papilionoideae, floral organs normally are initiated in the order of sepals, carpel, petals, and stamens; within each whorl, the organs are initiated unidirectionally from the abaxial to adaxial side. Another difference is the heterogeneous whorl seen in some species, such as pea and *M. truncatula*. Prior to the initiation of petal and stamen primordia, common primordia are formed between sepals and the carpel, which will then give rise to primordia of both petals and outer stamens and, eventually, to the primordia of inner stamens formed in another whorl (Ferrandiz et al., 1999; Benlloch et al., 2003). Thus, heterogeneous organs with different identities can be initiated simultaneously in the same whorl, and homogeneous organs with the same identity can also be initiated from different whorls. In contrast, petals and stamens are initiated individually from different whorls in most other eudicots. Floral organ number, arrangement, and initiation timing, therefore, are unique in the Papilionoideae.

Consistent with this observation, molecular and genetic evidence has indicated that the underlying molecular mechanisms diverge to some degree between Papilionoideae and other well-studied eudicots. According to studies in Arabidopsis and Antirrhinum, *LFY* and *FLO* are floral meristem identity homologs that are expressed throughout the floral primordium and are responsible for specifying floral fate (Coen et al., 1990; Weigel et al., 1992). They play an additional role in the recruitment of floral organs and the activation of downstream organ identity genes. However, loss of function of *Uni*, a pea *LFY/FLO* homolog, led to development of flowers lacking petals and stamens and

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* Corresponding author; e-mail dluo@sibs.ac.cn; fax 86–021–54924106.

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growth of additional mutant flowers in the axils of the sepals (Hofer et al., 1997). In Arabidopsis and Antirrhinum, *UFO* or *FIM* acts synergistically with *LFY/FLO* (Simon et al., 1994; Ingram et al., 1995; Levin and Meyerowitz, 1995). In *ufo* and *fim* mutants, homeotic transformations of petals to sepals and stamens to carpels were found, as well as some loss of lateral and apical determinacy during floral organ development (Simon et al., 1994; Ingram et al., 1995). Similar phenotypes were observed in the loss-of-function mutants, *stp* and *pfo*, of pea and *Lotus japonicus UFO/FIM* homolog, respectively (Taylor et al., 2001; Zhang et al., 2003). The *pfo* phenotype was strikingly similar to those of *ufo*, *fim*, and *stp* plants, although *pfo* produced many more ectopic flowers. Furthermore, the pea *Uni* and *Stp* genes were also shown to function in compound leaf development (Hofer et al., 1997; Taylor et al., 2001). In both *uni* and *stp* mutant plants, the complexity of compound leaves decreased, suggesting that these orthologs may be recruited to different regulatory pathways and/or may target different biochemical factors in Papilionoideae than in the other model organisms.

The molecular mechanisms controlling floral organ specification tend to be highly conserved between species (Soltis et al., 2002). Based on genetic studies in Arabidopsis and Antirrhinum, the ABC model (Bowman et al., 1991; Coen and Meyerowitz, 1991) has been proposed, in which the combination of three functions specifies floral organ identity. Later, the function E was found to act along with the B and C functions to specify petal, stamen, and carpel identity (Pelaz et al., 2000). Many organ identity genes, which play a central role in these different functions, have been isolated, and most of them encode MADS-domain proteins (Ng and Yanofsky, 2001; Lohmann and Weigel, 2002). In Arabidopsis, *LFY* plays a role in activating these organ identity genes directly (Parcy et al., 1998; Busch et al., 1999; Wagner et al., 1999; Lenhard et al., 2001; Lohmann et al., 2001), and *UFO* acts synergistically with *LFY* to activate B function genes in whorls 2 and 3 (Simon et al., 1994; Ingram et al., 1995; Levin and Meyerowitz, 1995). Several homeotic mutants have been identified in pea and *M. truncatula* (Ferrandiz et al., 1999; Penmetsa and Cook, 2000; Taylor et al., 2002), showing that A, B, and C functions are necessary for floral patterning in legumes.

Although the ABC model is expected to be at least partially valid in legumes, characterization of ABC function orthologs is needed to examine how the ABC model can be used to explain the unique floral patterning in Papilionoideae. Functional genomic studies in the model plant, *L. japonicus* (Handberg and Stougaard, 1992), have taken advantage of its relatively small genome to identify several genes involved in nodulation or floral patterning (for review, see Perry et al., 2003; Somers et al., 2003; VandenBosch and Stacey, 2003). We have used similar approaches to study floral pattern formation in *L. japonicus* as the basis for comparing the floral regulation networks of Arabidopsis and *L. japonicus*. We report here the cloning of 13 homologs of *LFY/FLO*, *UFO/FIM*, and A, B, C, D, and E function genes in *L. japonicus*. Phylogenic studies indicated that duplication of B and C function genes frequently occurs in *L. japonicus*. A mutant, *proliferating floral meristem* (*pfm*), was identified from an ethyl methanesulfonate (EMS) mutant collection and associated with a nonsense mutation in *LjLFY*, and an allele of *proliferating floral organ* (*pfo*) was also found. Moreover, RNA in situ hybridization analysis revealed that the expression patterns of these homeotic genes during *L. japonicus* floral ontogeny differed from those in Arabidopsis and Antirrhinum, indicating molecular explanations for the species-specific differences in floral ontogeny.

RESULTS

Ontogeny of *Gifu* Flowers

Under our growth conditions, plants of *Gifu*, an ecotype of *L. japonicus* (Handberg and Stougaard, 1992), initiated reproductive growth and their shoot apical meristems (SAM) acquired primary inflorescence (I1) meristem identity 40 d post germination. Subsequently, the secondary inflorescence (I2) meristems were produced in the peripheral regions of the shoot apices and 1 to 3 flowers developed in each I2. As described in a previous study on floral ontogeny in *L. japonicus* (Zhang et al., 2003), each flower consisted of 21 concentrically arranged organs: the outermost 5 sepals, then 5 petals and 10 stamens, with a single carpel in the center (Fig. 1, a and b). The five sepals were fused at their bases. The corolla contains three types of petals, one adaxial standard, two lateral wings, and two abaxial keels. Nine stamen filaments were joined into a tube around the carpel, whereas the adaxial stamen was separate. The stamens and carpel were enclosed by the keels, which were fused along their adjacent edges. We have divided the floral development into eight stages according to changes observed in morphology (Fig. 1, c–j).

