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The *ASK1* gene regulates B function gene expression in cooperation with *UFO* and *LEAFY* in *Arabidopsis*

Dazhong Zhao[1], Qilu Yu[1,2], Min Chen[1] and Hong Ma[1,2,*]

[1]Department of Biology and the Life Sciences Consortium, 504 Wartik Laboratory, and [2]The Intercollegiate Graduate Program in Plant Physiology, Pennsylvania State University, University Park, PA 16802, USA
*Author of correspondence (e-mail: hxm16@psu.edu)

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SUMMARY

The *Arabidopsis* floral regulatory genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) are required for the B function according to the ABC model for floral organ identity. *AP3* and *PI* expression are positively regulated by the *LEAFY* (*LFY*) and *UNUSUAL FLORAL ORGANS* (*UFO*) genes. *UFO* encodes an F-box protein, and we have shown previously that *UFO* genetically interacts with the *ASK1* gene encoding a SKP1 homologue; both the F-box containing protein and SKP1 are subunits of ubiquitin ligases. We show here that the *ask1-1* mutation can enhance the floral phenotypes of weak *lfy* and *ap3* mutants; therefore, like *UFO, ASK1* also interacts with *LFY* and *AP3* genetically. Furthermore, our results from RNA in situ hybridizations indicate that *ASK1* regulates early *AP3* and *PI* expression. These results support the idea that UFO and ASK1 together positively regulate *AP3* and *PI* expression. We propose that the UFO and ASK1 proteins are components of a ubiquitin ligase that mediates the proteolysis of a repressor of *AP3* and *PI* expression. Our genetic studies also indicate that *ASK1* and *UFO* play a role in regulating the number of floral organ primordia, and we discuss possible mechanisms for such a regulation.

Key words: *ASK1*, Floral organ identity, *LEAFY*, *AP3*, *PI*, Gene regulation

INTRODUCTION

Genetic and molecular studies in *Arabidopsis* and *Antirrhinum* have led to the proposal of the ABC model for control of floral organ identity (Haughn and Somerville, 1988; Coen and Meyerowitz, 1991; Meyerowitz et al., 1991; Ma, 1994; Weigel and Meyerowitz, 1994; Yanofsky, 1995; Ma and dePamphilis, 2000). Particularly, the *Arabidopsis* B function genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) are required to specify petal and stamen identities (Bowman et al., 1989; Hill and Lord, 1989; Jack et al., 1992; Goto and Meyerowitz, 1994). Both *AP3* and *PI* are expressed in specific regions of the floral meristem prior to the initiation of petal and stamen primordia (Jack et al., 1992; Goto and Meyerowitz, 1994) at stage 3 of *Arabidopsis* flower development (Smyth et al., 1990). The stable spatial pattern of *AP3* and *PI* expression is directly correlated with the control of organ identity, as further supported by the fact that ectopic expression of both *AP3* and *PI* leads to the formation of ectopic petals and stamens (Jack et al., 1994; Krizek and Meyerowitz, 1996).

The *Arabidopsis* floral meristem identity gene *LEAFY* (*LFY*) is required for normal levels of *AP3* and *PI* expression (Weigel and Meyerowitz, 1993), consistent with the lack of petals and stamens in severe *lfy* mutants (Schultz and Haughn, 1991; Huala and Sussex, 1992; Weigel et al., 1992). However, flowers of weak *lfy* mutants, such as *lfy-5*, can still produce petals and stamens. Another *Arabidopsis* gene, *UNUSUAL FLORAL ORGANS* (*UFO*), also plays a role in controlling floral meristem development and the B function (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995; Samach et al., 1999). Moreover, the activation of *AP3* expression by *LFY* requires *UFO* (Lee et al., 1997; Parcy et al., 1998), although how *UFO* interacts with *LFY* is not known. The *SUPERMAN* (*SUP*, or *FLO10*) gene can also regulate B function in *Arabidopsis* and is expressed shortly after the onset of *AP3* and *PI* expression (Schultz et al., 1991; Bowman et al., 1992; Sakai et al., 1995; Jacobsen and Meyerowitz, 1997; Sakai et al., 2000). In addition, *AP3* and *PI* are expressed ectopically in the *sup* mutant floral meristems (Bowman et al., 1992; Sakai et al., 1995). These observations led to the hypothesis that *SUP* acts to maintain the boundary between whorl 3 and 4, possibly by controlling differential cell division in different domains of the floral meristems (Sakai et al., 1995; Sakai et al., 2000).

The cell division cycle is regulated by both the synthesis and degradation of key regulatory proteins. Proteolysis is essential for many normal cellular functions, but its role in plant development is not clear. A major pathway for protein degradation is the ubiquitin-dependent pathway by the 26S proteosome (Ciechanover et al., 2000). Ubiquitin is a highly conserved small protein that is covalently attached to proteins through a three-step process requiring the E1, E2, and E3 enzymes (Jentsch and Pyrowolakis, 2000). Whereas the E1 and E2 enzymes are rather non-specific, the E3 ubiquitin ligase confers substrate specificity. The SCF E3 ubiquitin ligase

complexes are named after the three subunits: SKP1, cullin (CDC53 in yeast), and one of the F-box containing proteins, which are the substrate specificity factors (Feldman et al., 1997; Skowyra et al., 1997; Peters, 1998; Craig and Tyers, 1999). The yeast *SKP1* gene is essential for the mitotic cell cycle (Bai et al., 1996; Connelly and Hieter, 1996).

The *Arabidopsis* UFO protein (Ingram et al., 1995) contains an F-box, suggesting that it may be a subunit of a SCF ubiquitin ligase. Furthermore, UFO and its *Antirrhinum* homologue FIM have been found using yeast two-hybrid assays to interact with homologues of the yeast and human SKP1 proteins, including the *Arabidopsis ASK1* gene product (Ingram et al., 1997; Samach et al., 1999). *ASK1* was shown to be expressed in dividing cells, including meristems and floral organ primordia (Porat et al., 1998), consistent with a potential role in cell division. We have previously isolated a male-sterile transposon insertion, *ask1-1*, in the *ASK1* gene (Yang et al., 1999). The *ask1-1* mutant also has mild defects during vegetative and reproductive development (Zhao et al., 1999). Furthermore, some *ask1-1* flowers exhibit abnormality in petals and stamens, including reduced number and size of petals and reduced stamen filament lengths, suggesting a weak defect in B function (Zhao et al., 1999). We further showed that *ASK1* and *UFO* interact genetically with each other, consistent with the observed interaction using the yeast two-hybrid method (Samach et al., 1999). These results support the hypothesis that UFO and ASK1 may be subunits of a SCF ubiquitin ligase required for normal *Arabidopsis* flower development, particular for regulating B functions.

To further investigate the function of *ASK1*, we have constructed additional double and triple mutants between *ask1* and other mutations, including *ufo*, *ap3*, *pi*, *sup*, and *lfy*. Our results support the idea that ASK1 interacts with UFO to regulate B function genes *AP3* and *PI*. To more directly test this idea, we have performed RNA in situ hybridization experiments and found that indeed *ask1* mutation can cause a reduction of *AP3* and *PI* expression when *LFY* gene function is reduced by a weak mutation. We propose that ASK1 and UFO together control *AP3* and *PI* expression via a negative regulator of these genes. In addition, we describe results indicating a role for *ASK1* in regulating the number of floral organ primordia, and discuss their implications.

MATERIALS AND METHODS

Plant materials and growth conditions

The wild type and mutants used were in the Landsberg *erecta* (L*er*) backrgound. The *ask1-1* mutant was isolated as a male sterile mutant and it has a *Ds* transposon insertion in the middle of the protein-coding region upstream of a highly conserved domain (Yang et al., 1999; Zhao et al., 1999). The other mutants have been described previously: *ap3-1* and *pi-1* (Bowman et al., 1989), *ap3-3* (Jack et al., 1992), *lfy-5* and *lfy-6* (Weigel et al., 1992), *ufo-2* and *ufo-6* (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995), *sup-1* (Bowman et al., 1992). Seeds were sown onto Metro-Mix 360 (Scotts-Sierra Horticultural Products Co., Maryville, OH), incubated for 4 days at 4°C and then grown at 23°C with long-day cycles (16 hours light and 8 hours dark).

Construction of double and triple mutants

All single mutants used for phenotypic comparison were derived from self-pollination of either homozygous (e.g., *ufo-2*) or heterozygous (e.g., *ap3-3*/+) plants. To construct double and triple mutants, the male sterile and female fertile *ask1-1* mutant (Yang et al., 1999) was used as the female in crosses whenever possible. For crosses with *ap3-1*, *ap3-3*, or *pi-1*, pollen from *ask1-1*/+ heterozygous plants was used. We had previously generated partially fertile *ufo-2*/*ufo-2 ask1-1*/+ plants (Zhao et al., 1999), which were used as male for crosses to generate triple mutants with *ap3-3* and *pi-1* mutations. In addition, *ufo-2*/*ufo-2 ask1-1*/*ask1-1* plants were pollinated with pollen from *sup-1*, *lfy-5*, or *lfy-6*/+ plants to generate the *sup-1 ufo-2*, *lfy-5 ufo-2*, and *lfy-6 ufo-2* double mutants and the triple mutants. The *ask1-1 Ds* insertion confers kanamycin resistance, allowing the selection on MS kanamycin plates for double heterozygous F_1 plants from crosses using *ask1-1*/+ plants. For crosses with *lfy-6*/+, F_2 seeds from multiple F_1 plants were harvested and tested for segregation of each relevant single mutants. All F_1 plants that were doubly or triply heterozygous were normal.

The *ask1-1* mutant has a shorter stature than normal (Zhao et al., 1999); this characteristic is unique among the mutants studied here and was used to identify candidate *ask1-1* homozygous plants. The *ask1-1* mutant has many morphologically normal flowers and can be easily distinguished from the *ap3-1*, *ap3-3*, *pi-1*, *sup-1*, and *lfy-6* mutants; furthermore, *sup-1* is male fertile but usually female sterile. The *ufo-2*, *ufo-6*, and *lfy-5* mutants also have mild floral phenotypes, but are male and female fertile, are of normal height and lack normal flowers, unlike *ask1-1* plants. Therefore, all known single floral mutants can be distinguished from *ask1-1* based on a combination of plant stature, floral morphology and fertility. Furthermore, the *ASK1* allele was confirmed by a PCR product using the *ASK1* gene-specific primers oMC221 (5′-AAG GTG ATC GAG TAT TGC AAG AG-3′) and oMC 383 (5′-GAA GAT AGT CAT GAT TCA TGA AG-3′); the *ask1-1* mutant allele was verified by the oMC221 primer and the *Ds*-specific primer Ds 5-2 (5′-CGT TCC GTT TTC GTT TTT TAC C-3′).

