Regulation of Flower Development in Arabidopsis by SCF Complexes[1]

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SCF complexes are the largest and best studied family of E3 ubiquitin protein ligases that facilitate the ubiquitylation of proteins targeted for degradation. The SCF core components Skp1, Cul1, and Rbx1 serve in multiple SCF complexes involving different substrate-specific F-box proteins that are involved in diverse processes including cell cycle and development. In Arabidopsis, mutations in the F-box gene *UNUSUAL FLORAL ORGANS* (*UFO*) result in a number of defects in flower development. However, functions of the core components Cul1 and Rbx1 in flower development are poorly understood. In this study we analyzed floral phenotypes caused by altering function of Cul1 or Rbx1, as well as the effects of mutations in *ASK1* and *ASK2*. Plants homozygous for a point mutation in the *AtCUL1* gene showed reduced floral organ number and several defects in each of the four whorls. Similarly, plants with reduced *AtRbx1* expression due to RNA interference also exhibited floral morphological defects. In addition, compared to the *ask1* mutant, plants homozygous for *ask1* and heterozygous for *ask2* displayed enhanced reduction of B function, as well as other novel defects of flower development, including carpelloid sepals and an inhibition of petal development. Genetic analyses demonstrate that *AGAMOUS* (*AG*) is required for the novel phenotypes observed in the first and second whorls. Furthermore, the genetic interaction between *UFO* and *AtCUL1* supports the idea that UFO regulates multiple aspects of flower development as a part of SCF complexes. These results suggest that SCF complexes regulate several aspects of floral development in Arabidopsis.

An Arabidopsis flower has four concentric whorls that contain four sepals, four petals, six stamens, and two carpels. After the transition from vegetative to reproductive development, the Arabidopsis apical meristem (inflorescence meristem) produces the floral meristem, which in turn undergoes a series of developmental stages to form a flower (Smyth et al., 1990). Genetic and molecular studies have uncovered a large number of genes that control different steps in flower development including flowering time, flower meristem identity, and flower organ identity (Zhao et al., 2001a). In particular, the ABC model has been proposed for the specification of floral organ identity (Coen and Meyerowitz, 1991; Ma, 1994; Weigel and Meyerowitz, 1994; Ma and dePamphilis, 2000). The combinatorial expression of ABC genes defines the organ type that differentiates in each whorl: A function alone specifies the sepal identity; A and B function together controls petal identity; B and C function together specifies stamen identity; and C function alone directs carpel identity.

The *UNUSUAL FLORAL ORGANS* (*UFO*) gene is involved in multiple aspects of floral development, including regulating floral meristem identity and floral organ development (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). One known function of *UFO* in floral organ development is a positive regulation of the expression of B function gene *APETALA3* (*AP3*) in cooperation with the floral meristem identity gene *LEAFY* (Lee et al., 1997; Zhao et al., 2001b). Recently, a novel role of *UFO* in early petal formation was uncovered through the analysis of newly isolated *ufo* alleles (Durfee et al., 2003) and the transient restoration of *UFO* function in the strong *ufo-2* mutant (Laufs et al., 2003).

The *UFO* gene encodes one of the approximately 700 F-box proteins that are believed to be components of the SCF-type E3 ubiquitin ligase (Gagne et al., 2002; Kuroda et al., 2002). The ubiquitin ligase (E3) functions in a pathway with a ubiquitin-activating enzyme (E1) and a ubiquitin-conjugating enzyme (E2) to catalyze the ubiquitylation of proteins targeted for degradation (Koepp et al., 1999; Pickart, 2001). The SCF complexes are members of the largest and best-studied family of E3 ubiquitin ligases. In addition to the substrate-recognition factor F-box protein, an SCF complex consists of Skp1, Cul1/Cdc53, and a RING finger protein Rbx1/Hrt/Roc1. Cul1 functions as a scaffold protein linking Skp1 with Rbx1, which acts to recruit the E2 enzyme. Skp1 serves as an adaptor that bridges Cul1/Cdc53 and the F-box protein (Deshaies, 1999; Schulman et al., 2000; Jackson and Eldridge, 2002; Zheng et al., 2002).

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Homologs of the core components of the SCF ubiquitin ligase have been found in Arabidopsis. At least five *Cul* homologs are expressed in the Arabidopsis genome. Among these, AtCUL1 and AtCUL2 are able to interact with ASK1 and F-box proteins in a yeast (*Saccharomyces cerevisiae*) two-hybrid assay (Risseeuw et al., 2003). However, AtCUL1 is the only Cul1 homolog in Arabidopsis that has been verified to be part of SCF complexes in vivo (Gray et al., 1999; Xu et al., 2002). In addition, a null mutant allele of *AtCUL1* exhibited embryo arrest at the single cell stage (Shen et al., 2002). Consistent with its broad expression pattern (Farras et al., 2001; del Pozo et al., 2002b; Shen et al., 2002), reduced AtCUL1 functions cause severe auxin related defects throughout plant development (Hellmann et al., 2003). Thus, it is likely that AtCUL1 is a component of multiple SCF complexes that play critical roles in development.

Two *Rbx1* homologs were uncovered in the Arabidopsis genome. The two Rbx1 proteins are highly similar to each other as well as to that of the human Rbx1 proteins. However, based on expression levels, AtRbx1a seems to be the dominant participant in SCF complexes (Gray et al., 2002; Lechner et al., 2002). Altered expression of *AtRbx1* causes severe defects in plant growth and development (Gray et al., 2002; Lechner et al., 2002; Schwechheimer et al., 2002; Xu et al., 2002), indicating that its function is essential.

Among the 21 Arabidopsis *Skp1* homologs (called *ASK*) in the Arabidopsis genome, at least 18 were found to be expressed under normal growth conditions, with a large subset of them detected in the inflorescence (Zhao et al., 2003b). The *ask1-1* mutant is male sterile and defective in both vegetative and reproductive development (Yang et al., 1999; Zhao et al., 1999). Recently, we have shown through mutations in *ASK1* and *ASK2* that these two genes are essential for normal embryo and seedling development (Liu et al., 2004). The relatively weak floral phenotypes of the *ask1-1* null mutant compared to that of strong *ufo* alleles suggest that other *ASK* genes might also interact with *UFO* to regulate flower development. ASK1 and ASK2 are very similar in sequence and expression patterns (Zhao et al., 2003b), and both can interact with a similar set of F-box proteins (including UFO) in yeast two-hybrid assays (Gagne et al., 2002; Risseeuw et al., 2003). Thus, *ASK1* and *ASK2* are also likely to share redundant functions in flower development.

ASK1 interacts genetically and in yeast two-hybrid assays with *UFO* (Samach et al., 1999; Zhao et al., 1999, 2001b). In addition, *ASK1* and *UFO* interact with *LEAFY* genetically to positively regulate B function gene expression (Zhao et al., 2001b). Furthermore, UFO interacts with ASK1 and AtCUL1 in an immunoprecipitation assay (Wang et al., 2003), supporting a role of the SCF^{UFO} complex in flower development. However, the functions of the SCF core components, Cul1, Rbx1, and *ASK2*, in flower development have not previously been demonstrated. We report here the role of *AtCUL1* and *AtRbx1* in flower development. We also provide the first genetic evidence that *UFO* interacts with *AtCUL1* to regulate several aspects of flower development. Moreover, genetic studies indicate that *ASK1* and *ASK2* share redundant functions in flower development, including the regulation of B function gene expression. Finally, we describe genetic evidence that supports a novel role of SCF^{UFO} in regulating C function.