At Stage 0, while the meristem of the I2 is degenerating, floral primordia are initiated from the peripheral region of I2 and trichomes are formed in the marginal area between the floral primordia (Fig. 1c). At Stage 1, bracts have developed on one side of each floral primordium. This is defined as the abaxial position of a floral primordium, just opposite of the adaxial position where the floral primordia are separated from each other (Fig. 1d). The bract adjacent to a developed floral primordium will degenerate quickly, and only the one adjacent to a degenerated floral primordium will develop fully. At Stage 2, a sepal primordium is formed in the abaxial position of the floral meristem, following the rule of unidirectional initiation order from abaxial to adaxial side (Fig. 1e). At Stage 3, one ellipsoid anlage (or abaxial common primordium, which will give rise to the abaxial petal and stamen primordia at later

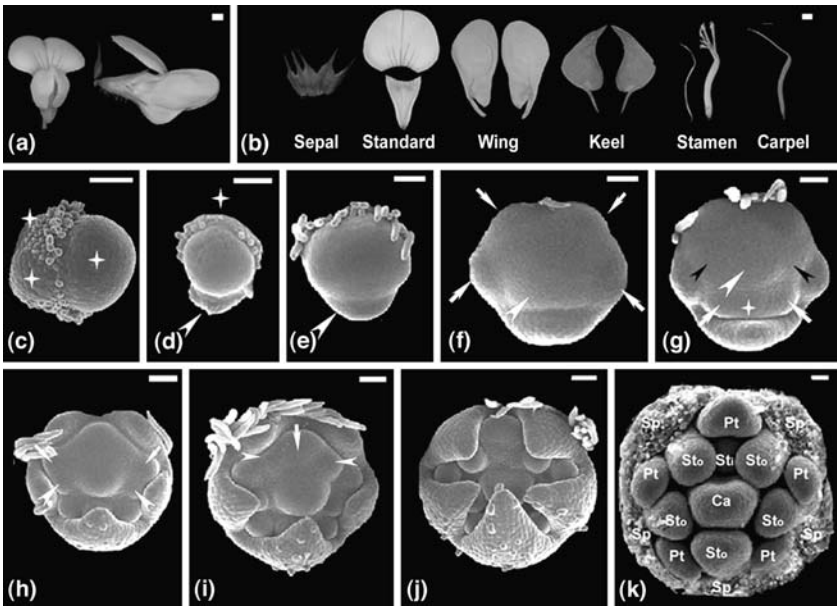

Figure 1. Ontogeny of *Gifu* flower. a, *Gifu* flower; bar = 1 mm. b, Dissection of *Gifu* flower; bar = 1 mm. c to k, SEM showing the floral ontogeny of wild-type *L. japonicus*. Sp, Sepal; Pt, petal; Sti, inner stamen; Sto, outer stamen; Ca, carpel. Bar = 50 μm. The adaxial side of all floral primordia faces up in all micrographs. c, Stage 0, Floral primordia (crosses) are initiated from the I2. d, Stage 1, A bract (arrowhead) develops from floral primordia opposite the adaxial position (cross). e, Stage 2, A sepal primordium (arrowhead) is initiated at the abaxial position of the floral meristem. f, Stage 3, One anlage (common primordium, arrowhead) forms in the axil of the abaxial sepal. The other four sepal primordia (arrow) appear at the lateral and adaxial positions of the floral meristem. g, Stage 4, A carpel primordium (arrowhead) appears in the center of the floral meristem. Two anlagen (black arrowhead) are initiated in the axils of the lateral sepals. Two abaxial petal primordia (keel, arrow) and one abaxial outer stamen primordium (cross) form from the flattened anlage. h, Stage 5, Two lateral petal primordia (wing, arrow) and two outer stamen primordia (arrowhead) form from the two lateral anlagen. i, Stage 6, One adaxial petal primordium (the standard, arrowed) and two outer stamen primordia (arrowhead) are initiated. j, Stage 7, The five inner stamen primordia are initiated. k, After Stage 7 organ primordia begin elongating.

stages) can be recognized in the axil of the abaxial sepal. At the same time, the other four sepal primordia appear at the lateral and adaxial regions of the floral meristem (Fig. 1f). At Stage 4, the carpel primordium appears at the center of the floral meristem. Two ellipsoid anlagen (or lateral common primordia) are initiated in the axils of the lateral sepals, and two abaxial petal (keel) primordia and one abaxial outer stamen primordium develops from the abaxial anlage. However, the shape of the lateral anlagen is less obvious than that of the abaxial ones (Fig. 1g). At Stage 5, two lateral petal (wing) primordia and two outer stamen primordia develop from the two lateral anlagen (Fig. 1h). At Stage 6, one adaxial petal (standard) primordium and two adaxial outer stamen primordia are initiated asynchronously (Fig. 1i). At Stage 7, the five inner stamen primordia are formed; the adaxial-most one has developed asynchronously and will be the only one not forming the fused stamen tube with the other nine stamens (Fig. 1j). After Stage 7, all organ primordia have developed and started elongating (Fig. 1k).

Hence, *L. japonicus* shows some features common to the Papilionoideae, including noncentripetal and unidirectional organ initiation order, a heterogeneous whorl with petal and stamen identity, and an inner whorl of stamens. However, unlike pea and *M. truncatula*, the standard (adaxial petal) and the two adjacent outer stamens were initiated separately in *L. japonicus*, and thus the common primordium in the adaxial position was not observed.

Phenotypic and Molecular Analyses of the *pfm* Mutant

To identify key regulators controlling floral organ identity specification and floral meristem determinacy, we isolated and analyzed several floral mutants induced by EMS mutagenesis. Two mutants, *pfm* and *pfo-2* (described in ''Materials and Methods''), were chosen in this study.

In wild-type plants, the compound leaf located above the sixth node was a complete leaf consisting of five leaflets, two basal leaflets, two lateral leaflets, and one terminal leaflet. In the corresponding positions of *pfm* mutants, about 85% of these complete compound leaves lacked 2 basal leaflets, 14% lacked 1 basal leaflet, and only 1% were normal. In *pfo-2* mutants, the phenotype was different, with about 70% of the complete compound leaves lacking at least 1 leaflet. However, the missing leaflets in *pfo-2* mutants were not contained in the basal position of the compound leaves, and petioles of the compound leaves were frequently missing (data not shown).