The double mutants with *ask1-1* and another mutation were identified using phenotypes and PCR tests for either *ASK1* or *ask1-1* alleles. For example, among the F_2 plants from the cross between *ap3-3* and the *ask1-1*/+ heterozygote, in addition to the *ask1-1* and *ap3-3* single mutants, a rare class of mutants produced *ap3-3* like flowers and was as short as *ask1-1* single mutant. These candidate double mutant plants were confirmed to be homozygous for the *ask1-1* allele by PCR. The *pi-1 ask1-1* and *lfy-6 ask1-1* double mutants were similarly identified. The *ap3-1 ask1-1* and *lfy-5 ask1-1* double mutants were more easily recognized because they had more severe floral phenotypes than either single mutant of the respective crosses. Additional *lfy-5 ask1-1* plants were obtained from progeny of *lfy-5*/*lfy-5 ask1-1*/+ plants. The *sup-1 ask1-1* double mutant had abnormal carpels, similar to *sup-1*; at the same time, it was also male sterile as is *ask1-1*. These double mutants were nevertheless confirmed by using PCR. Statistical analyses indicate that the segregation data can be accepted according to χ^2 tests (Table 1).

To identify triple mutants with *ufo-2*, *ask1-1* and either *ap3-3*, *pi-1*, *sup-1*, *lfy-5* or *lfy-6*, plants with floral phenotypes similar to, or more severe than those of the third mutant, were first confirmed as being *ask1-1*/*ask1-1* by PCR, and then tested for *UFO* genotype using PCR. Three primers were designed based on the *UFO* genomic sequence. Two of them were the same except for 3′-end nucleotides, one matched the wild-type *UFO* sequence (oMC396: 5′-TGG TAA GAT GGT TTA CGT GC-3′) and the other matched the sequence of the *ufo-2* allele (oMC 397 5′-TGG TAA GAT GGT TTA CGT GT-3′). The third primer (oMC410: 5′ TAA CCA CCG GTG TAG TAA GC 3′) was used with either of the other two primers. Both PCR experiments were performed with each candidate plant, and the *UFO* genotype of the plants was determined by comparing the relative amount of these two PCR products (Li et al., 1999). The *sup-1 ufo-2*, *lfy-5 ufo-2* and *lfy-6 ufo-2* double mutants were identified similarly among *ASK1*/*ASK1* plants.

Table 1. F_2 segregation of double mutants

Genotype	L*er*-like	*ask1-1*	The other single mutant	Double mutant	χ^2	P value
ap3-1/+ *ask1-1*/+	121	32	38*	12	1.42	0.70
ap3-3/+ *ask1-1*/+	163	41	52‡	16	2.69	0.44
pi-1/+ *ask1-1*/+	145	29	51§	13	7.54	0.06
sup-1/+ *ask1-1*/+	151	37	60¶	14	5.84	0.12
lfy-6/+ *ask1-1*/+	128	29	39**	11	3.94	0.27
lfy-5/+ *ask1-1*/+	97	24	26‡‡	7	3.07	0.38
lfy-5(–/–) *ask1-1*/+¶¶			137‡‡	39	0.76	0.86

**ap3-1*; ‡*ap3-3*; §*pi-1*; ¶*sup-1*; ***lfy-6*; ‡‡*lfy-5*; ¶¶genotype of F_2.

Light and scanning electron microscopy

Light microscopic images were recorded digitally using a Nikon dissecting microscope and Optronics camera, and processed using Photoshop. Additional flowers were examined using a Nikon dissecting microscope. Samples for scanning electron microscopy were fixed, dried, dissected and coated, and then the specimens were examined as previously described (Bowman et al., 1989) using a JSM 5400 (JEOL USA, Peabody, MA).

In situ RNA hybridization

RNA in situ hybridizations were performed on wild-type and mutant floral sections as previously described (Drews et al., 1991; Flanagan and Ma, 1994). The *AP3* and *PI* antisense and sense probes were synthesized using in vitro transcription reactions with the pD793 and pcPINX plasmids as templates, respectively (Jack et al., 1992; Goto and Meyerowitz, 1994).

RESULTS

Genetic interactions of *ASK1* with genes regulating B function

We compared the floral phenotypes of the wild type (Fig.1A), single mutants, the double and triple mutants of *ask1-1* with the following mutations: *ap3-1*, *ap3-3*, *pi-1*, *ufo-2*, *ufo-6*, and *sup-1* (Fig. 1; Table 2). Our results on the single mutants and the *ufo-2 ask1-1* double mutant are in agreement with previous reports (Bowman et al., 1989; Bowman et al., 1991; Bowman et al., 1992; Jack et al., 1992; Goto and Meyerowitz, 1994; Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995; Zhao et al., 1999).

ufo-2 ask1-1 and *ufo-6 ask1-1*

The *ask1-1* mutant flowers sometimes show a reduction of petal number and petal size (Fig.1B), reduced stamen filament length, and petal/anther chimeras (Zhao et al., 1999). The *ufo-2* flower (Fig. 1C) has abnormal floral organs interior to whorl one, including ectopic sepals, petals, stamens, carpels, filaments, or chimeric organs (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). The *ufo-2 ask1-1* double mutant flower (Fig. 1D) had a similar phenotype to the *ufo-2* single mutant (Zhao et al., 1999).

The *ufo-6* weak mutant has slightly affected petals and stamens (Levin and Meyerowitz, 1995), with variable flower phenotypes consisting of chimeric petals and reduction of petal number and size (Fig. 1E,F). To obtain further support for an interaction between *UFO* and *ASK1*, we constructed the *ufo-6 ask1-1* double mutant. In some *ufo-6 ask1-1* flowers, petals were similar to those in *ask1-1*,

Table 2. Comparison of floral organs among wild type, single and double mutants*

	Genotype									
Phenotype	Wild type	*ask1-1*	*ap3-1*	*ap3-1, ask1-1*	*ap3-3*	*ap3-3, ask1-1*	*pi-1*	pi-1, ask1-1	sup-1	*sup-1, ask1-1*
Whorl 1, Sepals	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00
Whorl 2,3 and 4										
Petal	4.00±0.00	3.24±0.08	0	0	0	0	0	0	4.00±0.00	2.86±0.10
Chimeric petals‡	0	0.52±0.06	0	0	0	0	0	0	0	0.53±0.08
Sepal and sepal-like§	0	0	3.98±0.01	3.98±0.01	4.00±0.00	1.11±0.08	4.02±0.01	1.18±0.12	0	0
Stamen and stamen-like¶	6.00±0.00	5.88±0.02	5.29±0.10	2.32±0.10	0	0	0	0	9.66±0.19	6.74±0.12
Filament and filament-like**	0	0	0.04±0.02	2.27±0.12	3.12±0.14	3.36±0.10	3.92±0.10	5.57±0.15	0.22±0.05	0.29±0.05
Fused: stamen and carpel	0	0	0.62±0.09	1.19±0.10	0	0	0	0	0.26±0.06	0.11±0.03
Carpel and carpel-like	2.00±0.00	2.00±0.00	2.00±0.00	2.06±0.02	3.15±0.10	2.89±0.10	2.60±0.07	2.56±0.08	0.45±0.07	2.07±0.05
Total number of Whorl 2,3 and 4	12.00±0.00	11.64±0.03	11.93±0.03	11.82±0.07	10.37±0.16	7.36±0.24	10.53±0.16	9.29±0.19	14.59±0.17	12.60±0.13

*All plants were grown under the same conditions and the average number of organs per flower is given ± standard errors. The first 10 flowers on each given plant were analyzed. A total of 100 flowers from 10 plants were examined for each genotype.

‡Chimeric organs included petal/stamen.

§Sepal-like organs included sepal/carpel, sepal/filament.

¶Stamen-like organs included stamen/filament and stamen/carpel/filament.