RESULTS

Floral Phenotypes of *axr6-2*, a Point Mutant in *AtCUL1*

A previous study showed that a null mutation in the *AtCUL1* gene causes embryo lethality (Shen et al., 2002); therefore, the null mutant cannot be used to investigate a possible *AtCUL1* function in flower development. Recently, a previously identified auxin resistant mutant, *axr6-2*, was found to carry a point mutation (replacement of Phe-111 by isoleucine) in *AtCUL1* that results in a reduced physical interaction between ASK1 and AtCUL1 (Hobbie et al., 2000; Hellmann et al., 2003). Furthermore, viable plants homozygous for the weak *axr6-2* allele have been obtained (G. Badrajan and L. Hobbie, unpublished), enabling analysis of the flowers. We have examined flower development in the homozygous *axr6-2* mutant and found that *axr6-2* flowers exhibited reduced organ numbers and/or various defects in all four whorls (with an average total organ number of 11.34 per flower; Table I).

Compared with the wild-type flower (Fig. 1A), a typical *axr6-2* mutant flower (Fig. 1B) has a slight reduction in the number of sepals, which are occasionally fused together (Table I). In the second whorl, the number of petals is reduced, and occasionally the size of petals is also reduced (Fig. 1B; Table I). In the third whorl, there are fewer than the normal number of stamens, and the stamen filaments are shorter than those of the wild type (data not shown). In addition, filamentous structures were also observed in the third whorl (Fig. 1B; Table I). Unlike *ufo* and *ask1* flowers, no petal/stamen chimeric organs were observed in the *axr6-2* flower (Table I), and whorls are clearly defined in the *axr6-2* flower. In the fourth whorl, the number of carpels seems to be normal. However, about 25% of the gynoecia are curled (Fig. 1B), and occasionally carpels are not fused.

In addition, we also observed some small flower buds in the *axr6-2* mutant which never opened (Fig. 1C). These flowers were found to contain aborted petals, stamens, and carpels inside relatively normal sepals.

Floral Phenotypes of Inducible *AtRbx1* RNAi Plants

Continuous silencing of *AtRbx1* expression by double-strand RNA interference caused severe defects in plant growth and development (Lechner et al., 2002; Xu et al., 2002; data not shown). Therefore, we

Table I. *Comparison of floral organs*

	Ler	axr6-2	ufo-6	axr6-2, ufo-6[a]	ask1-1	ask2-1	ask1-1, ask2-1/+
Whorl 1							
Sepal	4.00 ± 0.00	3.72 ± 0.35	4.00 ± 0.00	3.35 ± 0.81	4.00 ± 0.00	4.00 ± 0.00	3.75 ± 0.61
Carpel-like	0	0	0	0.05 ± 0.21	0	0	0.07 ± 0.30
Whorl 2							
Petal	4.00 ± 0.00	1.51 ± 0.88	1.70 ± 0.42	0.07 ± 0.26	2.94 ± 0.61	4.00 ± 0.00	0.14 ± 0.41
Sepal-like petal	0	0	0.10 ± 0.21	0.02 ± 0.15	0	0	0
Whorls 2 and 3							
Petal-stamen chimera	0	0	2.06 ± 0.48	0.19 ± 0.45	0.76 ± 0.54	0	0.08 ± 0.29
Filament	0	0.13 ± 0.30	0.38 ± 0.62	0.86 ± 0.97	0	0	0.43 ± 0.70
Stamen	6.00 ± 0.00	4.02 ± 0.72	3.80 ± 0.88	3.16 ± 1.15	4.88 ± 0.61	6.00 ± 0.00	4.77 ± 1.39
Carpel-like[b]	0	0	0.05 ± 0.18	0.30 ± 0.56	0	0	0.23 ± 0.55
Whorl 4							
Carpel	2.00 ± 0.00	1.96 ± 0.10	2.03 ± 0.14	2.16 ± 0.37	2.00 ± 0.00	2.00 ± 0.00	2.08 ± 0.27
Sum of all organs	16.00 ± 0.00	11.34 ± 1.46	14.17 ± 0.73	10.16 ± 1.59	14.59 ± 0.69	16.00 ± 0.00	11.53 ± 1.52

All plants were grown under the same conditions and the average number of organs per flower is given ± SE. Unless otherwise indicated, the first 10 flowers on each given plant were analyzed, and a total of 100 flowers from 10 plants were examined. [a]A total of 43 flowers were analyzed. [b]Mosaic organs include carpel/sepal, carpel/stamen, carpel/filament, and carpel/sepal/stamen.

examined the effects of reducing *AtRbx1* expression during flower development by using inducible *AtRbx1* RNAi plants previously generated by Genschik and colleagues (Lechner et al., 2002). In agreement with previous results, we found that the transgenic plants treated for a period of 5 to 7 d with dexamethasone (Dex) can recover and develop further after being transferred to a Dex-free environment (see "Materials and Methods"). Therefore, this system provides another opportunity to study the role of SCF complexes in flower development.

All plants produce normal flowers without Dex treatment. Control plants transformed with an empty vector produced normal flowers after Dex treatment (Fig. 1D), except for occasional reductions in stamen number. In contrast, the flower of Dex-treated *Rbx1* RNAi plants exhibited slightly reduced petal number and reduced stamen filament length (Fig. 1E). In addition, petal/stamen chimeras, filaments, and carpelloid organs were also observed in the middle whorls of the Dex-treated *Rbx1* RNAi flowers (Fig. 1E; and data not shown), indicating a reduction of B function. Sometimes a curled gynoecium (Fig. 1E), or an axillary flower (Fig. 1F) was also observed in the *AtRbx1* RNAi flowers.

Genetic Interaction of *ASK1* and *ASK2*

The *ask1-1* mutant contains a Ds insertion in the middle of the coding region (Fig. 2A; Yang et al., 1999). Recently a mutation in the coding region of the *ASK2* gene (*ask2-1*) was isolated from a T-DNA insertional population (Fig. 2A; Liu et al., 2004). *ASK1* and *ASK2* expression was not detected by RNA blot in the respective single mutant. To test for possible genetic interactions between *ASK1* and *ASK2*, we compared flower development in different genotypes with wild-type and mutant alleles of these two genes (Fig. 2, A and B, see "Materials and Methods"). Consistent with previous results in our laboratory, we observed a slightly reduced number of petals and stamens, staminoid petals, and short stamen filaments in the flowers of the *ask1-1* mutant (Fig. 1H; Table I; Zhao et al., 1999).

The flower of *ask2-1* (Fig. 1I; Table I) was indistinguishable from that of the wild type. In the F2 progeny of a cross between *ask1* and *ask2*, we found that the *ask1 ask2* double mutant is defective in embryo development and is seedling lethal (Liu et al., 2004). Further examination revealed that some F2 plants displayed a novel floral phenotype characterized by the near absence of petals. From 400 F2 plants, we observed 22 (close to 1/15) plants with the *ask1-1*-like flower and 46 (close to 2/15) plants with this novel phenotype. All other plants were similar to the wild type. PCR analysis demonstrated that all F2 plants with the *ask1*-like flower were *ask1* single mutants (homozygous for the wild-type allele at the *ASK2* locus), whereas all F2 plants with the novel petalless phenotype were of the *ask1/ask1 ASK2/ask2* genotype. The *ASK1/ask1 ask2/ask2* plants produced normal flowers. In addition, we performed PCR analysis on 192 F2 plants with no apparent phenotype, and none of them were *ask1/ask1 ASK2/ask2*. The RNA expression levels of the *ASK2* gene were further tested using real-time PCR. Compared to the wild type, *ASK2* gene expression is close to normal in the *ask1* mutant, whereas its expression is reduced to about 48% in the *ask1/ask1 ASK2/ask2* plant (Fig. 2C). Residual *ASK2* expression was also detected in the *ask2-1* mutant using primers C-terminal relative to the T-DNA insertion (data not shown).