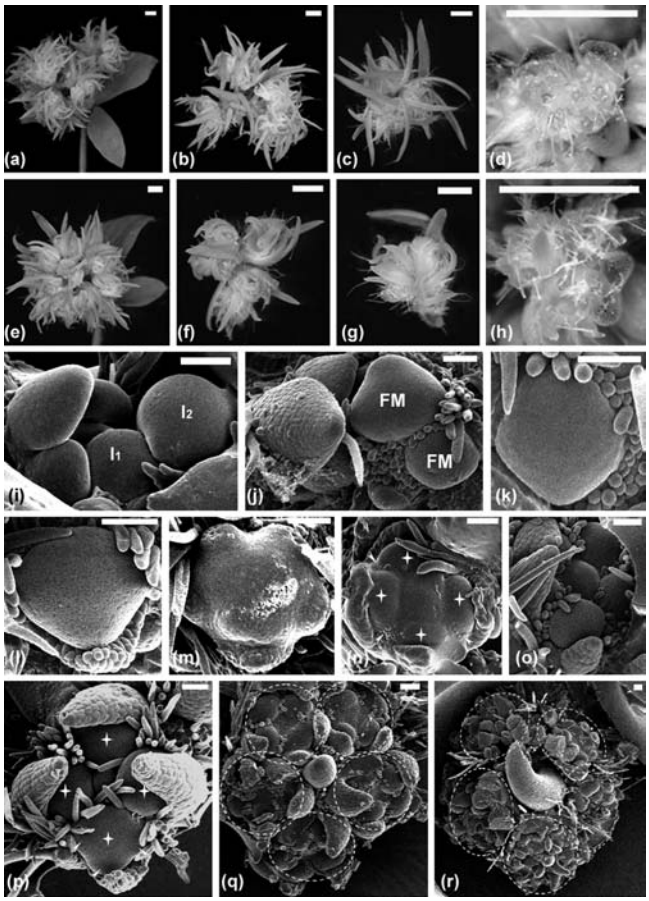

Figure 2. Ontogeny of mutant flowers. a, An inflorescence of *pfm*; bar = 1 mm. b to d, Dissection of a *pfm* mutant inflorescence; bar = 1 mm. e, An inflorescence of *pfo-2*; bar = 1 mm. f to h, Dissection of *pfo-2* mutant inflorescence; bar = 1 mm. i to r, SEM showing the floral ontogeny of the *pfm* mutant; bar = 50 μm. i, The I2 (I_2) initiates from the I1 (I_1). j, Primary floral meristems (FM) initiate progressively from the I2 meristem. k to m, Early stages of *pfm* primary floral development. n, The floral meristem of *pfm* initiates four to five anlagen or primordia (cross) between the sepals and the carpel. Trichomes (arrowhead) appear at the boundaries of the flattened primordia. o, The anlagen contract into ball-like structures. p, Another round of floral-like meristems (cross) form, at the periphery of which another whorl of sepals (arrowhead) initiates. q and r, Repeated pattern of floral-like meristems. Broken rings indicate the successive floral-like meristems.

During the reproductive phase, instead of producing flowers, the I2 in *pfm* plants produced sepal-like proliferating structures (Fig. 2a). These mutant flowers lacked petals and stamens and withered as the plant aged. Dissection of the *pfm* I2 under stereomicroscope revealed a few ball-like structures with pedicels, which are defined here as the primary flowers. The primary flowers each had 5 sepals, which were narrower than those from wild type (Figs. 2b and 1b, respectively). Removal of sepals revealed another round of floral-like structures, which were made up of sepals and a successive ring of floral-like structures (Fig. 2c). The sepals and nested floral-like structures were repeated continuously depending on the growth condition of the plants (Fig. 2d). Basically, the same phenotype was observed in *pfo-2* mutant plants (Fig. 2, e–h).

The phenotypic differences between the wild-type (*Gifu*) and *pfm* flowers were further analyzed by scanning electron microscopy. During the reproductive stage in both plants, the SAM became the I1 meristem, which initiated the I2 primordia at its periphery (Fig. 2i), with the floral meristems initiating progressively from the I2 meristems (Fig. 2j). There were no significant differences observed between *Gifu* floral and *pfm* primary floral development in the early stages (Figs. 1, d–f, and 2, k–m). When the floral meristems of *Gifu* and *pfm* had produced 5 sepal primordia at whorl 1 with a carpel (or a carpel-like) primordium at the center, there were obvious alterations in the mutant plants: trichomes initiated at the boundary of anlagen; the shape of the primary floral meristem was much rounder; the size of two lateral anlagen were obviously larger than the abaxial one; and 3 whorls of organ primordia were observed clearly (Fig. 2n).

Although these alterations indicated the primary floral meristems were developmentally abnormal in the mutants, the arrangement of the primordia in the middle whorl was basically the same as the wild type: three ellipsoid anlagen, one in the abaxial and two at the lateral position, with one or two primordia in the adaxial region (Fig. 2n). These primordia later contracted into four or five ball-like structures (Fig. 2o) and then were transformed into the secondary floral-like meristems (Fig. 2p). From then on, the pattern of floral-like structures was repeated and primordium initiation in the floral-like meristems was not initiated unidirectionally. In the mutants, therefore, numerous iterations of floral-like meristems proliferated in a highly organized manner. The repeated pattern of the typical floral-like meristems and their structures with three whorls (sepals, successive meristems, and carpel; Fig. 2, q and r) normally could be readily recognized for four to five rounds under scanning electron microscopy (SEM). Thus, our analysis of the mutant floral structures could be focused on the primary floral meristems. In a typical floral-like structure, the carpel-like structures produced at the center of the floral-like meristems had the characters of a carpel that cease developing at various stages but always failed to become pistils (data not shown). The SEM analysis confirmed that the phenotypes of *pfm* and *pfo-2* were similar (data not shown).

The phenotype of *pfm* and *pfo-2* was very similar to those of *stp* and *uni* in pea. It has been shown that *stp/pfo* and *uni* are the legume homologs of *UFO* and *LFY*, respectively, in Arabidopsis. Hence, we PCR amplified and sequenced the *L. japonicus* genomic sequences of *UFO* and *LFY* homologs from both the wild type and *pfm* mutants. Two genes, *LjUFO/Pfo* and *LjLFY*, from the wild type were obtained, respectively (see next section of this paper), and were used as the functional homologs, to conduct a comparison with the mutants (see ''Materials and Methods''). The *pfm* and *Gifu* genomic

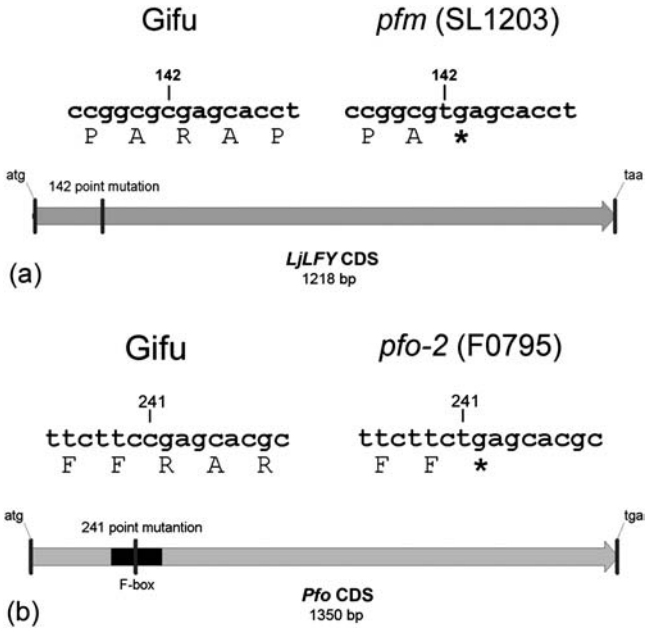

Figure 3. Point mutations in the *LjLFY* and *Pfo* sequences of the *pfm* and *pfo-2* mutants, respectively. a, Schematic representation of the *LjLFY* ORF. Nucleotide and predicted amino acid sequences are shown for the *pfm* mutant and the corresponding region in *Gifu*. b, Schematic representation of the *Pfo* ORF, with the F-box shaded black. Nucleotide and predicted amino acid sequences are shown for the *pfo-2* mutant and the corresponding region in *Gifu*.

sequences showed no differences in the *LjUFO/Pfo* gene, but those for the *LjLFY* gene contained a nonsense mutation in *pfm* at position 142 (cga to tga) that was predicted to truncate the LjLFY protein to 47 amino acids (Fig. 3a), indicating that the protein was nonfunctional in the *pfm* mutant plants, whereas the *LjLFY* sequence was unimpaired in the normal M2 plants whose progeny did not segregate a mutant phenotype.