**Filament-like organs included filament/stigma.

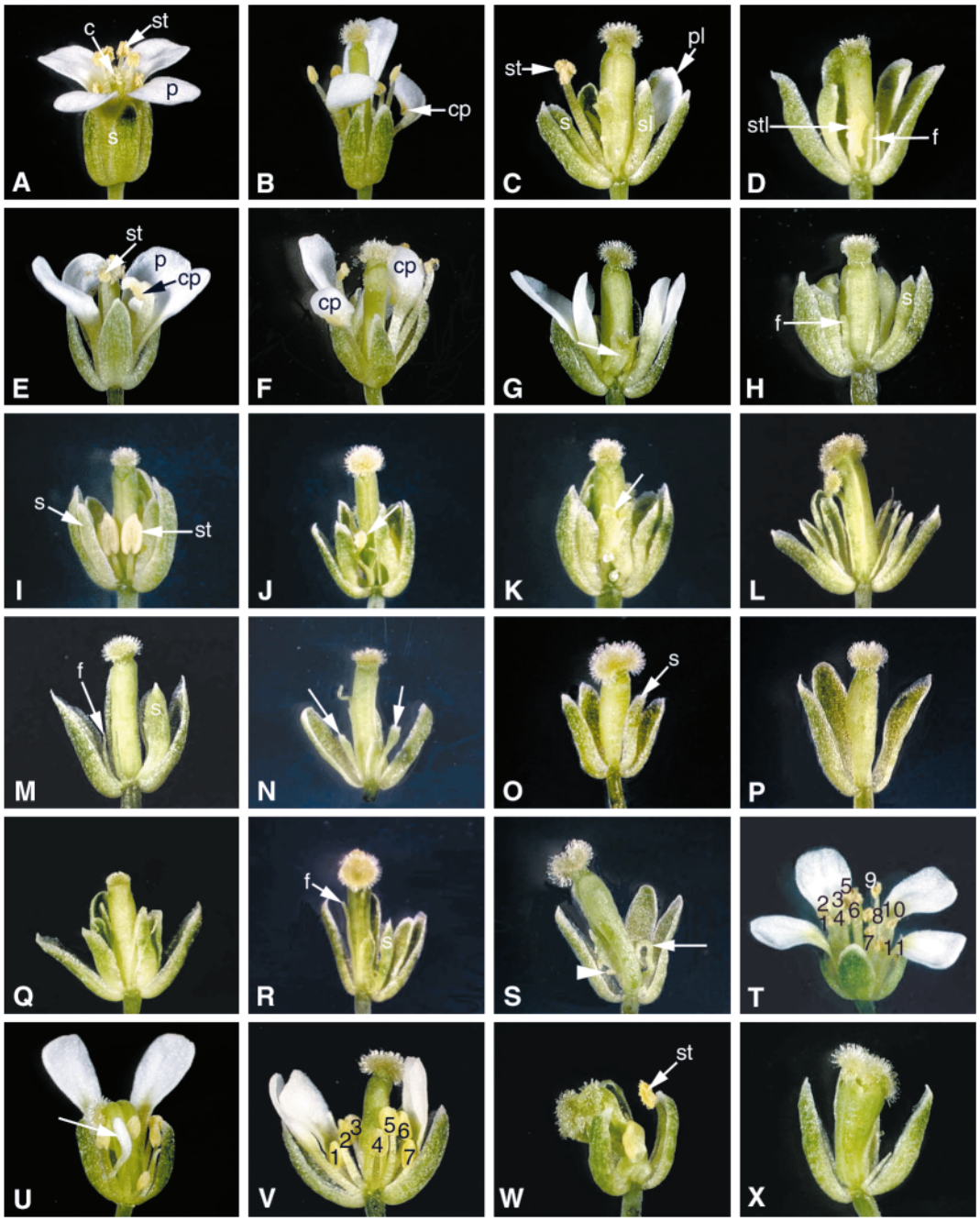

Fig. 1. The phenotypes of *Arabidopsis* wild-type and mutant mature flowers. All photographs are at the same magnification. One sepal was removed to show the interior organs except for flowers in A, B, E, F and T. (A) A wild-type flower showing sepals (s), petals (p), stamens (st), and carpels (c). (B) An *ask1-1* flower with 3 sepals; two of them are small and one is fused with stamen tissues (chimeric petal, cp). The stamens are shorter than normal. (C) A *ufo-2* flower with abnormal organs interior to whorl one, including sepals (s), sepal-like organs (sl), one petal-like organ (pl) and one normal stamen. (D.) A *ufo-2 ask1-1* flower showing sepal, sepal-like, filament (f) and stamen-like (stl) organs. (E) A *ufo-6* (weak allele) flower with normal petals, stamens, and one petal fused with stamen tissues. (F) A *ufo-6* flower with a normal petal, two chimeric petals and normal stamens. (G) A *ufo-6 ask1-1* flower showing a stamen-like organ with carpel tissues (arrow). (H) A *ufo-6 ask1-1* flower with sepals and filaments interior to whorl one. (I) An *ap3-1* flower with four sepals in whorl two and stamens in whorl three. (J) An *ap3-1 ask1-1* flower showing sepals and filament fused with carpel tissues (arrow) interior to whorl one. (K) An *ap3-1 ask1-1* flower with sepals and carpel-like organs (arrow) interior to whorl one. (L) An *ap3-3* flower with sepals, filaments and carpel-like organs interior to whorl one. (M) An *ap3-3 ask1-1* flower with a similar phenotype to the *ap3-3* but with fewer sepals. (N) An *ap3-3 ask1-1* flower showing two small sepal-like organs (arrows). (O) An *ap3-3 ufo-2 ask1-1* triple mutant flower with only one sepal between whorls one and four. (P) An *ap3-3 ufo-2 ask1-1* flower showing no organs between whorls one and four. (Q) A *pi-1* single mutant flower. (R) A *pi-1 ask1-1* flower with fewer sepals than the *pi-1*. (S) A *pi-1 ufo-2 ask1-1* triple mutant flower showing only one filament-like organ (arrow) and carpels fused with ovule-like tissues (arrowhead). (T) A *sup-1* single mutant flower showing 11 stamens. (U,V) Two *sup-1 ask1-1* double mutant flowers showing a reduced number of stamens and an increase in carpel structure. The flower in U has fewer, smaller stamens and more carpel-like organs than the *sup-1* (Arrow indicates a small petal, which is often found in the *ask1-1* mutant flower). The flower in V has normal carpels in the center and seven stamens with short filaments, as in the *ask1-1* flower. (W) *sup-1 ufo-2* flower with a reduced number of stamens (arrow points to a normal stamen with pollen grains) and a carpel structure larger than that in *sup*-1. (X) A *sup-1 ufo-2 ask1-1* triple mutant flower showing a large gynoecium in the center and a near absence of organs between the outer sepals and the central gynoecium.

but carpelloid organs and filaments were often found (Fig. 1G). Furthermore, the *ufo-6 ask1-1* could sometimes produce flowers with a phenotype very similar to that of *ufo-2* (Fig. 1H). The enhancement of the *ufo-6* phenotype by *ask1-1* supports a genetic interaction between these two genes.

ap3-1 ask1-1, ap3-3 ask1-1 and *ap3-3 ufo-2 ask1-1*

Because both *UFO* and *ASK1* affect organ identity in whorls two and three, we wanted to analyze double and triple mutants with *ap3* mutations. The *ap3-1* mutant is a temperature sensitive weak mutant (Bowman et al., 1989); we observed that at 23°C *ap3-1* flowers had sepals in whorl two and stamens, staminoid or carpelloid organs in whorl three (Fig. 1I). In contrast, *ap3-1 ask1-1* flowers (Fig. 1J,K) had filaments or carpelloid organs interior to whorl one but no stamens at all, similar to the strong *ap3-3* mutant flower which also has filaments, carpelloid organs and/or carpels (Fig. 1L; Bowman et al., 1989; Bowman et al., 1991; Jack et al., 1992).

We had previously generated the *ap3-3 ask1-1* double mutant (Zhao et al., 1999) and briefly reported its flower phenotype, which is similar to that of *ap3-3* in terms of organ type. We show here that *ap3-3 ask1-1* flowers have fewer floral organs than the *ap3-3* flower (Table 2). Specifically, *ap3-3 ask1-1* flowers had fewer than four sepals interior to whorl one (Fig. 1M; Table 2), which was significantly different from *ap3-3* (T value=24.41). Also sepals in some *ap3-3 ask1-1* flowers were small (Fig. 1N). Interior to whorl one, the number of filaments in *ap3-3 ask1-1* flowers was not significantly different from that in *ap3-3* flowers (Table 2, T value=0.49). The total organ number in *ap3-3 ask1-1* flowers was significantly reduced compared to that in *ap3-3* flowers (Table 2, T value=10.87).

To test for genetic interaction between *AP3*, *UFO* and *ASK1*, we also examined the *ap3-3 ufo-2 ask1-1* triple mutant and found that it was similar to *ap3-3 ask1-1*, except that the organ number was perhaps further reduced slightly. In some *ap3-3 ufo-2 ask1-1* flowers, we found only one or two sepals and no filament interior to whorl one (Fig.1O). Some *ap3-3 ufo-2 ask1-1* flowers did not form any organs at all between whorls one and four (Fig. 1P). These results indicate that the effect of *ap3-3 ask1-1* and *ap3-3 ufo-2 ask1-1* on flowers were more severe than *ap3-3* alone in terms of organ number.

pi-1 ask1-1 and *pi-1 ufo-2 ask1-1*

We also characterized double and triple mutants involving *pi-1*, which causes the formation of abnormal organs interior to whorl one, similar to *ap3-3* (Bowman et al., 1989; Bowman et al., 1991; Fig. 1Q). Although the *pi-1 ask1-1* flowers showed a similar phenotype to that of *pi-1* flowers, the double mutant flowers had fewer floral organs interior to whorl one than *pi-1* flowers (Fig. 1R; Table 2). Most *pi-1 ask1-1* flowers had fewer than four sepals interior to whorl one, which was significantly different from that of *pi-1* (T value=23.02). In addition, the number of filaments in the double mutant flower was significantly greater than that of the *pi-1* single mutant (T value=7.31), but the total number of floral organs in the *pi-1 ask1-1* flower was significantly smaller than in the *pi-1* flower (Table 2, T value=4.68).

Although *ap3-3 ask1-1* and *pi-1 ask1-1* flowers had similar number of sepals interior to whorl one, *pi-1 ask1-1* flowers produced more filaments or filament-like organs than *ap3-3 ask1-1* flowers (Table 2). The *pi-1 ufo-2 ask1-1* triple mutant flower seemed to have a slightly more severe phenotype than either the *pi-1 ask1-1* double mutant or the *pi-1* single mutant in terms of the total floral organ number (Fig. 1S). In addition, *pi-1 ufo-2 ask1-1* flowers made fewer filaments than *pi-1 ask1-1* flowers (Fig. 1S and data not shown).

sup-1 ask1-1, sup-1 ufo-2 and *sup-1 ufo-2 ask1-1*

We also analyzed double and triple mutants with the *sup-1* mutation, which causes the production of flowers with extra stamens interior to whorl two and a reduced carpelloid organ in the center (Bowman et al., 1992; Fig. 1T). Most *sup-1* flowers had about ten stamens and a dramatically reduced carpelloid organ (Bowman et al., 1992; Table 2). However, the *sup-1 ask1-1* double mutant flowers usually produced approximately seven stamens, significantly different from *sup-1* (Table 2, T value=13.24). In addition, *sup-1 ask1-1* flowers had a larger carpelloid organ in the center than did *sup-1* (Fig. 1U; Table 2, T value=18.83). About 10% of the double mutant flowers could even make a normal pistil in the fourth whorl (Fig. 1V). Furthermore, *sup-1 ask1-1* flowers had reduced number and size of petals, short stamen filaments and sterile anthers, similar to the *ask1-1* flowers.

Compared to the *sup-1* mutant, the *sup-1 ufo-2* flowers also had a reduction in stamen number and an enlargement of the carpelloid organs, similar to the *sup-1 ask1-1* flowers (Fig. 1W; Levin and Meyerowitz, 1995). But the *sup-1 ufo-2* flower had fewer petals and stamens than the *sup-1 ask1-1* flowers and was male fertile. The *sup-1 ufo-2 ask1-1* triple mutant flower was slightly more similar to *sup-1 ufo-2* than *sup-1 ask1-1* flowers (Fig. 1X). Compared with the two double mutants, the number of petals and stamens in the triple mutant was even smaller and the central carpelloid organ was slightly larger.

Early floral development in double and triple mutants

Our observations with mature flowers described above indicate that *ask1-1* and *ufo-2* mutations alone or together caused a reduction of organ number in the *ap3-3*, *pi-1*, and *sup-1* backgrounds. We were interested to determine when the effect of *ask1-1* and *ufo-2* can be detected during flower development and whether these mutations affect floral organ primordium initiation; therefore, we examined early floral development of the double and triple mutants using scanning electron microscopy.

ap3-1 ask1-1, ap3-3 ask1-1 and *ap3-3 ufo-2 ask1-1*

First we compared flower development between *ap3-1* single and *ap3-1 ask1-1* double mutants. There was no detectable difference in the inflorescence meristem and early floral primordia before stage 5 between *ap3-1* and *ap3-1 ask1-1* mutants (not shown). At stage 6, the *ap3-1* floral bud (Fig. 2D) showed sepal primordia in whorl two and stamen primordia in whorl three, but the size was smaller than wild type (Fig. 2A; Bowman et al., 1989). Although the stage-6 *ap3-1 ask1-1* bud (Fig. 2F) had four sepal primordia interior to whorl one, similar to that of *ap3-1*, it lacked the characteristic stamen primordia. In addition, some peripheral regions of the central carpel primordia were enlarged (Fig. 2F). In the stage-7 *ap3-1 ask1-1* floral bud, the carpel primordia continued to enlarge, but there were no stamen primordia (Fig. 2G). The *ap3-1* floral bud at about stage 10 could form stamen primordia (Fig. 2E) which were smaller than the wild-type ones (Fig. 2C). However, the *ap3-1 ask1-1* floral bud at stage 10 only produced sepals, filaments or carpelloid organs (Fig. 2H), without any stamens.