As mentioned above, the most dramatic phenotype in the *ask1/ask1 ASK2/ask2* plants is the absence of second whorl organs in most flowers (Fig. 1, J–N; Table I), with an average number of 0.14 petals per flower. In addition, carpelloid stamens and filaments were observed in the third whorl of the *ask1/ask1 ASK2/ask2*

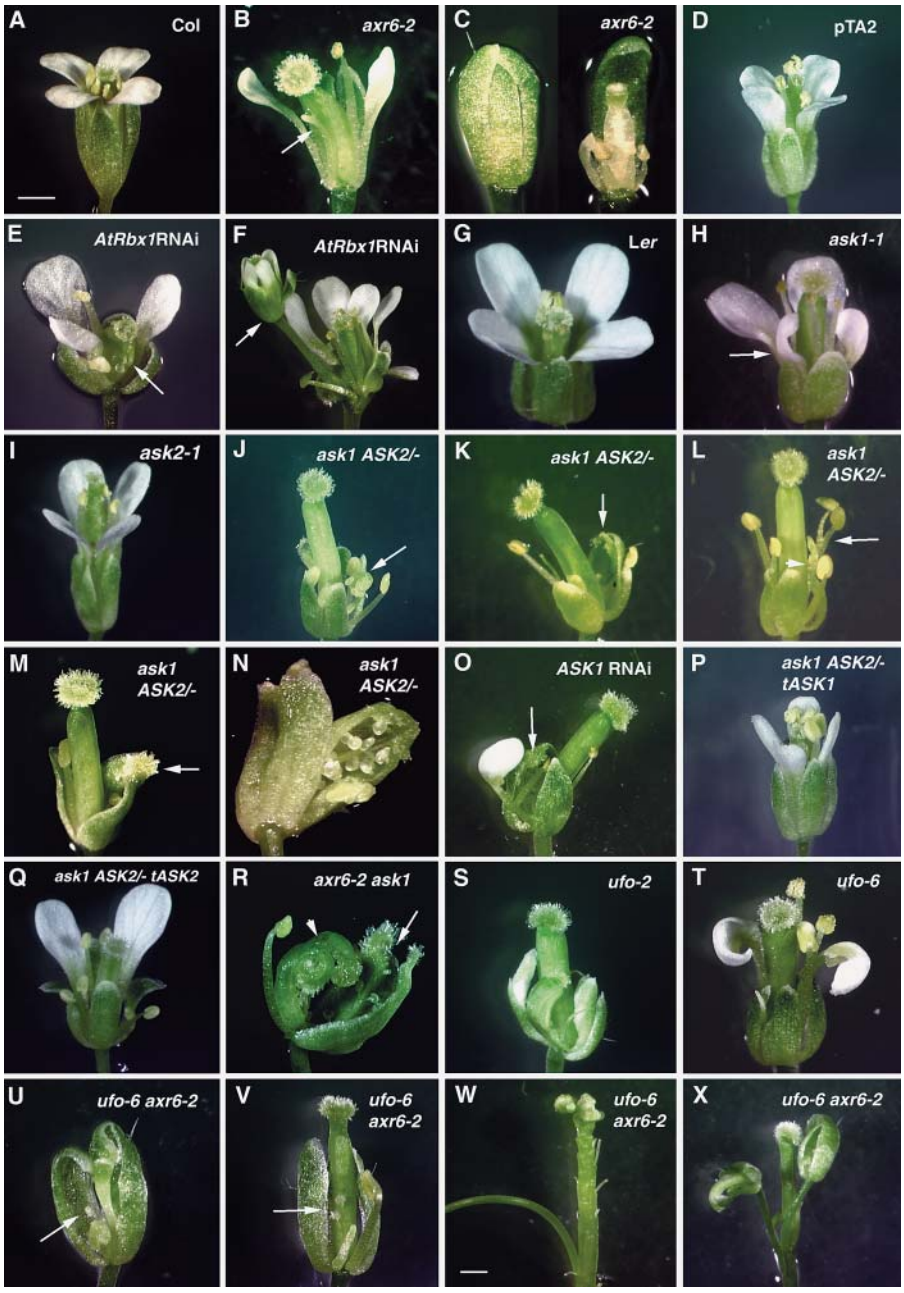

Figure 1. The phenotypes of Arabidopsis wild-type and mutant flowers. One sepal was removed to show the interior organs in B, E, F, K, L, M, O, R, S, and V. A, A wild-type flower of the Columbia ecotype. B, An *axr6-2* flower with reduced number of petals and stamens, a filament (arrow), and curled gynoecium. C, An aborted *axr6-2* flower that did not open. The right flower is the same flower as the left one, but three sepals were removed to show the interior organs. D, A Dex-treated pTA2 control flower of WS ecotype. E, A Dex-treated *AtRbx1* RNAi flower with three petals and ovule-like organs which fuse to the fourth whorl carpel (arrow). F, A Dex-treated *AtRbx1* RNAi flower showing a second flower (arrow). G, A wild-type flower of L*er* ecotype. H, An *ask1* (L*er* ecotype) flower with small staminoid petals (arrow). I, An *ask2* flower (WS ecotype) with normal floral organs. J to N, *ask1/ask1 ASK2/ask2* flowers with no petals, short stamens, and a carpel-like anther (arrow in J), a filament, a carpel-like sepal (arrow in K), curled carpels (K), a filament fused to a carpel (arrowhead in L) and fused stamens (arrow in L), papillae-like structures in the first whorl (arrow in M), or fused sepals and unfused carpels (N). O, An *ASK1* RNAi flower (L*er* ecotype) with papillae-like structures in the first whorl (arrow), one petal, and curled carpel. P, A normal *ask1/ask1 ASK2/ask2 tASK1* flower. Q, An *ask1/ask1 ASK2/ask2* 35S:*ASK2* flower with *ask1*-like phenotypes. R, an *axr6-2 ask1* flower with carpelloid first whorl sepals (arrow) and curled, unfused fourth whorl carpels (arrowhead). S, A *ufo-2* flower with sepal-like petals, filaments, and carpel-like structure in the middle whorls. T, A *ufo-6* flower showing reduced petal number and staminoid petals U, A *ufo-6 axr6-2* flower with ovule-like organ (arrow) in the first whorl, greatly reduced organ number in middle whorls and aborted carpel. V, A *ufo-6 axr6-2* flower showing papillae-like structures (arrow). W, A *ufo-6 axr6-2* inflorescence with a filament-like structure instead of a normal flower. X, A terminated *ufo-6 axr6-2* inflorescence with pistil-like structure occupying the inflorescence meristem. Scale bars = 0.5 mm. A–V have the same magnification; W and X have the same magnification.