In *pfo-2* (line F0795), we identified a nonsense mutation at position 241 (cga to tga) causing a truncation of the deduced LjUFO/PFO protein within the putative F-box (Fig. 3b). This point mutation gave rise to a cleaved amplified polymorphic sequence (CAPS) marker that was used to distinguish the *Pfo* alleles in both the *pfo-2* mutant and the wild type (see ''Materials and Methods''). The homozygous point mutation cosegregated with the mutant flower phenotype in the M3 descendents of F0795. However, there was no observable difference in the *LjLFY* genomic sequence between *pfo-2* and *Gifu*. Taken together, these data indicate that we had identified another allele (*pfo-2*) of the *Pfo* gene. We refer to the original *pfo* mutant (Zhang et al., 2003), therefore, as *pfo-1* from here on.

Isolation of Homologs Controlling Floral Patterning in *L. japonicus*

To compare floral development with Arabidopsis and Antirrhinum, we isolated 13 homologs of *LFY/FLO*, *UFO/FIM*, and A, B, C, D, and E function genes from *Gifu*. Sequence similarity comparison of the putative gene products showed that they were highly homologous to their counterparts in Arabidopsis, with 60% to 70% identity and 71% to 85% similarity (Supplemental Table I).

LjLFY consists of three exons that are conserved with *LFY*, *FLO*, and *Uni* in terms of exon size and number. Southern blotting (data not shown) indicated that *L. japonicus* contained only one copy of the *LFY/FLO* homolog. *LjUFO* encodes an F-box protein, and the amino acid sequence is consistent with that of *Pfo* (Zhang et al., 2003). Southern blotting (data not shown) of *LjUFO* and *Pfo* showed the same pattern as each other, suggesting that *LjUFO* is *Pfo*, the *UFO/FIM* ortholog in *L. japonicus*. From here on, therefore, we refer to this gene as *Pfo*.

Most of the A, B, C, D, and E function genes belong to the plant-specific MIKC-type MADS box gene superfamily, which encodes transcriptional regulators with MADS domain, intervening domain, keratin-like domain, and C-terminal domain. We obtained sequences for 11 MIKC-type MADS box genes/fragments from *L. japonicus*. Based on phylogenic analysis and comparison of gene structures, we grouped them into different homologs of A, B, C, D, and E function genes, respectively (Fig. 4a; Supplemental Table I). All of the identified A, B, C, D, and E homologs possessed typical MADS domains (Fig. 4b) due to our primer-designing strategy, but varied to different extents in their C-terminal sequences (Fig. 4c). Two of them, *LjAP1a* and *LjAP1b*, were identified as homologs of *AP1/SQUA* (a floral meristem identity and A function gene) and *CAL*. Like *PEAM4*, the *AP1/SQUA* homolog in pea, *LjAP1a* and *LjAP1b* encode proteins that lack the characteristic C-terminal CaaX motif and are thus unable to be prenylated (Fig. 4c). *LjPIa* and *LjPIb* are homologs of *PI/GLO* (a B function gene) that share 90% similarity through the 170 amino acids at their N termini, including the M, I, and K domains. The C domain of LjPIb is about 30 amino acids shorter than that of LjPIa and other PI/GLO homologs (Fig. 4c). Therefore, LjPIb, but not LjPIa, lacks the canonical PI motif that was shown to be necessary for PI as a B function protein in Arabidopsis (Lamb and Irish, 2003). Analysis of soybean (*Glycine max*) expressed sequence tags (ESTs) in silico showed a similar phenomenon: GmPI2 (deduced from soybean ESTs together with GmPI1) has the PI motif in its C terminus, whereas GmPI1 lacks the PI motif. The *PI/GLO* gene duplication and C-terminal truncation appears to have occurred widely in legumes. The phylogenic tree shown in Figure 4a indicates that the LjAP3-like protein is located at the root of the AP3/DEF (another B function protein) subfamily, while LjAP3, ALFBMP (the AP3 homolog in *Medicago sativa*), and GmAP3 (deduced from soybean ESTs) form a legume clade in the AP3/DEF subfamily. Moreover, LjAP3 contains the PI-derived motif in its C terminus, but the LjAP3-like protein does not. *LjAGa* and *LjAGb* appear to have resulted from a recent duplication in *L. japonicus*; we