Similar to the *ap3-1 ask1-1* floral bud (Fig. 2F), the *ap3-3* floral bud at stage 6 had four sepal primordia interior to whorl

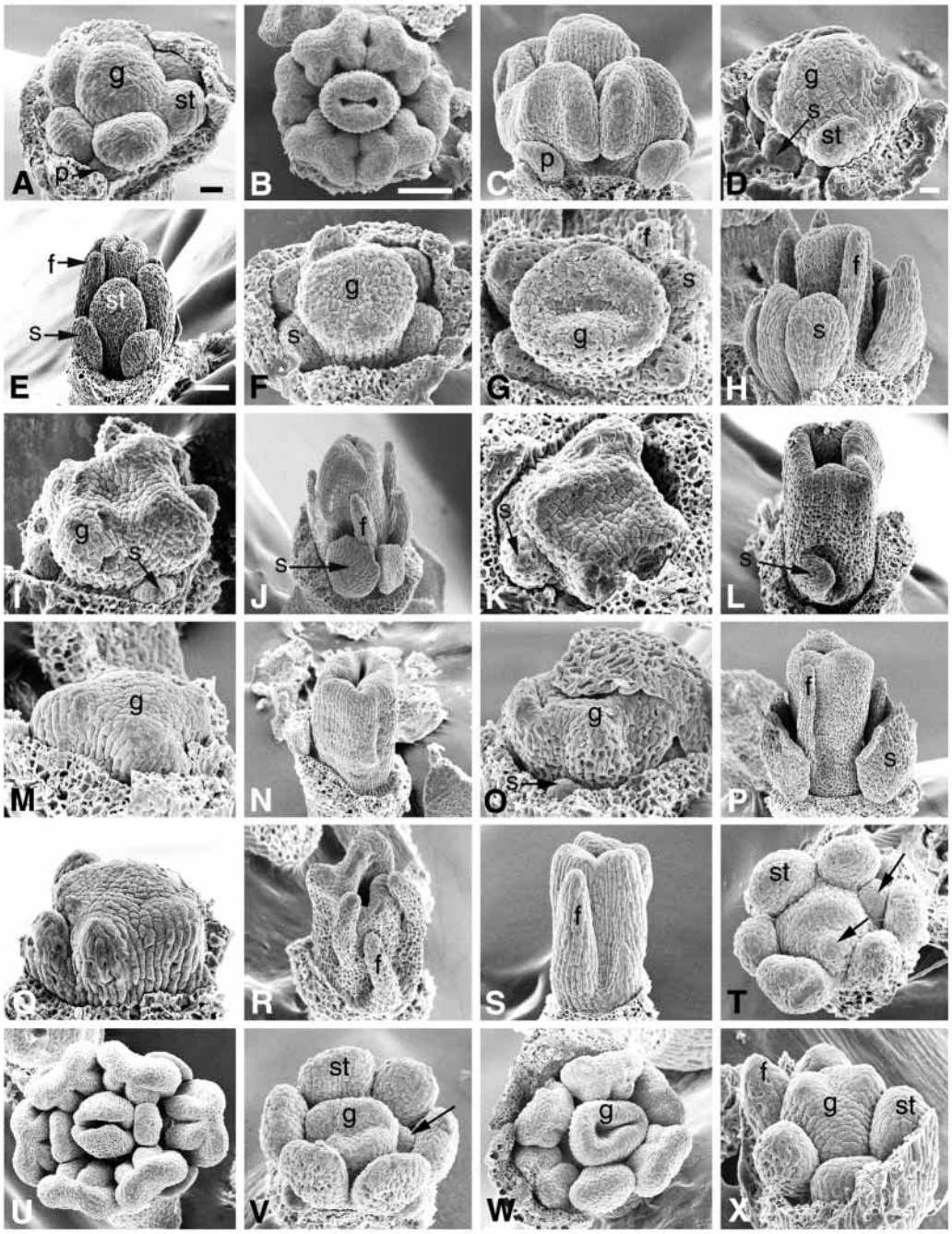

Fig. 2. Morphology of early flowers of wild-type and mutant plants carrying *ap3*, *pi*, and *sup* mutations. In all flowers, the whorl one sepals or sepal primordia were removed. (A) A wild-type bud at stage 6 showing six stamen primordia (st), carpel primordia (gynoecium, g), and a tiny petal primordium (p). (B) A wild-type bud at stage 8 with clearly visible stamens and fused carpels. (C) A wild-type bud at stage 9 showing petal primordia becoming more prominent than before. (D) A stage 6 *ap3-1* floral bud with sepal primordia and stamen primordia that are smaller than the normal. (E) An *ap3-1* bud at about stage 10 with sepals (s), stamens (st) and filaments (f). (F) A stage 6 *ap3-1 ask1-1* bud showing no stamen primordia but with sepal primordia and the central gynoecium primordium that had become flattened and enlarged at the periphery. (G) An *ap3-1 ask1-1* bud at about stage 7 showing sepal, filament primordia (f) and enlarged carpel primordia. (H) An *ap3-1 ask1-1* bud at about stage 10 showing sepals and filaments. (I) A late stage 6 *ap3-3* bud without stamen primordia, but with sepal primordia and a gynoecium primordium that is enlarged and misshapen. (J) An *ap3-3* bud at about stage 10 showing sepals and filamentous organs. (K) A stage 6 *ap3-3 ask1-1* bud with sepal primordia which are fewer than in *ap3-3*, and the enlarged gynoecium primordium is similar to *ap3-3*. (L) An *ap3-3 ask1-1* bud at about stage 10 with one small sepal-like (s) organ but no filaments. (M) A stage 6 *ap3-3 ufo-2 ask1-1* triple mutant bud showing enlarged carpel primordia but no sepal primordia. (N) An *ap3-3 ufo-2 ask1-1* bud at about stage 10 having only the gynoecium interior to the removed whorl one sepals. (O) A *pi-1* bud at stage 6 showing sepal primordia and anenlarged gynoecium primordium. (P) A *pi-1* bud at about stage 10 showing sepals and filaments. (Q) A stage 6 *pi-1 ask1-1* bud showing enlarged carpel primordia but no sepal primordia. (R) A *pi-1 ask1-1* bud at about stage 10 with filaments but without sepals. (S) A *pi-1 ufo-2 ask1-1* bud at about stage 10 with one filament but no sepals. (T) A stage 6 *sup-1* bud that has six stamen primordia and is beginning to form two more stamen primordia (arrows). (U) A *sup-1* bud at stage 9 showing 6 large stamens and two small stamens, but no detectable carpel structure. (V) A *sup-1 ask1-1* bud at late stage 6 that has six stamen primordia and is begimming to form one more stamen primordium (arrow). (W) A *sup-1 ask1-1* bud at stage 8 showing six stamens and fused carpels (g). (X) A *sup-1 ufo-2 ask1-1* triple mutant bud at late stage 6 showing a phenotype similar to that of the *sup-1 ask 1-1* bud with filament primordia and fewer stamen primordia. The gynoecium primordium was also larger than that of the *sup-1 ask1-1*. Scale bars, in A (A,T,V,X) 10 μm; in D (D,F,G,I,K,M,O,Q) 10 μm; in B (B,C,H,L,N,R,S,W) 50 μm; in E (E,J,P,U) 50 μm.

one and enlarged carpel primordia in the center (Fig. 2I). In contrast, the *ap3-3 ask1-1* floral bud at stage 6 formed fewer sepal primordia than the *ap3-3* single mutant, even though both of them could produce similarly enlarged carpel primordia (Fig. 2K). At about stage 10, the *ap3-3* bud usually had sepals, filaments, or carpelloid organs interior to whorl

one (Fig. 2J), again similar to *ap3-1 ask1-1* buds (Fig. 2H). In comparison, *ap3-3 ask1-1* flowers produced fewer floral organs interior to whorl one than *ap3-3* flowers. Some *ap3-3 ask1-1* flowers had fewer than four sepals and no filament structure (Fig. 2L). Some *ap3-3 ask1-1* flowers had no sepals or sepal-like organs (not shown). The *ap3-3 ufo-2 ask1-1* triple mutant flower was similar to the *ap3-3 ask1-1* flower, except that the triple mutant flower had slightly fewer floral organs than the *ap3-3 ask1-1* flower (Fig. 2M,N). Our observations indicate that the *ask1-1* mutation could enhance the *ap3-1* phenotype and the *ask1-1* and *ufo-2* mutations reduced the number of floral organ primordia in the *ap3-3* background.

pi-1 ask1-1 and *pi-1 ufo-2 ask1-1*

We also examined the early floral morphology of double and triple mutants with the *pi-1* mutation. We observed that at stage 6 both *pi-1* single (Fig. 2O) and *pi-1 ask1-1* double mutant (Fig. 2Q) floral buds formed enlarged carpel primordia at the center; however, the *pi-1 ask1-1* bud at this stage showed fewer sepal primordia than the *pi-1* bud. At a later stage the *pi-1 ask1-1* flower produced fewer floral organs than *pi-1*(Fig. 2P), sometimes lacking sepals interior to whorl one (Fig. 2R). The *pi-1 ufo-2 ask1-1* floral buds at stage 6 (not shown) and approximately stage 10 (Fig. 2S) had fewer floral organ primordia than the *pi-1 ask1-1* floral buds. Therefore, the *ask1-1* and *ufo-2* mutations also caused a reduction of floral organ primordia in the *pi-1* background.

sup-1 ask1-1, sup-1 ufo-2 and *sup-1 ufo-2 ask1-1*

Finally, we analyzed the early floral morphology of double and triple mutants with the *sup-1* mutation. Before stage 5, there was no detectable difference between the *sup-1* single and *sup-1 ask1-1* double mutant floral buds (not shown). The stage-6 *sup-1* floral bud (Fig. 2T) formed six stamen primordia in whorl three and began to form more stamen primordia. The gynoecium primordium at the center was shorter than in the wild type. At stage 9, the *sup-1* flower produced more than six stamens and no obvious carpel structures (Fig. 2U). The *sup-1 ask1-1* bud at stage 6 (Fig. 2V) was similar to that of *sup-1*. In some late *sup-1 ask1-1* flowers, we found six stamens and fused carpels (Fig. 2W), which was similar to the wild type at this stage (Fig. 1C). The *sup-1 ufo-2 ask1-1* triple mutant flower had a similar floral phenotype to that of *sup-1 ask1-1*, but produced fewer stamens and a slightly larger carpel-like structure (Fig. 2X).