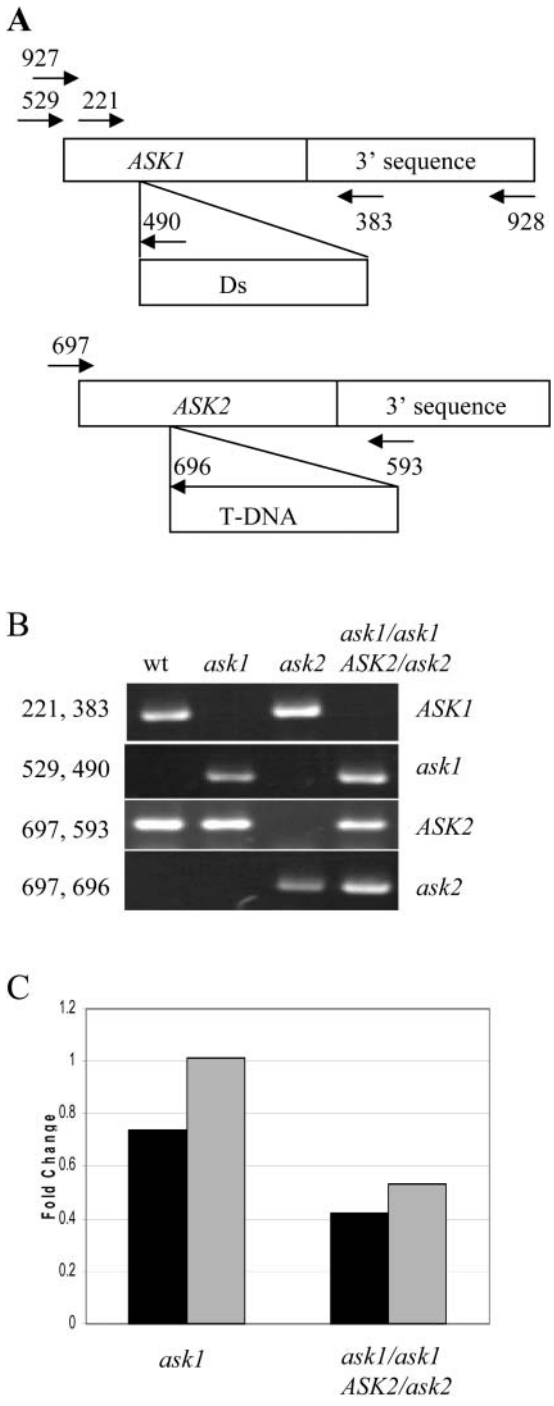

Figure 2. Characterization of *ask2* and *ask1 ASK2/ask2*. A, Schematic map of the *ask1-1* and *ask2-1* mutations and primers for identifying genotypes in F2 plants. B, Specificity of the primer combinations (left side of the panel) used in PCR reaction to amplify either *ASK1*, *ask1-1*, *ASK2*, or *ask2-1* alleles (right side). C, Relative mRNA expression levels of *ASK2* gene in mutants determined by real-time PCR. Black and gray bars represent results from two independent experiments. *ASK2* expression in the wild type was considered as 1.

flowers (Fig. 1, J and K; Table I). Other defects that were also observed in whorls 2 and 3 include a variable number of stamens, both fewer and more than six, and chimeras of petal/stamen and sepal/carpel (Fig. 1K). Sometimes organs in the same whorl, or between different whorls, were fused together (Fig. 1L). The first and fourth whorls of the *ask1/ask1 ASK2/ask2* flower were mostly normal, with a slightly reduced number and variable size of sepals, and a slightly increased number of carpels (Table I; and data not shown). Occasionally, carpelloid sepals (Fig. 1M; Table I), fused sepals (Fig. 1N), curled carpels or unfused carpels (Fig. 1, K and N) were also observed; these defects were more severe in late flowers (data not shown). The carpelloid sepals and absence of organs in whorl 2 in the *ask1/ask1 ASK2/ask2* flower are similar to the phenotype of *ap2* mutants, in which the C function gene *AGAMOUS* (*AG*) expands to the first and second whorls (Kunst et al., 1989; Bowman et al., 1991; Drews et al., 1991).

We have also generated *ASK1* RNAi transgenic plants (Zhao et al., 2003b). Similar floral phenotypes to those mentioned above in the *ask1/ask1 ASK2/ask2* plants were also observed in strong *ASK1* RNAi plants, including a further reduction in petal number compared to the *ask1* single mutant; carpelloid organs in the first and middle whorls; and enlarged, curled, or unfused carpels (Fig. 1O; and data not shown; Zhao et al., 2003b). In the inflorescences of strong *ASK1* RNAi plants, *ASK1* expression was not detected, while *ASK2* expression was reduced (Zhao et al., 2003b).

To further verify that the enhanced and novel phenotypes in *ask1/ask1 ASK2/ask2* flowers were caused by the combination of *ask1* and *ask2* mutations, we carried out a functional complementation of the *ask1/ask1 ASK2/ask2* mutant with either a genomic *ASK1* or a fusion of the *ASK2* cDNA with the 35S promoter of the cauliflower mosaic virus (CaMV). The flowers of *ask1/ask1 ASK2/ask2* mutant with a transgenic genomic *ASK1* were restored to normal (Fig. 1P; and data not shown). In addition, the flowers of *ask1/ask1 ASK2/ask2* mutant with the 35S-*ASK2* construct exhibited phenotypes similar to those of the *ask1* single mutant, including less reduction in petal number compared to the *ask1/ask1 ASK2/ask2* flowers and correct organ identities for sepal, stamen, and carpel (Fig. 1Q). However, filaments were still occasionally observed in these plants, and the petal number was less than that in the *ask1* mutant. This may be due to a possible difference in the expression pattern of the 35S promoter from that of the endogenous *ASK2* regulatory elements. In conclusion, our results demonstrated that reduced expression of *ASK1* and *ASK2* is responsible for the *ap2*-like phenotype in the outer two whorls, the enhanced reduction of B function in the third whorl, and the defects in carpel identity in the fourth whorl of the *ask1/ask1 ASK2/ask2* flower.

We have also made a cross between *axr6-2* and *ask1-1*. Similar to the *ask1/ask1 ASK2/ask2* flower, carpelloid

stamens (data not shown), carpelloid first whorl sepals, and unfused fourth whorl carpels (Fig. 1R) were also observed in the *axr6-2 ask1* flower.

Genetic Interaction of *UFO* and *AtCUL1*

Previous studies in our laboratory have shown that *UFO* interacts with *ASK1* genetically to positively regulate B function gene expression (Zhao et al., 2001b). To further investigate if *UFO* interacts with *AtCUL1* to regulate flower development, we generated a double mutant between *axr6-2* and *ufo-6* (a weak *ufo* allele) and compared the floral phenotypes of the double mutant with those of the single mutants and *ufo-2*, a strong *ufo* allele. Our results from the *ufo* single mutants are in agreement with previous reports (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995).

The strong *ufo-2* mutant flower exhibited obvious defects in organ identity in the middle whorls, with sepal-like petals and filament-like structures occupying most of the organ positions in the second and third whorl, respectively (Fig. 1S). The first whorl sepals are largely normal. Occasionally two sepals are fused together. Enlarged or increased number of carpels was observed in the fourth whorls. In the weak *ufo-6* mutant, sepals and carpels are generally normal, whereas the number and identity of petals and stamens are somewhat altered (Fig. 1T; Table I). Filaments and occasionally sepal-like petals were observed (Table I), suggesting a slightly reduced B function. In addition, petal/stamen chimeric organs were frequently observed. The inflorescence of the *ufo*-6 mutant is normal.

In *ufo-6 axr6-2* double mutant flowers, fused sepals were more frequently observed compared to the *axr6-2* single mutant. Occasionally carpel-like sepals were observed in the first whorl (Fig. 1U; Table I). Compared to the *axr6-2* and two *ufo* single mutants, the number of second whorl organs in the *ufo-6 axr6-2* double mutant was greatly reduced, similar to those found in *ask1/ask1 ASK2/ask2* flowers (0.09 per flower; Table I). Most flowers in the double mutant had no petals (Fig. 1, U and V). Occasionally a petal, or sepal-like petal, was observed in the second whorl (Table I). In the third whorl, the number of stamens was further reduced compared to the *ufo-6* and *axr6-2* single mutants, whereas carpel-like structures and filaments were increased in the double mutant (Fig. 1V; Table I). In the fourth whorl, the phenotypes of double mutant carpels were similar to those of *axr6-2* single mutant. The increased carpelloid organs and filaments in the third whorl of *ufo-6 axr6-2* double mutant flower support the hypothesis that these two genes act together to promote B function; there also seems to be a unique aspect of the phenotypes that may not result from B function, suggesting additional roles for AtCUL1, particularly in organ formation and carpel development.