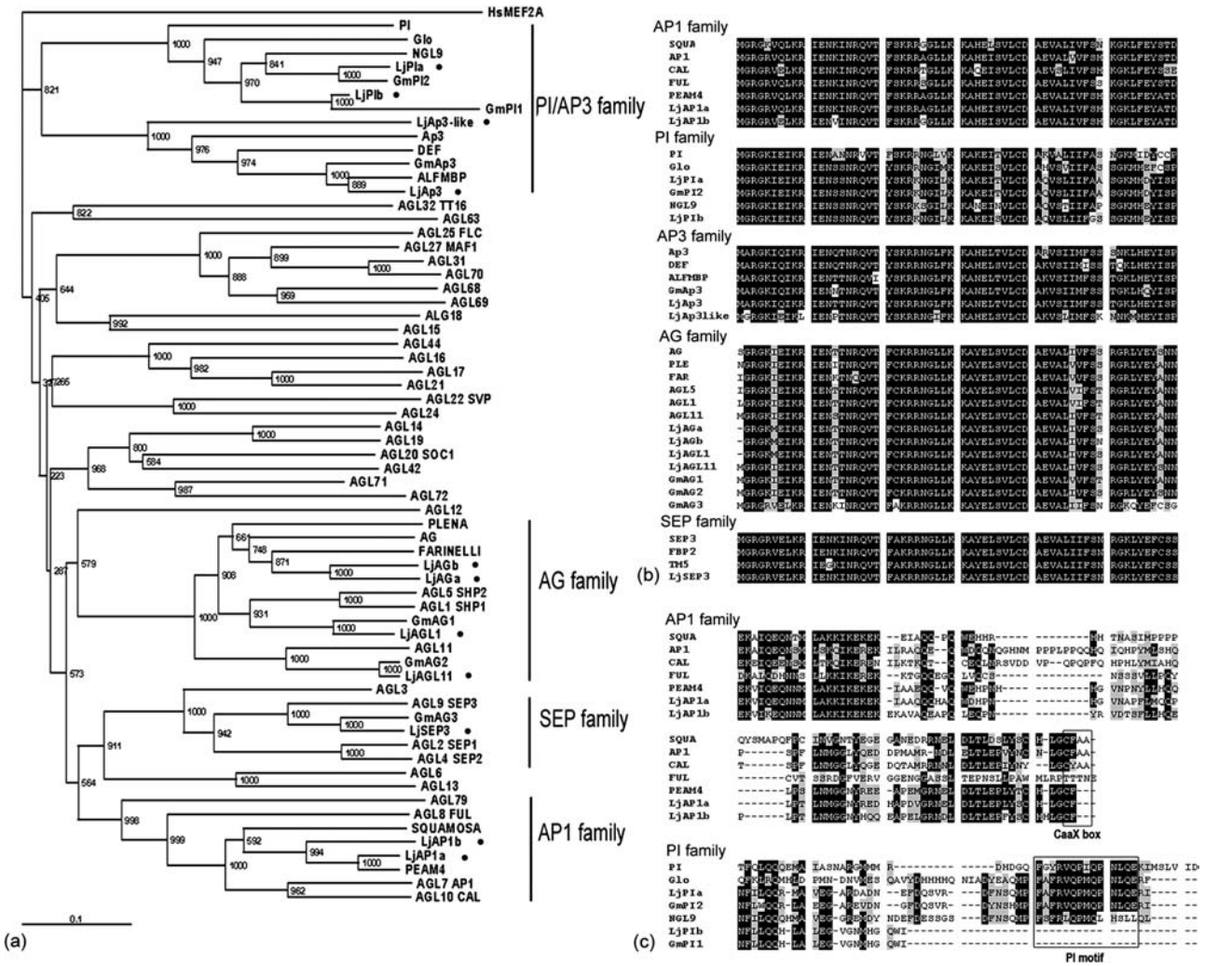

Figure 4. Sequence analysis of ABC function gene homologs in *L. japonicus*. a, A phylogenetic tree generated using neighbor-joining method from the predicted amino acid sequences of genes in the MIKC subfamily of MADS from Arabidopsis, Antirrhinum, pea, *M. sativa*, soybean (for accession no., see Supplemental Table II), and *L. japonicus* (dot). b, Alignment of the predicted MADS domain amino acid sequences from the *AP1*, *PI*, *AP3*, *AG*, and *SEP* subfamilies. c, Alignment of the predicted C-terminal amino acid sequences from the *AP1* and *PI* subfamilies.

found partial genomic sequences for *LjAGa* and *LjAGb* in GenBank (accession nos. AP004549 and AP004519) that contained 5 of their 3′ exons. The exon positions and sizes were conserved among *LjAGa*, *LjAGb*, and *AG/PLE*. A Q-rich motif and extra 20 amino acids were found at the C terminus of LjAGa but not in the AG/PLE protein. Such motifs are considered to belong to transcriptional activators (Tiwari et al., 2003), implying that LjAGa may act as an activator. The sequence of *LjSEP3* was deduced from the *L. japonicus* genomic sequence (accession no. AP004516). Comparisons between the cDNA and genomic sequences revealed that *LjSEP3* had 8 exons conserved in number and size with *SEP3* (an E function gene) from Arabidopsis.

In summary, based on the analysis of 13 homologs of *LFY/FLO*, *UFO/FIM*, and the ABCDE function genes, it appears that gene duplication must have taken place in the *PI/GLO* and *AG/PLE* clades in the *L. japonicus* genome, and some duplication products, such as LjPIb and LjAGa, have lost or gained protein domains that are functional in Arabidopsis.

Expression Patterns of Floral Patterning Genes during Floral Ontogeny in *Gifu* and Mutants

To check the regulatory network on floral patterning in *L. japonicus*, RNA in situ hybridization was conducted to analyze the expression patterns of these genes during floral ontogeny in wild-type and mutant plants. *LjLFY* expression could be detected in the compound leaf primordium (Fig. 5a, subsection 1). When the floral primordium was initiated from the I2, *LjLFY* was highly expressed in the floral anlagen until floral Stage 1, and then the expression pattern shifted to the peripheral region of the floral meristem (Fig. 5a, subsection 2). Later, *LjLFY* was expressed in the in-

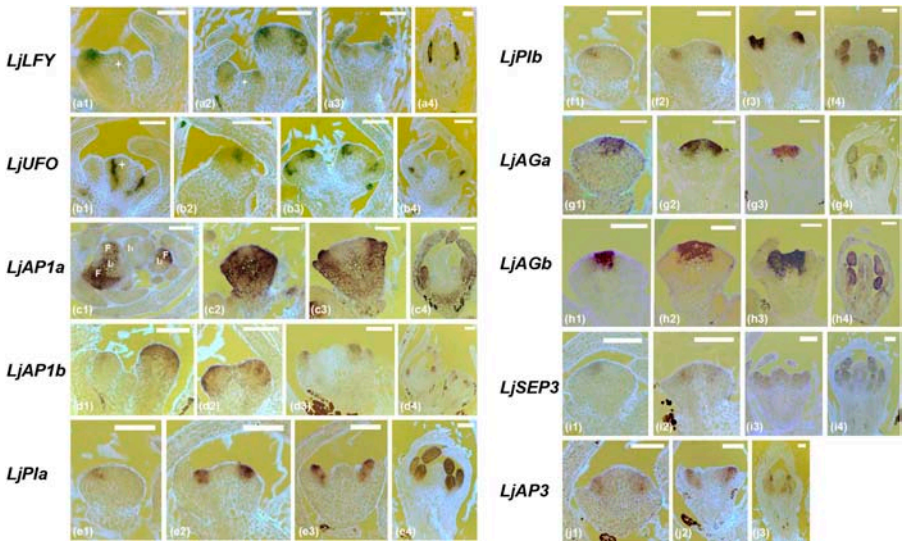

Figure 5. Expression patterns of floral patterning genes in *Gifu*. Probe: a1 to 4, *LjLFY*; b1 to 4, *Pfo*; c1 to 4, *LjAP1a*; d1 to 4, *LjAP1b*; e1 to 4, *LjPla*; f1 to 4, *LjPlb*; g1 to 4, *LjAGa*; h1 to 4, *LjAGb*; i1 to 4, *LjSEP3*; and j1 to 3, *LjAP3*. SAM was labeled as a cross. (c1), Transverse section of a *Gifu* florescence; F, floral primordium; I_1, I1 meristem; I_2, I2 meristem. Bar = 50 μm.

cipient sepals, petals, and stamens (Fig. 5a, subsection 3). After all the floral organs were initiated, expression was confined to the petals (Fig. 5a, subsection 4). In *pfm* mutants, *LjLFY* transcripts were undetectable by RNA in situ hybridization in either the compound leaf or flower (data not shown).

Like *Stp*, the *UFO/FIM* homolog in pea plants (Taylor et al., 2001), *Pfo* was also expressed at the margin between the compound leaf primordium and the SAM (Fig. 5b, subsection 1), and later at the boundary of the leaflets (data not shown). *Pfo* was expressed in the floral meristem with a similar pattern to that of *FIM*, *UFO*, and *Stp* (Simon et al., 1994; Ingram et al., 1995; Taylor et al., 2001). At floral Stage 0, *Pfo* was expressed at the center of the floral meristem; by Stage 1, the expression domain had extended throughout the floral meristem (Fig. 5b, subsection 2). By Stage 2, the transcript had ceased at the center and edge of the floral meristem and retained where the petal and stamen later formed (Fig. 5b, subsection 3). During Stage 3, *Pfo* expression was confined to the base of floral organs (Fig. 5b, subsection 4). Interestingly, *Pfo* was expressed similarly in both wild-type and *pfm* mutant plants. In the primary floral-like meristems, *Pfo* expression appeared first at the center of the meristem, then changed to be expressed in the peripheral region (Fig. 6h) and eventually formed a ring. This pattern was repeated in the secondary floral-like meristem (Fig. 6i). Almost the same expression pattern was seen for *Pfo* in *pfo-2* mutant plants (Fig. 6, q and r). Moreover, RNA in situ hybridization showed consistent results between *pfo-1* and *pfo-2* plants.