The analyses of these double and triple mutants indicate that *ask1-1* and *ufo-2* mutations cause a reduction of organ primordium initiation interior to whorl one in the *ap3*, *pi* and *sup* mutant backgrounds. In addition, the combination of both *ask1-1* and *ufo-2* mutations results in a slightly greater reduction in organ initiation.

Genetic interaction between *ASK1, UFO* and *LFY*

Phenotypes of double and triple mutant mature flowers

Because *lfy* mutations affect floral organ identity in a way consistent with a defect in B function (Schultz and Haughn, 1991; Huala and Sussex, 1992; Weigel et al., 1992), we tested for possible interaction between *ASK1*, *UFO* and *LFY* by comparing the floral phenotypes of single, double and triple mutants. The strong *lfy-6* mutant flowers only had leaf-like and carpel-like organs (Fig. 3A; Weigel et al., 1992). Flowers of the *lfy-6 ask1-1*and *lfy-6 ufo-2* double mutants (Fig. 3B, C) and the *lfy-6 ufo-2 ask1-1*triple mutant flower (Fig. 3D) had similar phenotypes, suggesting that *ask1-1* and *ufo-2* mutations have no effect in the *lfy-6* background.

We then analyzed double and triple mutants between *ask1-1*, *ufo-2* and the weak allele *lfy-5*. Flowers of the weak *lfy-5* mutant had well-developed petals, stamens and carpels (3.0 petals, 2.7 stamens, and 2.2 carpels, *n*=30; Fig. 3E; Weigel et al., 1992). In contrast, the *lfy-5 ask1-1* double mutant flower had a much more severe phenotype than that of *lfy-5*, and closely resembled that of *lfy-6*. Most of the *lfy-5 ask1-1* flowers only produced leaf-like and carpel-like organs (7.2 and 2.9, respectively, *n*=30; Fig. 3F). Nevetheless, we occasionally found that the *lfy-5 ask1-1* flower had stamen or stamen-like organs (0.2, *n*=30), which were never found in the *lfy-6* flower. The *lfy-5 ufo-2* flower was similar to that of *lfy-5 ask1-1* (Fig. 3G); futhermore, the *lfy-5 ufo-2 ask1-1*triple mutant had no detectable difference from *lfy-6* (Fig. 3H). These results suggest that the combination of a partial loss of *LFY* function (*lfy-5*) and *ask1-1* and *ufo-2* mutations can cause a similar floral defect to the complete loss of *LFY* function (*lfy-6*).

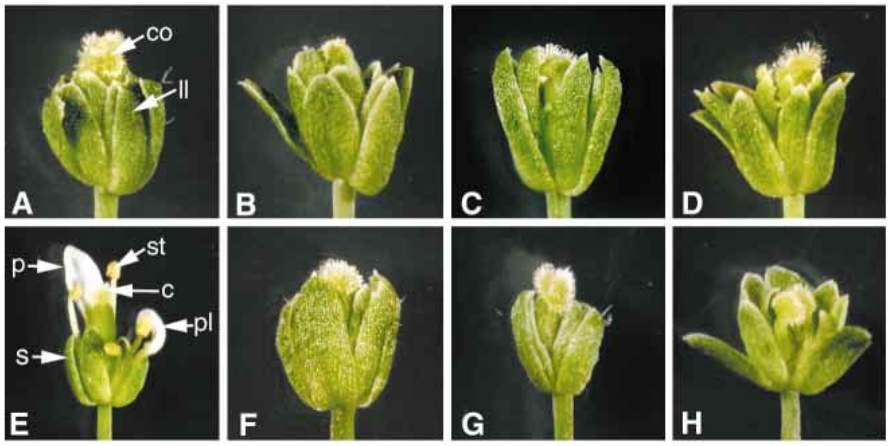

Fig. 3. Mature floral phenotypes of mutants with *lfy-6* or *lfy-5* alleles. (A) A *lfy-6* flower with leaf-like (ll) and carpelloid (co) organs. (B) A *lfy-6 ask1-1* flower showing a similar phenotype to the *lfy-6* mutant. (C) A *lfy-6 ufo-2* flower with the similar phenotype to *lfy-6* and *lfy-6 ask1-1* flowers. (D) A *lfy-6 ufo-2 ask1-1* flower, similar to *lfy-6*, *lfy-6 ask1-1*, and *lfy-6 ufo-2* flowers. (E) A *lfy-5* flower showing sepals (s), petal (p), petal-like organ (pl), stamens (st), and carpels (c), unlike the *lfy-6* flower. (F) A *lfy-5 ask1-1* flower with a phenotype similar to that of *lfy-6*, and much more severe than that of *lfy-5*. (G) A *lfy-5 ufo-2* flower exhibiting a phenotype similar to those of *lfy-6* and *lfy-5 ask1-1* flowers. (H) A *lfy-5 ufo-2 ask1-1* triple mutant flower, showing a similar phenotype to those of *lfy-6*, *lfy-5 ask1-1*, *lfy-5 ufo-2*, *lfy-6 ufo-2*, *lfy-6 ask1-1*, and *lfy-6 ufo-2 ask1-1* flowers. All photographs were taken at the same magnification.

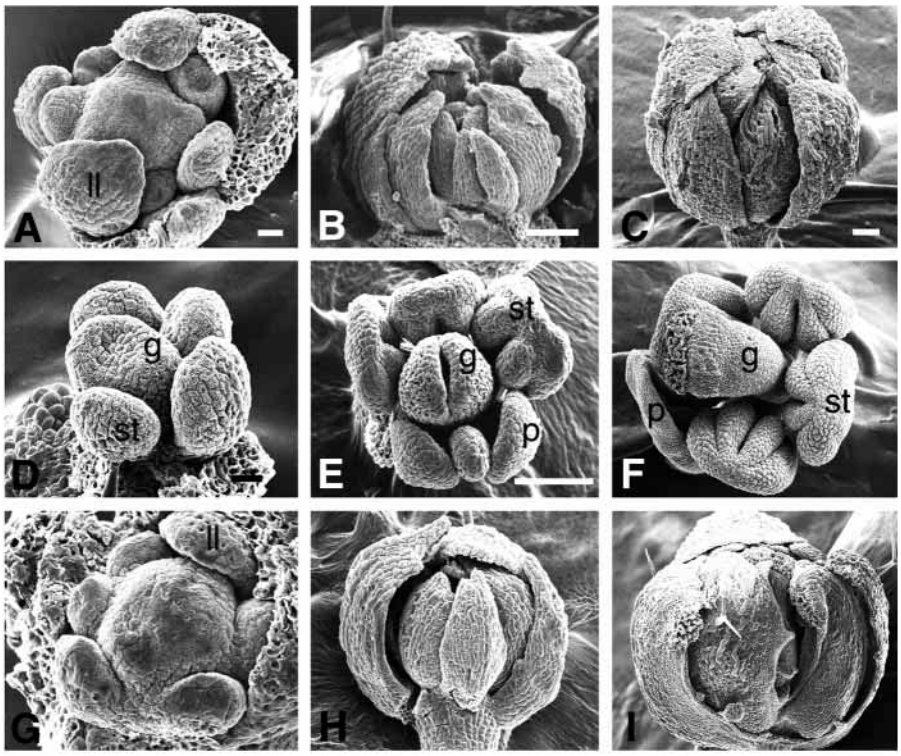

Fig. 4. Early floral morphology of mutants with *lfy-6* or *lfy-5* alleles. (A) A *lfy-6* bud at about late stage 6 showing leaf-like primordia (ll). (B,C) Two *lfy-6* flowers with leaf-like organs. The flower in B is younger than the flower in C. (D) A *lfy-5* bud at about late stage 6 showing stamen primordia (st) and carpel primordia (g). (E) A stage 9 *lfy-5* bud with obvious stamens (st), petals (p) and carpels (g) in the center. (F) A stage 11 *lfy-5* bud with normal stamens, petal and two curled carpels. (G) A *lfy-5 ask1-1* bud at about stage 6 showing spiral leaf-like primordia that were similar to those in the *lfy-6* bud at the same stage. (H,I) Two *lfy-5 ask1-1* flowers showing a similar phenotype to that of the *lfy-6* flower. The flower in H is younger than the flower in I. Scale bars, A, 10 μm; B (B,H), 50 μm: C (C,F,I), 50 μm; D (D,G), 10 μm; E, 50 μm.

Early floral development in double and triple mutants

We have examined the early floral morphology of single, double and triple mutants carrying *lfy* mutations. In the *lfy-6* floral bud at about stage 6, the first four leaf-like primordia formed a whorl, but the other leaf-like primordia developed in a spiral pattern (Fig. 4A). At later stages *lfy-6* flowers produced leaf-like organs with branched trichomes (Fig. 4B,C). Both in early and late stages, *lfy-6 ask1-1*and *lfy-6 ufo-2 ask1- 1* floral buds had similar phenotypes to that of the *lfy-6* single mutant (data not shown). Therefore, the *ask1-1* and *ufo-2* mutations did not affect early flower development in the *lfy-6* background.

We further compared early flower development in the weak *lfy-5* mutant and corresponding double and triple mutants. The stage 6 *lfy-5* floral bud (Fig. 4D) had stamen primordia that were nearly normal in size, but their number was reduced compared to the wild type. In addition, we observed nearly normal carpel primordia at the center of the *lfy-5* floral bud (Fig. 4D). The late *lfy-5* flower clearly showed well developed petals, stamens and carpels (Fig. 4E,F). However, the development of *lfy-5 ask1-1* flower was quite different from *lfy-5* flowers. The *lfy-5 ask1-1* floral bud at about stage 6 (Fig. 4G) produced leaf-like primordia in a spiral pattern, similar to the *lfy-6*. The leaf-like primordia eventually developed into leaf-like organs (Fig. 4H,I). The *lfy-5 ufo-2 ask1-1* flowers had similar phenotypes to that of *lfy-5 ask1-1*, and were not detectably different from the *lfy-6* flower (data not shown). We conclude that when *LFY* function is reduced, *ASK1* and *UFO* function are important for the specification of floral organ primordia identities and phyllotaxy.

AP3 and *PI* expression in wild-type and mutant flowers

Our results from phenotypic studies suggest that *ASK1* and *UFO* interact with B function genes and *LFY* genetically. It is known that *LFY* and *UFO* positively regulate the expression of B function genes *AP3* and *PI*. Therefore, it is possible that *ASK1* also contributes to the positive regulation of *AP3* and *PI* expression. To test this idea, we performed RNA in situ hybridization to determine *AP3* and *PI* expression in wild-type, single and double mutant inflorescence sections. Our results for *AP3* expression in wild-type and *lfy-6* and *PI* expression in the wild type were in agreement with previous findings (Jack et al., 1992; Weigel and Meyerowitz, 1993; Goto and Meyerowitz, 1994).