Moreover, we observed inflorescence defects in the *ufo-6 axr6-2* double mutant, including a filament-like structure instead of a normal flower (Fig. 1W), and termination of an inflorescence by a pistil-like structure (Fig. 1X), after the production of only four to five flowers. These defects were not observed in *ufo-6* or *axr6-2* single mutant.

AP3 and *AG* Expression in Wild-Type and Mutant Flowers

Our phenotypic analysis of Dex-treated *AtRbx1* RNAi and *ask1/ask1 ASK2/ask2* plants suggests that B function gene expression may be reduced in these plants. To evaluate this possibility, we performed RNA in situ hybridization with an *AP3* probe. In the inflorescence of Dex-treated pTA2 control plants, *AP3* RNA was detected at a high level in petal and stamen primordia of a young floral bud (Fig. 3A). In the young buds of Dex-treated *AtRbx1* RNAi plants, the expression of *AP3* was detected at a lower level (Fig. 3B). In addition, compared to the wild type (Fig. 3C), the *ask1-1* flower showed slightly reduced *AP3* signals (Fig. 3D; Zhao et al., 2001b). Similarly, the *ask1/ask1 ASK2/ask2* flower also exhibited slightly reduced *AP3* signals (Fig. 3E).

To further confirm a reduction of *AP3* RNA expression in the *ask1* mutant and an enhanced reduction of *AP3* expression in the *ask1ask1/ ASK2/ask2* plant, we performed real-time PCR using the RNA isolated from young floral buds up to stage 8 (Smyth et al., 1990) from the wild-type, *ask1*, and *ask1/ask1 ASK2/ask2* plants. Compared to the wild type, *AP3* expression was reduced to approximately 45% in the *ask1* mutant, and further reduced to about 33% in the *ask1/ask1 ASK2/ask2* plant (Fig. 4).

In addition, the *ap2-1* like phenotype of *ask1/ask1 ASK2/ask2* flowers suggests an expansion of C function to the first and second whorl. To test whether C function is altered at the transcription level in the *ask1/ask1 ASK2/ask2* flower, we performed RNA in situ hybridization in the wild-type, *ask1*, and *ask1/ask1 ASK2/ask2* flowers with an *AG* probe. *AG* RNA was detected in the central dome of wild-type flowers starting at early stage 3, and thereafter was restricted to stamen and carpel primordia (Fig. 3F). Nonspecific signal was detected in the upper part of sepals in late stage flowers (starting from stage 7), as observed previously (Drews et al., 1991; data not shown). The *ask1* flower showed a normal spatial and temporal pattern of *AG* expression, and the expression level was also close to normal (Fig. 3G). The *ask1/ask1 ASK2/ask2* flower exhibits a slightly reduced *AG* expression level in the center (Fig. 3H), whereas the expression pattern was close to normal. No significant *AG* signal was detected in early sepal primordia (Fig. 3H). Compared to the wild-type flower, slightly earlier and stronger signals seemed to be detected at the upper part of sepals in the mutant flower (data not shown). However it is not certain whether these signals reflect altered *AG* expression pattern or are only nonspecific signals.

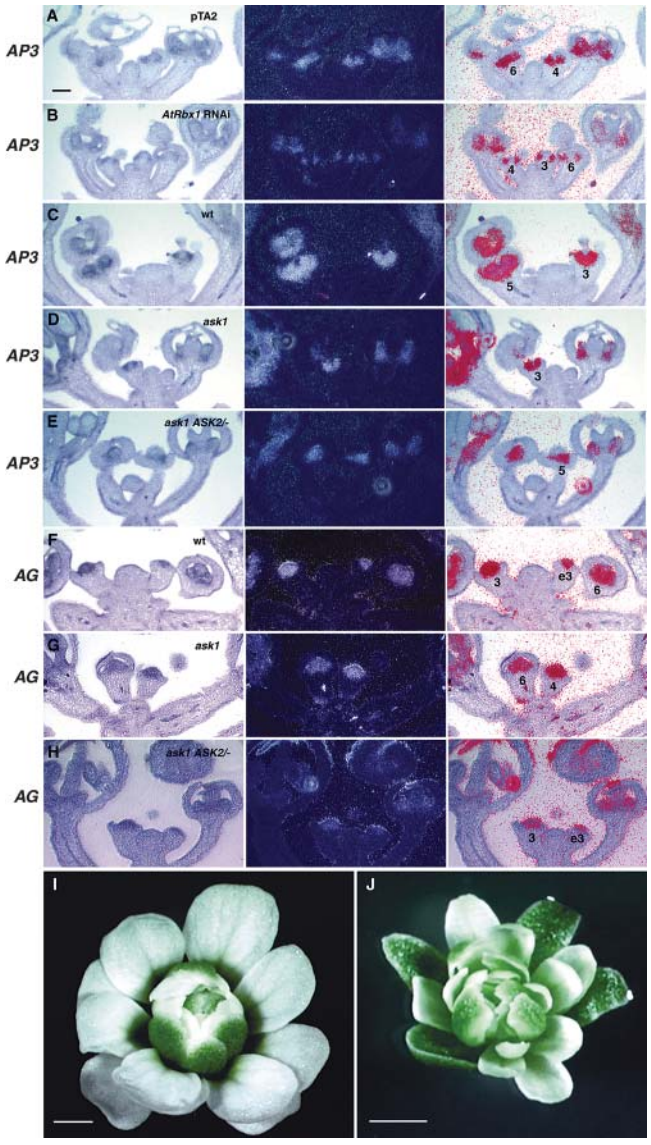

Figure 3. Expression of *AP3* and *AG* RNA in the inflorescence of wild type and mutants, and genetic interaction with *AG*. A to H, In situ RNA hybridization with an *AP3* probe (A–E) and an *AG* probe (F–H). The left panel in each triplet is a bright field image showing the tissues, the central panel is a dark field image, and the right panel is a composite image of both. Numbers indicate floral stages, and e3 means early stage 3. The sections in the following panels have been hybridized and developed at the same time: A and B; C, D, and E; F, G, and H. A, pTA2 control transgenic plant. B, *Rbx1* RNAi plant. C, Wild type. D, *ask1-1*. E, *ask1/ask1 ASK2/ask2*. F, Wild type. G, *ask1-1*. H, *ask/ask1 ASK2/ask2*. I, An *ag-1* flower with the third whorl stamens converted to petals and a new flower initiated in the fourth whorl. J, An *ask1 ASK2/ask2 ag-1* flower with normal organ identity in the first whorl and restored petal development in the second whorl. Scale bars = 50 μm (A–H), 0.5 mm (I and J).

Genetic Interaction with *AG*

To further test if *AG* is epistatic to *ASK1* and *ASK2* in terms of the *ap2*-like phenotypes in the *ask1/ask1 ASK2/ask2* flower, we crossed the *ask1/ask1 ASK2/ask2* into *ag-1*, a strong *ag* allele in which stamens are converted to petals, and whorl 4 is replaced by another flower (Bowman et al., 1989; Fig. 3I). The *ask1/ask1 ASK2/ask2 ag-1* flower is similar to the *ag-1* flower with normal whorl 1 organ identity and whorl 2 petals restored (Fig. 3J), suggesting that AG function is essential for the *ap2*-like phenotypes in the *ask1/ask1 ASK2/ask2* flower. Floral organs in the *ask1/ask1 ASK2/ask2 ag-1* plant are smaller than those in the *ag-1* mutant, suggesting that the defect in organ size of the *ask1/ask1 ASK2/ask2* flower is not dependent on AG function.