In a similar manner to those of *LjLFY*, transcripts of *LjAP1a* were first detected in a group of cells in the I2s (Fig. 5c, subsection 1) where the first floral anlage formed. Thereafter, transcripts were confined to the next floral anlagen. During Stages 1 to 3, the transcripts gradually decreased in the center of the floral

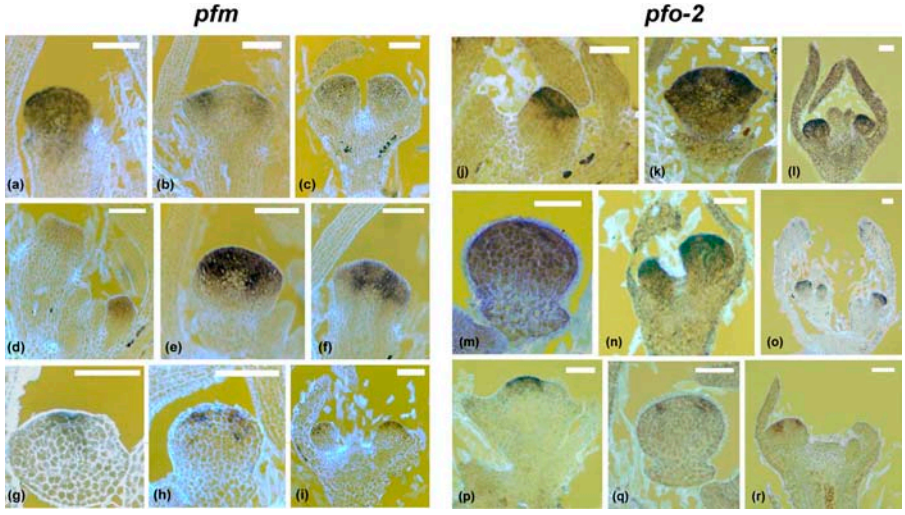

Figure 6. Expression patterns of floral patterning genes in *pfm* and *pfo-2*. a to i, *pfm* and j to r, *pfo-2*. a to c and j to m, *LjAP1a* probe; d to f, n, and o, *LjAP1b* probe; g and p, *LjAGa* probe; and h, i, q, and r, *Pfo* probe. Bar = 50 μm.

meristem (Fig. 5c, subsection 2). At Stage 4, *LjAP1a* was expressed in the sepals and petals, but was undetectable in the outer and inner stamens (Fig. 5c, subsection 3). This pattern was maintained until the flower matured (Fig. 5c, subsection 4). *LjAp1b* showed a similar expression pattern to that of *LjAP1a* (Fig. 5d, subsection 1–4), although its signal was always weaker in sepals and petals. However, the expression patterns of *LjAP1a* and *LjAP1b* were altered in *pfm* mutant plants. The expression of *LjAP1a* and *LjAP1b* in *pfm* I2s was similar to that in *Gifu* (Fig. 6d). When abaxial sepal primordium was initiated in the primary floral meristem, *LjAP1a* and *LjAP1b* were expressed throughout the floral meristem (Fig. 6, a and e). Later on, *LjAP1a* and *LjAP1b* were ectopically expressed in the region next to the sepal primordia, where another round of floral-like meristems would form, whereas their expression decreased in the sepal and carpel primordia (Fig. 6, b and f). The expression of *LjAP1a* and *LjAP1b* was maintained in the primary floral meristem until the formation of successive rings of floral-like meristems (Fig. 6c). The expression patterns of *LjAP1a* and *LjAPb* in *pfo-2* mutant plants were similar to those in *pfm*. *LjAP1a* and *LjAP1b* were expressed in the *pfo-2* I2, similar to the expression pattern in wild type (Fig. 6, j and n). Later, *LjAP1a* and *LjAP1b* were expressed throughout the primary floral meristem (Fig. 6, k and n), and they were ectopically expressed next to the sepal primordia (Fig. 6, l and o) where the secondary floral-like meristems initiated, whereas their expression decreased in the sepal and carpel primordia.

Interestingly, *LjPIa* was expressed in a unidirectional pattern over time. At Stage 2, *LjPIa* transcripts first appeared at the abaxial side of the floral meristem, covering an area 2 to 3 cells wide (Fig. 5e, subsection 1) where abaxial anlage would form. By Stage 3, the expression pattern had shifted into the region where lateral anlagen would form (Fig. 5e, subsections 2 and 3). *LjPIa* was consistently expressed in petals and stamens throughout flower development (Fig. 5e, subsection 4). *LjPIb* displayed almost the same expression pattern as *LjPIa* (Fig. 5f, subsections 1–4). *LjAP3* was first expressed at Stage 4 between the sepals and the carpel (Fig. 5j, subsection 1), where the petals and stamens would form. Thereafter, the *LjAP3* transcripts were found strictly within the petals and stamens as the flower matured (Fig. 5j, subsections 2 and 3). Expression of the 3 B function orthologs, *LjPIa*, *LjPIb*, and *LjAP3*, were not detected in either *pfm* or *pfo-2* mutant plants (data not shown). This is consistent with the absence of petals and stamens in *pfm* and *pfo-2* mutants, indicating that their expression was linked to the identity of petals and stamens in *L. japonicus*.

LjAGa and *LjAGb* were expressed in a similar pattern during floral ontogeny. Unlike the C function gene *AG* or *PLE* in Arabidopsis and Antirrhinum, these genes were first expressed at Stage 2, in the center of the floral meristem in an area of 5 to 6 cells (Fig. 5g, subsection 1, and 5h, subsection 1). At Stage 3, the expression domains of both genes extended toward a position where the inner and outer stamen primordia would form and decreased at the center where the carpel primordium had initiated (Fig. 5g, subsections 2 and 3, and 5h, subsections 2 and 3). In the heterogeneous whorl, *LjAGa*, *LjAGb*, and *LjAP1a*, *LjAP1b* displayed complementary expression patterns: *LjAGa* and *LjAGb* were expressed only where the outer stamen primordia would form, whereas *LjAP1a* and *LjAP1b* were expressed only where the petal primordia would form (Fig. 7). *LjAGa* and *LjAGb* were continually expressed in the outer and inner stamens until the flower matured (Fig. 5g, subsection 4, and 5h, subsection 4). In the *pfm* and *pfo-2*, *LjAGa* was transiently expressed at the center of the primary floral meristem at the Stage 2 (Fig. 6, g and p) at the time when the carpel-like primordia were initiated, but then its expression subsequently disappeared. Our observations that *LjAP1a/LjAP1b* and *LjAGa* were expressed in a complementary manner in all the *Gifu*, *pfm*, and *pfo-2* plants, indicates the antagonistic relationship between the A and C function genes and further supports that the identity conferred at the center of the floral-like meristems in the mutants was carpel like.