The onset of *AP3* expression has been shown to occur at stage 3 in the wild-type floral meristem (Fig. 5A; Jack et al., 1992). During stages 5-8, *AP3* was present in petal and stamen primordia at a high level. After stage 9, the level of *AP3* mRNA was reduced, but still detectable. The *ask1-1* flower showed a normal *AP3* expression pattern (Fig. 5B), but the expression level in some mutant flowers was slightly reduced (not shown). In the *lfy-5* flower, *AP3* mRNA was clearly detectable in stage-3 to -5 floral meristems, but the level was considerably lower than normal (Fig. 5C,D). After stage 6, the *AP3* mRNA was present in the *lfy-5* bud at a slightly lower level than either the wild-type or the *ask1-1* mutant buds (data not shown). *AP3* mRNA was not detectable in most *lfy-6* flowers and only occasionally found at the base in some *lfy-6* flowers (Fig. 5E,F). The *lfy-5 ask1-1* flowers showed an *AP3* expression pattern very similar to those of *lfy-6*. In most *lfy-5 ask1-1* flowers, the *AP3* mRNA was not detectable, although a very limited amount of *AP3* signal was observed at the base of some flowers (Fig. 5G,H).

The *PI* mRNA was first detected in the wild-type stage 3 bud and it remained present at a high level in the developing petals and stamens (Goto and Meyerowitz, 1994; Fig. 5I). The *ask1-*

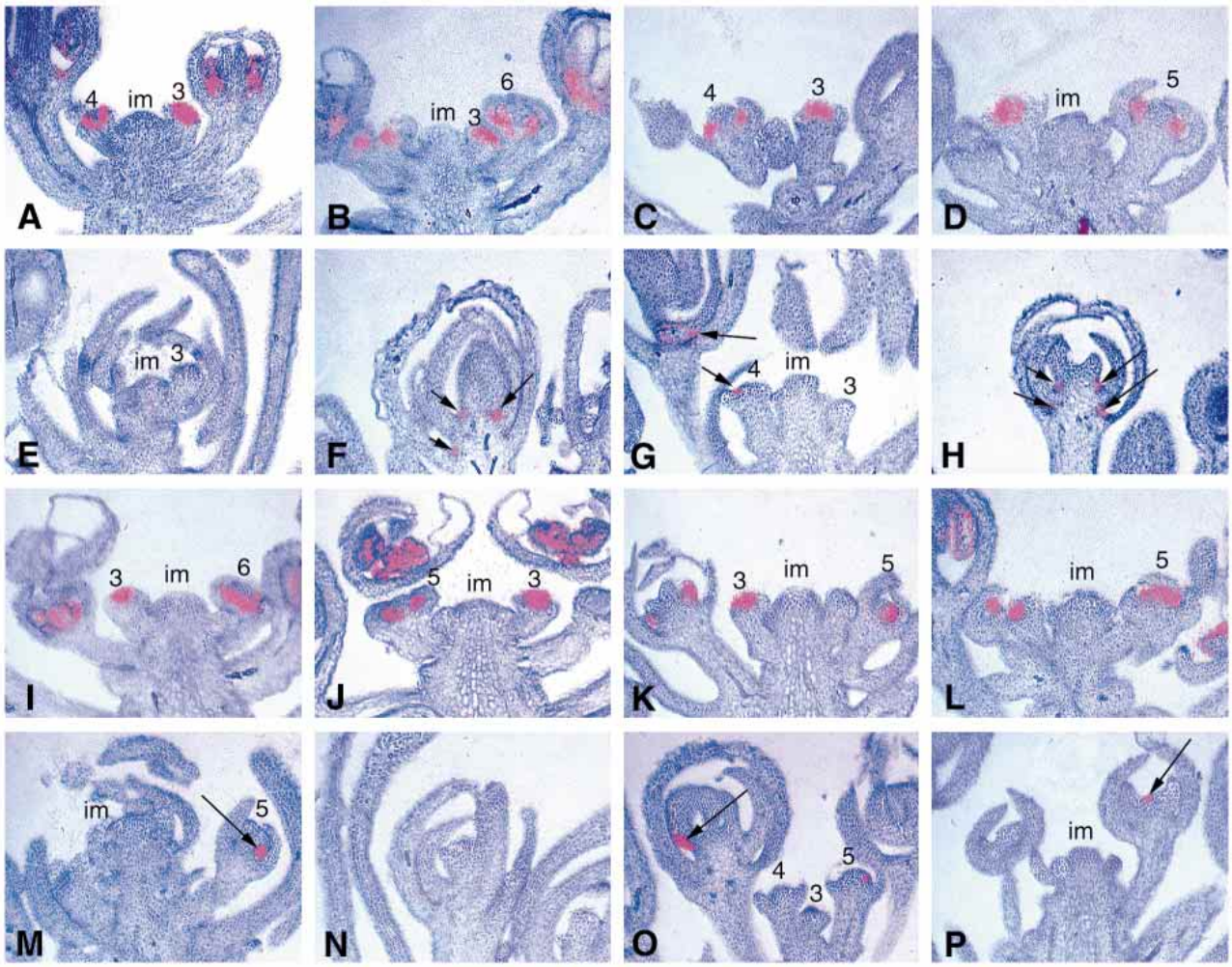

Fig. 5. *AP3* and *PI* expression in wild-type and mutant flowers. Sections of inflorescences in A-H were hybridized with an *AP3* probe and in I-P were hybridized with a *PI* probe. All photographs are at the same magnification. The numbers indicate the bud stage; im, inflorescence meristem. (A) Wild-type showing *AP3* RNA expression in the floral meristem at a high level at stages 3 and 4 and in whorl two and three in an old flower. (B) *ask1-1* exhibiting *AP3* RNA expression at the same position and nearly the same level as in the wild-type flower. (C,D) *lfy-5* has a similar expression pattern of *AP3* RNA to that in the wild type, but the expression level is slightly reduced. (E,F) *lfy-6* showing no or very low *AP3* RNA expression (arrows) in a young and an old bud, respectively. (G,H) *lfy-5 ask1-1* double mutant showing that *AP3* RNA expression is much reduced and largely undetectable. Only very low *AP3* RNA expression in limited areas was observed in some buds (arrows). (I) Wild-type showing that *PI* RNA is present at a high level in a stage-3 floral meristem and in whorl two and three of old flowers. (J) *ask1-1*; the *PI* RNA signal shows a similar pattern to that in the wild-type flower. (K,L) *lfy-5*; the *PI* RNA expression pattern is similar to that in the wild type, but the level of expression is slightly lower than the normal. (M,N) *lfy-6* showing very limited *PI* RNA expression in a floral bud (arrow). (O,P) *lfy-5 ask1-1* ; *PI* RNA expression is also much reduced. Only an occasional small region of *PI* expression could be observed in some buds (arrows).

1 (Fig. 5J) and *lfy-5* (Fig. 5K,L) flowers exhibited similar *PI* expression pattern to that of the wild-type flower, but the *PI* mRNA level in some *ask1-1* and *lfy-5* flowers was slightly lower than that of wild type. In contrast, the *PI* expression was not detectable in most areas of *lfy-6* flowers, except for a limited amount of *PI* signal at the base of some flowers (Fig. 5M,N). Similarly, *PI* mRNA was not detectable in most regions of *lfy-5 ask1-1* flowers, with only a small amount of *PI* signal at the base of some flowers (Fig. 5O,P). The results from the *AP3* and *PI* in situ hybridization experiments indicate that the *ask1-1* and *lfy-5* mutations together cause a much more severe reduction of *AP3* and *PI* mRNA levels and domains than either single mutations.

DISCUSSION

ASK1 and *UFO* interact with *LFY* genetically to regulate B function genes

ASK1 and *UFO* both affect petal and stamen identities in whorls two and three, respectively, and interact with each other genetically; furthermore, the ASK1 and UFO proteins have been shown to interact physically (Samach et al., 1999; Zhao et al., 1999). These findings suggested that *ASK1* and *UFO* may act together to regulate B function. In this report we show that *ask1-1* can further enhance the floral organ identity phenotype of *ap3-1*. At the same time, our observations indicate that *ask1-1* does not enhance the floral organ identity

defects of *ap3-3* and *pi-1* mutations. Moreover, triple mutants with *ask1-1*, *ufo-2* and *ap3* or *pi* mutations, showed similar organ identity phenotypes to *ap3-3* and *pi-1* mutants. It has been argued that although null alleles of genes in the same genetic pathway should not enhance each other's phenotypes, partially functional mutations and/or mutations in functionally redundant genes could enhance each other's phenotypes (Martienssen and Irish, 1999). Therefore, our double and triple mutant phenotypes support the idea that *ASK1* and *UFO* may function in the same regulatory network that requires *AP3* and *PI* gene functions, i.e., the B function of the ABC model for the specification of floral organ identity.

However, *ask1-1* and *ufo-2* single mutants, even the *ufo-2 ask1-1* double mutant, are less severe than the *ap3-3* and *pi-1* mutants. This may be due to functional redundancy because *Arabidopsis* has additional *SKP1* homologues (*ASK2~ASK9*, Gray et al., 1999; Samach et al., 1999; other *ASKs*, GenBank/*Arabidopsis* Sequencing Initiative). Three of these genes, *ASK2, ASK3* and *ASK18*, have 70% or more amino acid sequence identity to *ASK1* and might have similar functions to *ASK1* in flower development. Similarly, UFO is an F-box containing protein; there are dozens, if not hundreds, of putative F-box-containing proteins predicted by the *Arabidopsis* genome sequencing project (*Arabidopsis* Sequencing Initiative). The potential existence of functionally similar genes to both *ASK1* and *UFO* could explain why mutations in these genes cause less severe floral phenotypes. This is supported by the observed physical interaction between UFO and ASK2 (Samach et al., 1999) and by the observation that *ASK2* has a similar expression pattern in early floral buds to that of *ASK1* (D. Z. and H. M., unpublished data).