DISCUSSION

Ubiquitin-mediated protein degradation has been recognized as a very important mechanism for regulating many cellular events. In particular, the SCF ubiquitin-protein ligases are known to control cell cycle regulation, signal transduction, transcription, and other biological events (Bai et al., 1996; Hershko and Ciechanover, 1998; Schulman et al., 2000; DeSalle and Pagano, 2001; Conaway et al., 2002; Zheng et al., 2002). In Arabidopsis, several SCF complexes that are involved in hormone signaling or cell division have been characterized (Gray et al., 1999; del Pozo et al., 2002a; Xu et al., 2002). The existence of a large number of F-box genes in the Arabidopsis genome suggests that plants make extensive use of SCF complexes to regulate multiple biological processes (Gagne et al., 2002; Risseeuw et al., 2003).

Expression analysis indicates that many *ASK* genes are expressed in the inflorescence, suggesting that SCF complexes may play multiple roles in flower development (Zhao et al., 2003b). However, among the F-box and *ASK* genes, only *UFO* and *ASK1* have been shown to have roles in flower development. Functional redundancy among genes in these families may partially explain this limited characterization. *ASK1* and *ASK2* were found to be capable of interacting with many F-box proteins (Gagne et al., 2002; Risseeuw et al., 2003). In addition, *ASK1* and *ASK2* are both highly expressed

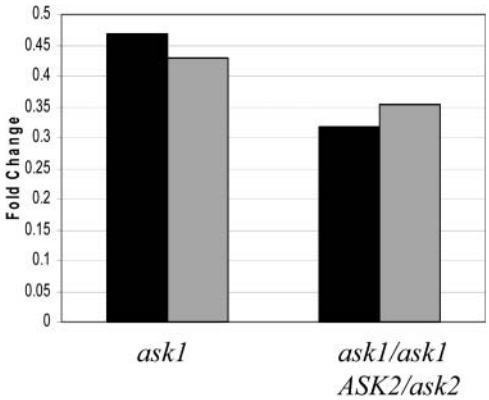

Figure 4. *AP3* transcript levels in mutant inflorescences. Relative mRNA expression levels of *AP3* gene in mutants were determined by real-time PCR. Black and gray bars represent results from two independent experiments. *AP3* expression in the wild type was considered as 1.

in all major tissues (Zhao et al., 2003b). Therefore, it is likely that *ASK1* or *ASK2* is a component of many SCF complexes, and they may share redundant functions throughout plant life cycle, including flower development. Indeed, we found that the *ask2* single mutant was indistinguishable from the wild type, whereas the *ask1 ask2* double homozygous mutant was seedling lethal (Liu et al., 2004), and the *ask1/ask1 ASK2/ask2* flower exhibited enhanced and additional novel phenotypes compared to the *ask1* flower. Although residual expression of the C terminus of the *ASK2* gene was detected in the *ask2-1* mutant by real-time PCR, the phenotypes in the *ask1/ask1 ASK2/ask2* flowers are not likely caused by altered ASK2 protein structure, as these phenotypes were also observed from strong *ASK1* RNAi plants, and were restored to normal with an additional copy of genomic *ASK1*.

Previous studies have suggested that *AtCUL1* and *AtRbx1a* encode core components of many SCF complexes in Arabidopsis and that they play critical roles throughout development (Gray et al., 2002; Lechner et al., 2002; Schwechheimer et al., 2002; Shen et al., 2002; Xu et al., 2002; Hellmann et al., 2003). Therefore, studies on the mutants or transgenic plants with a reduced function of these core components may also reveal the diverse roles of multiple SCF complexes in flower development. Indeed, we found diverse floral phenotypes in plants that are homozygous for a point mutation in the *AtCUL1* gene or carrying a Dex-induced *AtRbx1* RNAi construct.

Although several similar floral phenotypes were observed in all of the *ask1/ask1 ASK2/ask2*, *axr6-2,* and *AtRbx1* RNAi plants, distinctive floral phenotypes were also observed in plants of each of these genotypes. One of the explanations for the distinctive phenotypes is the partial functional redundancy within different members of *ASK* genes and cullin homologs. It is also possible that the *AtCUL1* point mutation might affect its interaction with some F-box proteins more so than that with others. In addition, although all of the *ASK1, ASK2*, *AtCUL1*, and *AtRbx1* genes encode core components of SCF complexes, we cannot rule out the possibility that these proteins can also function as a subunit of non-SCF complexes so that each gene may have its distinctive function.

Regulation of B Function Gene Expression by the SCF^{UFO} Complex

The floral phenotypes of *axr6-2*, *axr6-2 ask1*, and Dex-induced *AtRbx1* RNAi plants suggest a reduction of B function in these flowers. In situ results further suggested a reduction of *AP3* gene expression in the Dex-induced strong *AtRbx1* RNAi flower. Furthermore, the *ufo-6 axr6-2* double mutant showed an increased number of carpelloid organs and filaments in the third whorl, suggesting a further reduction in B function compared to either single mutant. Previous studies in our laboratory indicated that *UFO* interacts with *ASK1* and *LEAFY* genetically to regulate B function gene expression (Zhao et al., 1999, 2001b). In addition, UFO interacts with ASK1 and AtCUL1 physically (Samach et al., 1999; Wang et al., 2003). We have recently confirmed the physical interaction between UFO and ASK1 by coimmunoprecipitation using an anti-myc antibody and inflorescence extracts from 35S:UFO-myc transgenic plants (data not shown). Taken together, these results support the idea that B function gene expression is positively regulated by SCF^{UFO} complex consisting of UFO, AtCUL1, AtRbx1, and ASK1.

Consistent with previous results, we observed a slight reduction of B function in the *ask1-1* mutant flower (Zhao et al., 1999, 2001b). A further reduction of *ASK2* levels in the *ask1/ask1 ASK2/ask2* plants and the strong *ASK1* RNAi plants produced an additional reduction of B function in the flowers, which was further confirmed by complementation with either the *ASK1* or *ASK2* transgene. Both ASK1 and ASK2 can interact with UFO in a yeast two-hybrid assay (Samach et al., 1999). Therefore, it is likely that ASK1 and ASK2 share a redundant function in promoting B function through their interaction with UFO. Our results also suggest that *ASK1* plays a much more important role than *ASK2* in promoting B function, as the *ask1* flower exhibits phenotypes of reduced B function, whereas the *ask2* flower as well as the *ASK1/ask1 ask2/ask2* flower is normal.

Regulation of C Function by the SCF^{UFO} Complex and Other Possible SCF Complexes

The carpelloid sepals in the first whorl and near absence of petal in both the *ufo-6 axr6-2* and *ask1/ask1 ASK2/ask2* flowers were similar to those observed in *ap2* mutants, in which expression of the C function gene *AG* expands to the first and second whorls (Bowman et al., 1991; Drews et al., 1991). The similar phenotypes in strong *ASK1* RNAi flowers and genetic complementation of *ask1/ask1 ASK2/ask2* plants further verified that *ASK1* and *ASK2* are responsible for the *ap2*-like phenotype. In addition, carpelloid sepals were also observed in the *axr6-2 ask1* flowers. Genetic analysis suggests that *AG* function is essential for the *ap2*-like phenotypes in the *ask1/ask1 ASK2/ask2* flowers. Recently, new *ufo* alleles characterized by the absence of petals were isolated, which uncovered an additional role for UFO in promoting organ formation in the second whorl (Durfee et al., 2003). Genetic data suggested that UFO functions to inhibit an AG-dependent activity to promote early petal formation (Durfee et al., 2003). Intriguingly, the *ufo-6* mutation (P299 to L) maps immediately adjacent to the *ufo-14* mutation (S298 to A), which is required for petal formation (Lee et al., 1997; Durfee et al., 2003). In conjunction with this, petal/stamen mosaic organs were frequently observed in the second whorl of the *ufo-6* flower. One alternative explanation is that C function expands into the second whorl of the *ufo-6* flower and that this phenotype was enhanced by the *axr6-2* mutant. Taken together, these

results support a role for the SCF^{UFO} complex in promoting early petal formation through a negative regulation of AG function.