Like *LjPIa* and *LjPIb*, expression of *LjSEP3* was first observed at Stage 2 in an area 4 to five cells wide near the abaxial side of the floral meristem (Fig. 5I, subsection 1) where the abaxial anlage would be formed later. At Stage 4, its expression disappeared from the carpel primordium but was maintained in the petals and stamens until the flower matured (Fig. 5I, sub-

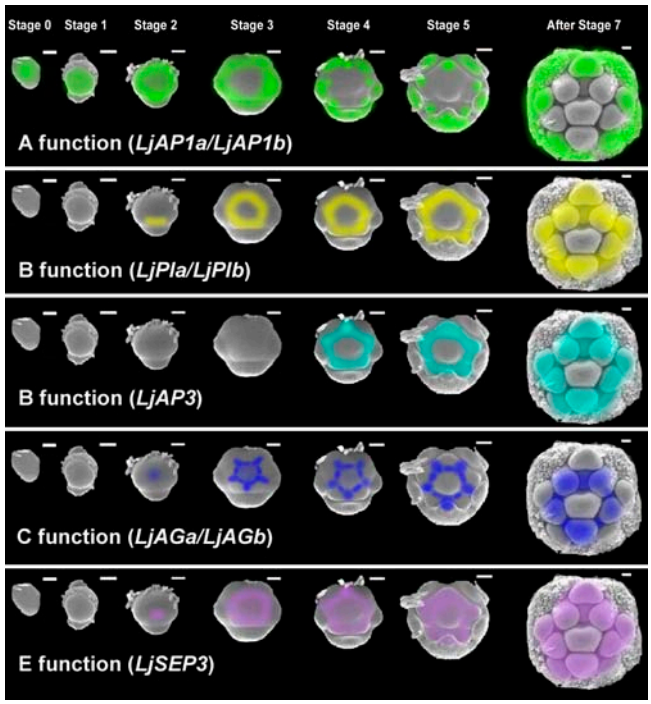

Figure 7. Summary of the expression patterns of A, B, C, and E function genes during floral ontogenesis. The false colorings superimposed on SEM of the SAM indicate the expression patterns of different function genes. Bar = 50 μm.

sections 2–4). The transcripts of this *SEP* homolog were not detected in either *pfm* or *pfo-2* (data not shown), suggesting that *LjLFY* and *Pfo* were also necessary for the transcription of this *SEP* homolog.

DISCUSSION

Floral Ontogeny in *L. japonicus*

Initiation of *Gifu* petals and stamens was reported to occur individually rather than from common primordia, as seen in pea plants (Zhang et al., 2003). Consistently, we found that the initiation of petals and stamens occurred independently at the adaxial region of floral meristems. We also found, however, that the abaxial and lateral anlagen gave rise to both petal and stamen primordia (Fig. 1, f–h), suggesting that common primordia do exist at these positions. Tucker (1989, 2003) noted that the common primordium development in pea might represent an evolutionary specialization as an extreme case of temporal overlap of petal and stamen initiation. According to our results, this overlap should be suppressed in the adaxial region, but should occur in the abaxial and lateral regions when the heterogeneous whorl formed in *L. japonicus*. The petal and stamen ontogeny in *L. japonicus*, therefore, can be considered an intermediate between that of pea or *M. truncatula* and that of other species in Papilionoideae, where petals and stamens arise directly without a common primordia stage (Tucker, 2003). This suggests that the appearance of common primordia could be variable among papilionoids. It is reasonable to speculate that there could be an interaction between the mechanism controlling the adaxial-abaxial identity and the one controlling organ identities, which contributes to the initiation timing, position, and identities of floral organs. The differences between *L. japonicus* and pea/*M. truncatula* indicate the divergence in the mechanisms controlling the unidirectional floral organ initiation in Papilionoideae.

LjLFY and Pfo Function

We identified a point mutation in the *LjLFY* sequence, which should cause a truncation of the LjLFY protein and down-regulation of its mRNA in *pfm* mutant. It is expected that the genetic background of *pfm* contains more mutations, which we were not able to explore due to the loss of heterozygous plant in the small M2 family we obtained. We are currently conducting an experiment using 35S::*LjLFY* to complement *pfm* mutant plants to demonstrate conclusively that the *LjLFY* loss-of-function mutation alone is responsible for the *pfm* phenotype. However, we believe that the floral meristem proliferation and leaflet loss from compound leaves in the main would be caused by *LjLFY* loss-of-function, because of the similarities between this mutant phenotype and that of *uni*, the equivalent mutant in pea. We also identified an allele at *Pfo*, the *UFO/FIM* ortholog in *L. japonicus*. In both *pfm* and *pfo* mutant plants, floral meristem defects are similar, having three consequences: a reduction in whorl numbers (only three whorls in a *pfm*/*pfo* primary flower, in contrast to four whorls in wild-type flower), a failure of petal and stamen initiation, and a loss of floral determinacy. Consistent with the functional analysis of *Uni* and *Stp* (Hofer et al., 1997; Taylor et al., 2001), our data supports the idea that the *LFY* and *UFO* orthologs must play an indispensable role in the floral pattern formation in Papilionoideae.

LjLFY is functionally divergent from its homolog in Arabidopsis with respect to the determination of the floral meristem identity and activation of the floral organ identity genes. Shoot-like structures replaced flowers in the early stages of floral development of Arabidopsis *lfy* plants (Weigel et al., 1992), whereas in *pfm* plants, the I2 meristem could produce floral meristems with sepal and carpel primordia without a functional *LjLFY* gene. There are at least 2 explanations for this: (1) *LjLFY* is unnecessary for the formation of the floral meristem, or (2) some other components are involved, such as *AP1* homologs, but there is redundancy between them. In *pfm*, *LjAP1a* and *LjAP1b* are ectopically expressed in the floral meristem, and one of the *TFL1/CEN* (inflorescence identity genes in Arabidopsis and Antirrhinum) homologs, *LjCEN1* was expressed normally (Guo et al., 2004; data not shown), indicating that regulation among *LFY*, *TFL1*, and *AP1* homologs (Ratcliffe et al.,1999; Hempel et al., 2000) in *L. japonicus* differs from that in Arabidopsis. Furthermore, we found that 3 B and 1 E function genes were not expressed in *pfm* at detectable levels, whereas *LjAP1a/LjAP1b* were ectopically expressed and *LjAGa/LjAGb* expression was down-regulated in a spatially specific manner. These data suggest that *LjLFY* may be required for the activation of B function genes in a similar manner to *LFY* in Arabidopsis (Parcy et al., 1998), but, unlike *LFY*, it is not necessary for the initiation of A and C function genes. *LjLFY* may be required, however, for maintenance of their correct expression patterns and levels.

In *L. japonicus*, *pfm* and *pfo* mutants give rise to repetitive floral meristems between sepals and carpel. However, the determinacy in *L. japonicus* may still require the C function since (1) *LjAGa* and *LjAGb* maintain their expression in the outer and inner stamen primordia during carpel formation, (2) in *petalous*, the pea C function loss mutant, floral meristem determination is lost, and (3) *LjAGa* was found to be down-regulated in *pfo* and *pfm* where an indeterminate ectopic floral meristem was produced. It has been observed that ectopic flowers could be produced to different extents in *fim* mutant of Antirrhinum, *stp* of pea, and *pfo* of *L. japonicus*, respectively, and these differences could be truly species specific (Zhang et al., 2003). The very stable indeterminacy in both *pfm* and *pfo*, showing a highly regulated repetitive pattern of floral meristems, is in contrast to the phenotype of *lfy* and *ufo* mutants in Arabidopsis, where only some loss of lateral and apical determinacy during floral organ

development was observed in *ufo* (Simon et al., 1994; Ingram et al., 1995). This difference is probably due to the functional differences between both *Uni/LjLFY* and *Stp/Pfo* in the two species.