The *ask1-1* mutation enhances the phenotype of the weak *lfy-5* mutant, but not that of the strong *lfy-6* mutant, suggesting that *ASK1* likely functions in the same regulatory pathway as *LFY*. Previous studies showed that LFY is a positive regulator of *AP3* and *PI* expression and that UFO is an important co-regulator of LFY (Lee et al., 1997; Parcy et al., 1998). Our results suggest that *ASK1* may also be a co-regulator of LFY for the activation of *AP3* and *PI* expression. Indeed, this hypothesis was further supported by our findings that the expression of both the *AP3* and *PI* genes was reduced to a much greater extent by the combination of *ask1-1* and *lfy-5* mutations than by either mutation alone. Furthermore, the reduction of *AP3* and *PI* expression in the *lfy-5 ask1-1* double mutant flowers was very similar to that in *lfy-6*, a presumed null allele. This result and the fact that *lfy-6* single, *lfy-6 ask1-1* and *lfy-6 ufo-2* double, and *lfy-6 ask1-1 ufo-2* triple mutants all have nearly identical floral phenotypes suggest that the regulation of B function by *ASK1* and *UFO* requires *LFY* function.

AP3 and *PI* have slightly different domains of expression initially, with the *PI* expression domain closer to the center of the floral meristem (Jack et al., 1992; Goto and Meyerowitz, 1994). In addition, it was shown that the *ufo-1* mutation causes a reduction of early *AP3* expression, but not *PI* expression (Samach et al., 1999). This and the fact that the *35S-AP3*, but not the *35S-PI*, transgene could rescue the *ufo-1* mutant phenotype in whorl three led to the idea that *UFO* positively regulates *AP3* expression, but not that of *PI* (Samach et al., 1999). However, the *35S-PI* transgene also did not rescue the *pi-1* mutant in whorl three, suggesting that the transgene might not provide enough *PI* function (Krizek and Meyerowitz, 1996; Samach et al., 1999). In addition, the lack of reduction of *PI* expression in the *ufo-1* mutant could be explained by a possible functional redundancy of UFO and other F-box proteins. Furthermore, the observation that *PI* is expressed throughout *35S-LFY 35S-UFO* seedlings strongly supports the idea that LFY and UFO also positively regulate *PI* expression (Honma and Goto, 2000). Our results support the hypothesis that ASK1 positively regulates the expression of both *AP3* and *PI* with LFY, as well as UFO.

ASK1 regulates floral organ primordium initiation

We observed that the *ap3-3* and *pi-1* mutations caused an reduction of organ number interior to whorl one, consistent with earlier studies (Bowman et al., 1989; Bowman et al., 1991; Jack et al., 1992; Goto and Meyerowitz, 1994). Furthermore, ectopic expression of *AP3* and *PI* resulted in extra whorls of stamens (Krizek and Meyerowitz, 1996). Therefore, in addition to their roles in specifying organ identity, the *AP3* and *PI* genes also promote cell proliferation, especially near the center of the floral meristem (Jack et al., 1992; Krizek and Meyerowitz, 1996). In addition, it was previously observed that *sup-1* mutants have reduced floral meristem determinacy, resulting in additional whorl(s) of stamens (Schultz et al., 1991; Bowman et al., 1992; Sakai et al., 2000). The ectopic expression of *AP3* and *PI* in *sup-1* floral meristem also supports a role for *AP3* and *PI* in cell proliferation and initiation of floral organ primordia.

We showed previously that the *ask1-1* mutant flowers had a slightly reduced number of petals and a nearly normal number of other organs (Zhao et al., 1999; Table 2). *ASK1* is homologous to the yeast *SKP1* gene, which is an essential regulator of cell division and encodes a subunit of the SCF ubiquitin ligase. Therefore, *ASK1* may also regulate cell proliferation during flower development. We further observed that flowers of the *sup-1 ask1-1* double mutant and the *sup-1 ufo-2 ask1-1* triple mutant had fewer stamens or stamen-like organs and more carpels than the *sup-1* single mutant. Therefore, the increased whorl-three cell proliferation in *sup-1* mutant requires *ASK1* and *UFO* functions. We found that *sup-1 ask1-1* flowers had a nearly normal number of carpels, more than the *sup-1* mutant; therefore, relative to *sup-1*, reduction of whorl three is balanced by an increase in whorl four.

Because the *ask1-1* mutation can cause a reduction in *AP3* and *PI* expression in the *lfy-5* background, the opposite of the effect of the *sup-1* mutation, part of *ASK1* function in regulating cell proliferation may be mediated by *AP3* and *PI*. Furthermore, the *ask1-1* mutation could enhance the phenotype of *ap3-3* or *pi-1* mutants in the reduction of floral organ number interior to whorl one, particularly the number of sepal or sepal-like organs (Table 2). Therefore, the *ASK1* and *AP3*/*PI* genes seem to have redundant functions in regulating cell proliferation in this region of the flower. This suggests that part of *ASK1*'s function in cell proliferation is independent of *AP3* and *PI*.

Models for ASK1 and UFO actions in regulating *AP3* and *PI* expression

UFO is an F-box containing protein and ASK1 is a homologue of the yeast and human SKP1 protein (Ingram et al., 1995; Yang et al., 1999). Both SKP1 and F-box containing proteins

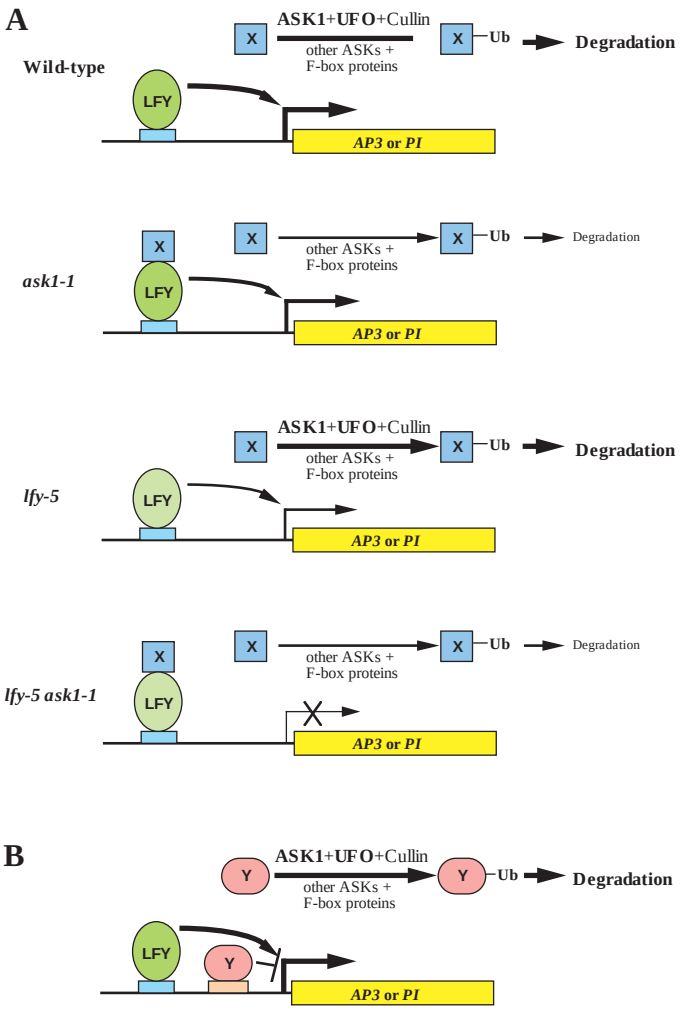

Fig. 6. Models for regulation of *AP3* and *PI* expression by LFY, ASK1 and UFO. (A) Model I. ASK1 and UFO facilitate the degradation of a negative modulator (X) of LFY protein activity. In the wild type, the level of X is low due to the function of ASK1, UFO and other ASK and F-box proteins, together with cullin, the third subunit of SCF. In the *ask1-1* mutant, the level of X may increase slightly but other *ASK* genes can still provide some needed function. Wild-type LFY protein is not obviously affected by the slight increase of X. In the *lfy-5* mutant, the mutant protein has reduced activity, leading to decreased *AP3* and *PI* transcription. In the *lfy-5 ask1-1* double mutant, the combination of a weak LFY protein and an increased level of X causes a dramatic reduction of *AP3* and *PI* expression. If LFY activity is completely eliminated, as in *lfy-6* (not shown here), then there is little *AP3* and *PI* expression regardless of the level of X. (B) Model II, ASK1 and UFO facilitate the degradation of a transcriptional repressor (Y) of *AP3* and *PI* genes. Again, the combination of *ask1-1* and *lfy-5* mutations would result in both an increase in the Y repressor and a decrease in the LFY activator, and a severe reduction in *AP3* and *PI* expression, whereas either single mutation would have less pronounced effects. In the absence of LFY activator (*lfy-6*), the Y repressor would have no effect.

are subunits of the SCF ubiquitin ligase complex, suggesting that ASK1 and UFO might be components of a SCF complex that facilitates the degradation of a negative regulator of B function gene expression. For example, ASK1 and UFO may control the level of a negative modulator (X) of LFY protein activity (Fig. 6A). When the ASK1 and UFO proteins are both functional, the level of X is low, and the LFY protein is fully active. However, if the *ASK* or *UFO* gene is mutated, then X is present at an increased level. When LFY protein is normal, the effect of X is minor, but when LFY protein activity is reduced by mutations such as *lfy-5*, then the negative effect of X becomes much more obvious. Alternatively, ASK1 and UFO may regulate a direct repressor (Y) of *AP3* and *PI* expression, whereas LFY is an activator of these genes (Fig. 6B). In this case, we need to postulate that when LFY is fully functional, the presence of Y, due to *ask1* or *ufo* mutations, cannot reduce *AP3* and *PI* expression substantially. In contrast, when LFY function is reduced by the *lfy-5* mutation, then Y repression of *AP3* and *PI* becomes effective. In either model, ASK1 and UFO could also interact with other partners to regulate *AP3* and *PI* expression; nevertheless, mutant phenotypes and RNA expression analysis suggest that ASK1 and UFO are the primary players in the proposed network of regulators.

These possibilities could be tested by analyzing *cis* elements in *AP3* and *PI* promoters that mediate regulation by LFY and UFO/ASK1. If the first scenario is true, then the same elements should mediate the effects of both LFY and UFO/ASK1 because X regulates LFY activity. If the second situation is true, then Y could bind to a different site from the LFY-binding site(s) in the *AP3* and *PI* upstream regions. Promoter studies of *AP3* revealed that a region from –328 to the transcriptional start seems to mediate the effect of UFO, and the –1500 to –300 region of the *PI* promoter mediates the effects of LFY and UFO (Hill et al., 1998; Honma and Goto, 2000). Furthermore, within the –328 to 0 region of the *AP3* promoter, there are three putative sites (CArG boxes) for binding by MADS proteins; mutational analysis suggests that two of these mediate activation, whereas the third (CArG3) mediates repression of *AP3* (Hill et al., 1998; Tilly et al., 1998). Because the precise sites mediating LFY and UFO regulation were not mapped, further analysis is required to distinguish the above models.