Unlike the *ufo-14* mutant, flowers in the *ufo-6 axr6-2* double mutant exhibited carpelloid sepals in the first whorl. Carpelloid sepals were also observed in strong *ufo* alleles in ecotype Landsberg *erecta* of Arabidopsis (L*er*) background (Wilkinson and Haughn, 1995). Similar carpelloid sepals were also observed in the *ask1/ask1 ASK2/ask2* flower. These results suggest that SCF^{UFO} complex(es) may also contribute to the inhibition of C function in the first whorl. The similar weak phenotypes found for the strong *ufo* alleles, the *ufo-6 axr6-2* double mutant, and the *ask1/ask1 ASK2/ask2* plant all indicate that other SCF complexes are also likely to be involved in inhibition of C function in the first whorl. Alternatively, UFO may retain some residual function even in these strong *ufo* alleles.

Our in situ results suggest that *AG* RNA did not expand to the sepal primordia in the *ask1/ask1 ASK2/ask2* flower. Similarly, *AG* RNA was not detected in the second whorl of the newly isolated petalless *ufo* alleles (Durfee et al., 2003), suggesting that AG might act noncell autonomously to play a role in the first and second whorls in those mutants. Alternatively, *AG* RNA may have been expressed at a very low level, or at late stages in the sepal of the *ask1/ask1 ASK2/ask2* flower. It is possible that the high level of nonspecific signals in the upper part of the late stage sepals makes it difficult to recognize the real *AG* signals.

SCF^{UFO} and other SCF complexes can repress AG protein function in the first and second whorls either through a direct repression of AG function, or through an indirect repression by activating a repressor of AG function. Because ectopic expression of UFO throughout the flower does not lead to an *ag* phenotype (Lee et al., 1997), it is not likely that UFO can act directly on AG, at least in the third and fourth whorl. Further support for the idea that UFO does not repress AG function directly in the first and second whorls can be obtained from an analysis on the double transgenic plant of 35S:*UFO* and 35S:*AG*. In addition to *AP2*, many other genes also contribute to the inhibition of AG function in the first and second whorls, including *AINTEGUMENTA* (Krizek et al., 2000), *LEUNIG* (Liu and Meyerowitz, 1995), *STERILE APETALA* (Byzova et al., 1999), *CURLY LEAF* (Goodrich et al., 1997), *INCURVATA2* (Serrano-Cartagena et al., 2000), and *SEUSS* (Franks et al., 2002). It is possible that the SCF^{UFO} complex and other SCF complexes contribute to the inhibition of AG function in the first and second whorls through the degradation of transcription repressors or other proteins to activate one or more of the negative regulators of AG function, although the SCF^{UFO} complex could also work separately to promote petal formation (Durfee et al., 2003; Laufs et al., 2003).

The curled or unfused carpel in the fourth whorl of the *ask1/ask1 ASK2/ask2* flowers suggests a reduced C function, as supported by the in situ hybridization results. Unfused carpels were also found in the *ufo-1* mutant when grown under short day (SD) condition (Wilkinson and Haughn, 1995), indicating that UFO might also participate in promoting C function in the fourth whorl. A role of UFO in the fourth whorl is also consistent with the expression of *UFO* in the center of a stage 2 flower primordia (Lee et al., 1997). Reduced C function was also observed in null mutants of *FIM* gene, the *UFO* homolog in *Antirrhinum majus* (Ingram et al., 1997). Thus, it is likely that the SCF^{UFO} complex and other SCF complexes might also function in promoting C function in the center of the flower.

Regulation of Other Aspects of Flower Development by the SCF^{UFO} Complex and Other SCF Complexes

The inflorescence of the *ufo-6 axr6-2* double mutant exhibited a filament-like structure instead of a normal flower and terminal inflorescence meristem, similar to those found in strong *ufo* mutants (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). Thus, it is likely that UFO also regulates floral and inflorescence meristem through an SCF^{UFO} complex containing AtCUL1.

An axillary flower similar to those found in *ap1* mutants (Bowman et al., 1993) was sometimes found in the *AtRbx1* RNAi flower. Similar secondary flowers were also found in *A. majus fim* null alleles (Ingram et al., 1997), indicating that the SCF^{UFO} complex or other SCF complexes are also involved in regulating floral meristem identity.

The *axr6-2* flower exhibited a reduction of floral organ number in all four whorls, indicating that SCF complexes also regulate floral organ number, probably through regulating cell division in the floral meristem. In addition, fused sepals, petals, and stamens were observed in *ask1/ask1 ASK2/ask2* and strong *ASK1* RNAi plants, which indicate that SCF complexes are also required for organ separation, probably through the regulation of cell division in each individual whorl. Furthermore, increased number or size of carpel was found in the *ask1/ask1 ASK2/ask2* flower. Similar phenotypes were also observed in strong *ufo* mutants. *UFO* seemed to be required for the restriction of cell division in the center of a stage 2 flower (Samach et al., 1999). Our results support that *UFO* interacts with *ASK1* or *ASK2* to restrict cell division in the central region.

In conclusion, our results indicate that SCF complexes regulate several aspects of floral development in Arabidopsis. Further functional studies on additional F-box proteins in flower development, as well as target proteins regulated by these SCF complexes, will provide insights into the network of flower development regulation by SCF complexes in Arabidopsis.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The *axr6-2* mutant is in the Columbia background (Hobbie et al., 2000). The following mutants and transgenic plants were in the Landsberg *erecta* (L*er*)

background: the *ufo-2*, *ufo-6* mutant (Levin and Meyerowitz, 1995), the *ask1-1* mutant (Yang et al., 1999), the *ASK1* RNAi plants (Zhao et al., 2003b), and the *ag-1* mutant (Bowman et al., 1989). The Dex-inducible *AtRbx1* RNAi lines (Lechner et al., 2002) and *ask2-1* mutant were of the Wassilewskija (Ws) ecotype.

The *axr6-2* mutant was backcrossed to the wild type eight times before phenotypic analysis. Among the rootless homozygous *axr6-2* seedlings cultured on Murashige and Skoog medium for 10 d, the few seedlings that developed roots were transferred to soil for further characterization.

For floral phenotypic analysis of *AtRbx1* RNAi plants, a strong line (dsRNA-2), a weak line (dsRNA-64), and a control line transformed with an empty vector were treated with Dex for 5 to 7 d at 2 to 3 weeks of age, then transferred to Dex-free conditions for further culture and analysis. Dex was dissolved in ethanol and kept at a concentration of 30 mM, and was directly added at a concentration of 1 μM to the medium or dripped onto plants in a solution at 10 μM (with 0.01% Tween 20). For RNA in situ hybridization, 3-week-old dsRNAi-2 plants and control plants were treated with Dex for 5 d, then fixed in formaldehyde-acetic acid fixative.

Plants were cultured on Murashige and Skoog medium, or grown on Metro-Mix 360 (Scotts-Sierra Horticultural Products, Marysville, OH) at 22°C (16 h light, 8 h dark).