ABC Function Homologs and Floral Patterning

We found duplications in B and C function genes of the *PI/GLO* and *AG/PLE* subfamilies in the *L. japonicus* genome. A recent study in petunia showed that duplicated B function genes played distinct roles in whorls 2 and 3 (Vandenbussche et al., 2004). We have showed the C-terminal PI domain was lacking in LjPIb. This suggests an alteration of the function of a *PI/GLO* gene following the duplication, since in Arabidopsis, PI proteins lacking the PI domain are unable to rescue *pi* mutants (Lamb and Irish, 2003). Further work to mutate *LjPIb*, therefore, will be necessary to explain its function fully. Similarly, we expect that *LjAGa* with two extra domains compared to *LjAGb* and other *AG/PLE* genes could be functionally different. *LjAGa* and *LjAGb* may have evolved from a recent gene duplication in *L. japonicus*, and the functional analysis of the extra domains might elucidate the role of *LjAGa* in specifying organgenesis in *L. japonicus*.

It is well recognized that the ABC function genes establish specific expression domains for floral patterning. We summarize the expression patterns of *L. japonicus* homologs of ABC function genes in Figure 7. In *L. japonicus*, sepal, carpel, and common primordia were initiated at Stages 3 and 4. *LjAGa* and *LjAGb* were expressed no later than the B function genes at Stage 2, potentially allowing the carpel primordium to be initiated earlier than the petal and stamen primordia. The expression of *LjAGa* and *LjAGb* weaken in the carpel primordium after Stage 2, further indicating that the determination of carpel identity could occur earlier and more quickly in *L. japonicus* than in Arabidopsis and Antirrhinum. Moreover, *LjAGa* and *LjAGb* are expressed in both whorls 2 and 3 beginning at Stage 3, leading to the development of 2 whorls of stamens. In whorl 2, *LjAP1a/LjAPb* and *LjAGa/LjAGb* are expressed in a complementary manner to each other, which is consistent with the heterogeneous nature of whorl 2. Finally, the unidirectional activation of *LjPIa*, *LjPIb*, and *LjSEP3* from the abaxial to adaxial sides is consistent with the observed initiation of petal and stamen primordia in the same direction.

CONCLUSION

In accordance with previous studies, we found that the molecular factors controlling floral organ development are well conserved between *L. japonicus*, Antirrhinum, and Arabidopsis. We have proposed, however, that four processes may contribute to the unique floral patterning in *L. japonicus*: alteration of the function of key genes, gene duplication, loss or gain of functional protein domains, and changes in gene expression patterns. These processes form the molecular basis for the specific order of initiation, the number of and the position of the petals and stamens during the floral ontogeny among Papilionoideae.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Lotus japonicus ecotype *Gifu* (Handberg and Stougaard, 1992) was used in this study. All plants were grown at 20°C to 22°C with a 16-h-light/8-h-dark photoperiod at 150 mE m^{-2} s^{-1}.

The *pfm* mutant was isolated from an EMS-mutagenized M2 population from The Sainsbury Laboratory and John Innes Center (Perry et al., 2003). This line, designated SL1203, contained 3 siblings in the M2 generation. One member (SL1203-1) displayed wild-type phenotype containing homozygous wild-type *LjLFY* allele, which did not segregate any mutants with floral or leaf phenotypes at the M3 generation (135 progenies). Another member (SL1203-2) displayed dwarf phenotype and was infertile. In contrast, SL1203-3 showed stable floral defects and a reduced compound leaflet number. SL1203-3 was propagated and maintained by cutting, and the resultant cuttings displayed phenotypes consistent with that of the original plant. Samples used for SEM, RNA in situ hybridization, and DNA analysis were harvested from cuttings.

The *pfo-2* mutant was isolated from a separate EMS-mutagenized M2 population (n = 4,000) generated in Shanghai. The mutant line was designated F0795; there were 11 siblings in the M2 line, designated F0795-1 to F0795-11. F0795-1 and F0795-2 showed phenotypes similar to that of SL1203-3 (above). Samples used for RNA in situ hybridization and DNA analysis were harvested from F0795-1, F0795-2, and mutant plants segregating in the M3 generations from the heterozygous plant (F0795-11) for *pfo-2*.

The *pfo* (renamed *pfo-1* in this paper) mutant plants were kindly provided by Dr. Pierre R. Fobert (National Research Council Canada).

SEM

Scanning electron micrographs were prepared according to the methods described by Green and Linstead (1990). Flower inflorescences were collected and leaves were removed as necessary. Plastic replicas were made and coated with gold palladium in an E-1010 ion sputter. SEM was performed with a Hitachi S-2460 scanning electron microscope (Hitachi, Tokyo) at 15 KV. SEM photographs were captured electronically and processed with the Adobe Photoshop 6.0 software (Adobe Systems, Mountain View, CA).

Isolation of cDNA and Sequence Analysis

Inflorescences with different stage flowers were collected from *Gifu* plants, and total RNA was prepared using the RNeasy Plant Midi kit (Qiagen, Valencia, CA). Total RNA (10 μg) was used for first-strand cDNA synthesis using AMV transcriptase (Promega, Madison, WI) primed with the B_{26} (5′-GACTCGAGTCGACATCGT$_{17}$-3′ = B_{25}+T_{17}) adapter.

The cloning of 13 homologs of *LFY/FLO*, *UFO/FIM*, and the ABCDE function genes was conducted by the reverse transcription-PCR and cDNA or genomic library screening (for details, see Supplemental Table I). The PCR products were cloned into the pGEM-T easy vector (Promega) and sequenced. Sequences were analyzed using the Vector NTI v.6.0.0.0 and homologous alignments were performed using Bioedit v.5.0.9. Amino acid alignments, including M, I, K, and C domains were used to obtain the phylogenetic with the neighbor-joining ClustalX program (version 1.83, February 2003).

Primers SL0805 (462 nucleotides upstream of the putative start codon) and SL0806 (198 nucleotides downstream of the putative stop codon) were designed according to the genomic sequence and used to PCR amplify the *LjUFO/Pfo* genomic fragment from mutant plants SL1203-1, SL1203-3, F0795-1, and F0795-2. The resulting PCR products were sequenced, and the identified point mutation gave rise to a CAPS marker and was subsequently used for the genetic linkage assay. The *LjLFY* genomic fragment was amplified from *Gifu* genomic DNA using degenerate primers SL0799 and SL0800. The genomic DNA fragment was additionally isolated 3 independent times from SL1203-3 cuttings as well as the *pfo*-2 mutant plants. Sequencing of these PCR products showed point mutation consistently. Simultaneously, *LjLFY* from the normally flowerings plant SL1203-1 showed unimpaired at the same site.

RNA in Situ Hybridization

RNA in situ hybridization with digoxigenin-labeled sense and antisense probes was performed on 8-μm sections of *Gifu*, *pfo-2*, and *pfm* flowering apices, as described by Coen et al. (1990). *LjLFY* transcripts were generated from a cDNA fragment corresponding to nucleotides 180 to 1,127 of the coding sequence, and transcripts of the ABCDE gene homologs were generated from cDNA fragments lacking the MADS boxes, so as to prevent cross-hybridization. Sections of both wild-type and mutant plants were placed on the same slide, which was hybridized and detected under the same conditions.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY770393 to AY770405.

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