Conclusion

We have shown here that the *ASK1* gene cooperates with *LFY* to activate *AP3* and *PI* expression, and it plays an important role in regulating floral organ primordia in whorls two and three. Our results also suggest that UFO also participates in these regulatory processes. The fact that ASK1 and UFO are both putative subunits of the SCF ubiquitin ligase suggests these proteins may regulate the level of other regulatory proteins that control cell division and/or transcription. These results support the idea that regulatory proteolysis can play important roles in controlling flower development.

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REFERENCES

Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J. W. and Elledge, S. J. (1996). SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. *Cell* **86**, 263-274.

Bowman, J. L., Sakai, H., Jack, T., Weigel, D., Mayer, U. and Meyerowitz, E. M. (1992). *SUPERMAN*, a regulator of floral homeotic genes in *Arabidopsis*. *Development* **114**, 599-615.

Bowman, J. L., Smyth, D. R. and Meyerowitz, E. M. (1989). Genes directing flower development in *Arabidopsis*. *Plant Cell* **1**, 37-52.

Bowman, J. L., Smyth, D. R. and Meyerowitz, E. M. (1991). Genetic interactions among floral homeotic genes of *Arabidopsis*. *Development* **112**, 1-20.

Ciechanover, A., Orian, A. and Schwartz, A. L. (2000). Ubiquitin-mediated proteolysis: biological regulation via destruction. *BioEssays* **22**, 442-451.

Coen, E. S. and Meyerowitz, E. M. (1991). The war of the whorls: genetic interactions controlling flower development. *Nature* **353**, 31-37.

Connelly, C. and Hieter, P. (1996). Budding yeast *SKP1* encodes an evolutionarily conserved kinetochore protein required for cell cycle progression. *Cell* **86**, 275-285.

Craig, K. L. and Tyers, M. (1999). The F-box: a new motif for ubiquitin dependent proteolysis in cell cycle regulation and signal transduction. *Prog. Biophys. Mol. Biol.* **72**, 299-328.

Drews, G. N., Bowman, J. L. and Meyerowitz, E. M. (1991). Negative regulation of the Arabidopsis homeotic gene *AGAMOUS* by the *APETALA2* product. *Cell* **65**, 991-1002.

Feldman, R. M. R., Correll, C. C., Kaplan, K. B. and Deshaies, R. J. (1997). A complex of Cdc4p, Skp1p, and Cdc53p/Cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. *Cell* **91**, 221-230.

Flanagan, C. A. and Ma, H. (1994). Spatially and temporally regulated expression of the MADS-box gene *AGL2* in wild-type and mutant *Arabidopsis* flowers. *Plant Mol. Biol.* **26**, 581-595.

Goto, K. and Meyerowitz, E. (1994). Function and regulation of the *Arabidopsis* floral homeotic gene *PISTILLATA*. *Genes Dev.* **8**, 1548-1560.

Gray, W. M., del Pozo, J. C., Walker, L., Hobbie, L., Risseeuw, E., Banks, T., Crosby, W., Yang, M., Ma, H. and Estelle, M. (1999). Identification of an SCF ubiquitin-ligase complex required for auxin response in *Arabidopsis thaliana*. *Genes Dev.* **13**, 1678-1691.

Haughn, G. W. and Somerville, C. R. (1988). Genetic control of morphogenesis in *Arabidopsis*. *Dev. Genet.* **9**, 73-89.

Hill, J. P. and Lord, E. M. (1989). Floral development in *Arabidopsis thaliana*: a comparison of the wild type and the homeotic *pistillata* mutant. *Can. J. Bot.* **67**, 2922-2936.

Hill, T. A., Day, C. D., Zondlo, S. C., Thackeray, A. G. and Irish, V. F. (1998). Discrete spatial and temporal cis-acting elements regulate transcription of the *Arabidopsis* floral homeotic gene *APETALA3*. *Development* **125**, 1711-1721.

Honma, T. and Goto, K. (2000). The *Arabidopsis* floral homeotic gene *PISTILLATA* is regulated by discrete cis-elements responsive to induction and maintenance signals. *Development* **127**, 2021-2030.

Huala, E. and Sussex, I. M. (1992). *LEAFY* interacts with floral homeotic genes to regulate Arabidopsis floral development. *Plant Cell* **4**, 901-913.

Ingram, G. C., Doyle, S., Carpenter, R., Schultz, E. A., Simon, R. and Coen, E. S. (1997). Dual role for *fimbriata* in regulating floral homeotic genes and cell division in *Antirrhinum*. *EMBO J.* **16**, 6521-6534.

Ingram, G. C., Goodrich, J., Wilkinson, M. D., Simon, R., Haughn, G. W. and Coen, E. S. (1995). Parallels between *UNUSUAL FLORAL ORGANS* and *FIMBRIATA*, genes controlling flower development in Arabidopsis and Antirrhinum. *Plant Cell* **7**, 1501-1511.

Jack, T., Brockman, L. L. and Meyerowitz, E. M. (1992). The homeotic gene *APETALA3* of Arabidopsis thaliana encodes a MADS box and is expressed in petals and stamens. *Cell* **68**, 683-697.

Jack, T., Fox, G. L. and Meyerowitz, E. M. (1994). Arabidopsis homeotic gene *APETALA3* ectopic expression: transcriptional and posttranscriptional regulation determine floral organ identity. *Cell* **76**, 703-716.

Jacobsen, S. E. and Meyerowitz, E. M. (1997). Hypermethylated *SUPERMAN* epigenetic alleles in *Arabidopsis*. *Science* **277**, 1100-1103.

Jentsch, S. and Pyrowolakis, G. (2000). Ubiquitin and its kin: how close are the family ties? *Trends Cell Biol.* **10**, 335-342.

Krizek, B. A. and Meyerowitz, E. M. (1996). The *Arabidopsis* homeotic genes *APETALA3* and *PISTILLATA* are sufficient to provide the B class organ identity function. *Development* **122**, 11-22.

Lee, I., Wolfe, D. S., Nilsson, O. and Weigel, D. (1997). A *LEAFY* co-regulator encoded by *UNUSUAL FLORAL ORGANS*. *Curr. Biol.* **7**, 95-104.

Levin, J. and Meyerowitz, E. M. (1995). *UFO*: an Arabidopsis gene involved in both floral meristem and floral organ development. *Plant Cell* **7**, 529-548.

Li, F., Flanagan, C. A., Zhao, Y., Ma, H. and Huang, H. (1999). Assignment of 44 Ds insertions to the linkage map of *Arabidopsis*. *Plant Mol. Biol. Rep.* **17**, 109-122.

Ma, H. (1994). The unfolding drama of flower development: recent results from genetic and molecular analyses. *Genes Dev.* **8**, 745-756.

Ma, H. and dePamphilis, C. (2000). The ABCs of floral evolution. *Cell* **101**, 5-8.

Martienssen, R. and Irish, V. (1999). Copying out our ABCs: the role of gene redundancy in interpreting genetic hierarchies. *Trends Genet.* **15**, 435-437.

Meyerowitz, E. M., Bowman, J. L., Brockman, L. L., Drews, G. N., Jack, T., Sieburth, L. E. and Weigel, D. (1991). A genetic and molecular model for flower development in *Arabidopsis thaliana*. *Development* **Supplement 1**, 157-167.

Parcy, F., Nilsson, O., Busch, M. A., Lee, I. and Weigel, D. (1998). A genetic framework for floral patterning. *Nature* **395**, 561-566.

Peters, J. M. (1998). SCF and APC: the Yin and Yang of cell cycle regulated proteolysis. *Curr. Opin. Cell Biol.* **10**, 759-768.

Porat, R., Lu, P. and O'Neill, S. D. (1998). *Arabidopsis SKP1*, a homologue of a cell cycle regulator gene, is predominantly expressed in meristematic cells. *Planta* **204**, 345-351.

Sakai, H., Krizek, B. A., Jacobsen, S. E. and Meyerowitz, E. M. (2000). Regulation of SUP expression identifies multiple regulators involved in Arabidopsis floral meristem development. *Plant Cell* **12**, 1607-1618.

Sakai, H., Medrano, L. J. and Meyerowitz, E. M. (1995). Role of *SUPERMAN* in maintaining *Arabidopsis* floral whorl boundaries. *Nature* **378**, 199-203.

Samach, A., Klenz, J. E., Kohalmi, S. E., Risseeuw, E., Haughn, G. W. and Crosby, W. L. (1999). The *UNUSUAL FLORAL ORGANS* gene of *Arabidopsis thaliana* is an F-box protein required for normal patterning and growth in the floral meristem. *Plant J.* **20**, 433-445.

Schultz, E. A. and Haughn, G. W. (1991). *LEAFY*, a homeotic gene that regulates inflorescence development in Arabidopsis. *Plant Cell* **3**, 771-781.

Schultz, E. A., Pickett, F. B. and Haughn, G. W. (1991). The *FLO10* gene product regulates the expression domain of homeotic genes *AP3* and *PI* in Arabidopsis flowers. *Plant Cell* **3**, 1221-1237.

Skowyra, D., Craig, K. L., Tyers, M., Elledge, S. J. and Harper, J. W. (1997). F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. *Cell* **91**, 209-219.

Smyth, D. R., Bowman, J. L. and Meyerowitz, E. M. (1990). Early flower development in *Arabidopsis*. *Plant Cell* **2**, 755-767.

Tilly, J. J., Allen, D. W. and Jack, T. (1998). The CArG boxes in the promoter of the *Arabidopsis* floral organ identity gene *APETALA3* mediate diverse regulatory effects. *Development* **125**, 1647-1657.

Weigel, D., Alvarez, J., Smyth, D. R., Yanofsky, M. F. and Meyerowitz, E. M. (1992). *LEAFY* controls floral meristem identity in Arabidopsis. *Cell* **69**, 843-59.

Weigel, D. and Meyerowitz, E. M. (1993). Activation of floral homeotic genes in *Arabidopsis*. *Science* **261**, 1723-1726.

Weigel, D. and Meyerowitz, E. M. (1994). The ABCs of floral homeotic genes. *Cell* **78**, 203-209.

Wilkinson, M. D. and Haughn, G. W. (1995). *UNUSUAL FLORAL ORGANS* controls meristem identity and floral organ primordia fate in Arabidopsis. *Plant Cell* **7**, 1485-1499.

Yang, M., Hu, Y., Lodhi, M., McCombie, R. and Ma, H. (1999). The *Arabidopsis SKP1-LIKE1* gene is essential for male meiosis and may control homologue separation. *Proc. Natl. Acad. Sci. USA* **96**, 11416-11421.

Yanofsky, M. F. (1995). Floral meristems to floral organs: Genes controlling early events in *Arabidopsis* flower development. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46**, 167-188.

Zhao, D., Yang, M., Solava, J. and Ma, H. (1999). The *ASK1* gene regulates development and interacts with the *UFO* gene to control floral organ identity in *Arabidopsis*. *Dev. Genet.* **25**, 209-223.