Construction and Identification of Double Mutants

The single mutants used for phenotypic analysis and comparison were from self-pollination of either homozygous (*ufo-2*, *ufo-6*, *ask2-1*) or heterozygous (*axr6-2*, *ask1-1*, *ag-1*) plants. To construct a *ufo-6 axr6-2* double mutant, a heterozygous *axr6-2* plant was used as the male parent in a cross with a homozygous *ufo-6* plant. The genotype of the double mutant was determined by sequencing PCR products. For determining the homozygous *ufo-6*, genomic DNA was amplified with primers oMC 834 (5′-CTTTGCCACGGCTTTG-TAGCTTG-3′) and oMC 835 (5′-GACCCACAGCCAGCTTTTTCTCA-3′). For determining the homozygous *axr6-2*, DNA was amplified with primers oMC 836 (5′-TGTGGTTAGGTTTTGCCTGCGTT-3′) and oMC 837 (5′-AGCAG-GGCCCTATCAATCTGCTC-3′).

To construct an *ask1-1 ask2-1* double mutant, pollens from a homozygous *ask2-1* plant were used to pollinate homozygous *ask1-1* pistils. The genotypes of F2 plants were determined by PCR. The *ask1-1* mutant harbors a *Ds* transposon in *ASK1* at the position 237 bp downstream of the ATG start codon (Yang et al., 1999). The *ask2-1* mutant carries a T-DNA in *ASK2* at the position 318 bp downstream of the ATG start codon. To determine the genotypes of F2 plants, gene-specific and allele-specific primers were designed to amplify *ASK1* (wild-type allele), *ASK2*, *ask1-1* (mutant allele), and *ask2-1* (Fig. 2). The wild-type *ASK1* allele was amplified with primers oMC221 (5′-AAGGT-GATCGAGTATTGCAAGAG-3′) and oMC383 (5′-GAAGATAGTCATGATT-CATGAAG-3′). The *ask1-1* allele was amplified with primers oMC529 (5′-TCACTAGTGAGCTCATAACCATGTCTGCGAAGAA-3′) and oMC490 (5′-CGTTCCGTTTTCGTTTTTTACC-3′) on Ds element. The wild-type *ASK2* allele was amplified with primers oMC697 (5′-TCCACGTCGTCTCTAAACT-CAG-3′) and oMC593 (5′-AAATGGGTCGAGGACATGAC-3′). The *ask2-1* allele was amplified with primers oMC696 (5′-CCATCATACTCATTGCT-GATCC-3′) on T-DNA boarder region and oMC697 (see above).

For the complementation of *ask1/ask1 ASK2/ask2* floral phenotypes with *ASK1*, we crossed the *ask2-1* mutant with an *ask1-1* mutant harboring an *ASK1* transgene (*tASK1*), which contains a 5,578 bp *Hin*dIII/*Eco*RV genomic fragment including the *ASK1* gene and 4,183 bp upstream of the ATG (Zhao et al., 2003a). The F2 plants were first screened with Liberty herbicide (AgrEvo, USA Company, Montvale, NJ), then genotyped for the *ask1/ask1 ASK2/ask2* allele. The wild-type *ASK1* allele was identified with primers oMC927 (5′-GAGTT-CCGATGGTGAATCTTTC-3′) at 27 bp downstream of the *ASK1* ATG start codon, and oMC928 (5′-TAGCTCTTTTCGAGTGACCACA-3′) at 1,466 bp downstream of the *ASK1* ATG. All other primers for identifying *ask1*, *ASK2* and *ask2* alleles were the same as above.

For the complementation of *ask1/ask1 ASK2/ask2* with *ASK2*, we performed a cross between the *ask2-1* mutant and an *ask1-1* mutant harboring an *ASK2* transgene (*tASK2*), which contains an *ASK2* cDNA fused with the 35S promoter (Zhao et al., 2003a). The F2 plants were first screened with Liberty herbicide as above, then screened for the *ask1/ask1 ASK2/ask2* genotype harboring *tASK2*. The *tASK2* was identified with primers oMC570 (5′-CCGACAGTGGTCCCAAAGATGGA-3′) specific to 35S promoter and oMC593 (see above). All the primers for identifying the *ask1/ask1 ASK2/ask2* genotype were the same as above.

To construct an *ask1-1 axr6-2* double mutant, pollens from a heterozygous *axr6-2* plant were used to pollinate homozygous *ask1-1* pistils. To construct an *ask1/ask1 ASK2/ask2 ag-1* mutant, pollens from a heterozygous *ag-1* plant were used to pollinate *ASK1/ask1 ask2/ask2* pistils. The genotypes of F2 plants were determined by PCR as described before.

In Situ RNA Hybridization

Inflorescences from wild-type and mutant plants were harvested from 3- to 4-week-old plants and immediately fixed in a formaldehyde-acetic acid fixative. RNA in situ hybridizations with radioactive probes were performed as previously described (Drews et al., 1991; Flanagan and Ma, 1994). The *AP3* and *AG* antisense probes were synthesized using pD793 (digested with *Bgl*II) and pCIT565 (digested with *Hin*dIII) as template, respectively (Yanofsky et al., 1990; Jack et al., 1992). Both probes were synthesized with T7 RNA polymerase (Promega, Madison, WI).

RNA Quantitation by Real-Time PCR

RNA was isolated from the young inflorescence including stage 0 to 8 young floral buds (Smyth et al., 1990). Total RNA was isolated using the RNeasy mini kit (QIAGEN, Valencia, CA) and was treated with DNase I (Life Technologies/Gibco-BRL, Carlsbad, CA). One microgram of RNA from different tissues was reverse transcribed into cDNA with oligo(dT), 16 mer, using Super Script II reverse transcriptase (Life Technologies/Gibco-BRL) in a total volume of 20 μL. The cDNA was then diluted 100 times, and 5 μL of the diluted cDNA was used as a template for real-time PCR analysis. The primers were designed with Primer Express version 1.0 (ABI, Foster City, CA). The primer sequences were as follows: *ASK2*FW (oMC1531), 5′-GGACTGTTGGACTTGACTTGCC-3′; *ASK2*RV (oMC1532), 5′-GAGACACAAATGGGTCGAGGA-3′; *AP3*FW (oMC1529), 5′-GATGTCGATGTTTGGGCCAC-3′; *AP3*RV (oMC1530), 5′-AG-ATTTGAACTTGCGCTCGC-3′. The primers are expected to produce 201-bp products. Primers for *ACTIN* genes were used as an internal control to normalize the expression data for each gene. The primers were designed so that the two genes *ACTIN2* and *ACTIN8* were amplified simultaneously (Charrier et al., 2002). The sequence for the control primers are as follows: *ACTIN*FW (oMC1533) 5′-GGTAACATTGTGCTCAGTGGTGG-3′; *ACTIN*RV (oMC1534) 5′-AACGACCTTAATCTTCATGCTGC-3′. They are expected to produce a product of 108 bp.

The cDNA was amplified using the SYBR Green PCR Master Mix (Stratagene, La Jolla, CA) on the ABI PRISM 7700 thermocycler (ABI). The PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 60°C for 1 min, 72°C for 1 min. The cycle threshold values were used to calculate differences in fold changes. At the end of PCR cycles, the data were analyzed with the ABI Sequence Detection Systems (SDS) version 1.7 (ABI). To check the specificity of annealing of the PCR products, a dissociation kinetics was performed by the machine at the end of the experiment. In addition, PCR products were verified by sequencing directly. Negative control using the same amount of RNA did not produce any PCR product. In one experiment, at least three replications were performed for each sample. The experiments were repeated at least twice independently.

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