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The *UNUSUAL FLORAL ORGANS* gene of *Arabidopsis thaliana* is an F-box protein required for normal patterning and growth in the floral meristem

Alon Samach[1,†], Jennifer E. Klenz[1,§], Susanne E. Kohalmi[2,‡,§], Eddy Risseeuw[2], George W. Haughn[1,*] and William L. Crosby[2]

[1]*Department of Botany, University of British Columbia, 3529–6270 University Blvd, Vancouver, BC V6T 1Z4, Canada, and*

[2]*Gene Expression Group, NRC Plant Biotechnology Institute, 110 Gymnasium Place, Saskatoon, SK S7N 0W9, Canada*

Summary

Genetic and molecular studies have suggested that the *UNUSUAL FLORAL ORGANS* (*UFO*) gene, from *Arabidopsis thaliana,* is expressed in all shoot apical meristems, and is involved in the regulation of a complex set of developmental events during floral development, including floral meristem and floral organ identity. Results from *in situ* hybridization using genes expressed early in floral development as probes indicate that *UFO* controls growth of young floral primordia. Transgenic constructs were used to provide evidence that *UFO* regulates floral organ identity by activating or maintaining transcription of the class B organ-identity gene *APETALA 3*, but not *PISTILLATA*. In an attempt to understand the biochemical mode of action of the *UFO* gene product, we show here that UFO is an F-box protein that interacts with *Arabidopsis* SKP1-like proteins, both in the yeast two-hybrid system and *in vitro*. In yeast and other organisms both F-box proteins and SKP1 homologues are subunits of specific ubiquitin E3 enzyme complexes that target specific proteins for degradation. The protein selected for degradation by the complex is specified by the F-box proteins. It is therefore possible that the role of UFO is to target for degradation specific proteins controlling normal growth patterns in the floral primordia, as well as proteins that negatively regulate *APETALA 3* transcription.

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*For correspondence (fax +1 604 822 6089; e-mail haughn@interchange.ubc.ca).

[†]Present address: John Innes Centre, Colney Lane, Norwich NR4 7UH, UK.

[‡]Present address: Department of Plant Sciences, University of Western Ontario, 1151 Richmond Street North, London, ON N6A 5B7, Canada.

[§]Both of these authors have contributed equally to this research.

Introduction

In *Arabidopsis thaliana* the formation of a wild-type flower requires floral organ fate to be specified by three classes of organ identity genes (A, B, C) encoding putative transcription factors (for reviews see: Coen and Meyerowitz, 1991; Haughn *et al.*, 1995; Weigel and Meyerowitz, 1994; Yanofsky, 1995). These genes are expressed in different and partially overlapping domains within floral primordia to effect the formation of different organ types. The function of class A genes alone results in sepals in the outer whorl, while the overlap of class A and B gene functions in the second whorl specifies petals. Classes B and C function in the third whorl to specify stamens, and class C alone directs carpel development in the innermost whorl. The class B organ identity genes encode two transcription factors *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) (Goto and Meyerowitz, 1994; Jack *et al.*, 1992), which form a heterodimer (Riechmann and Meyerowitz, 1997) and activate the necessary program to form petals and stamens. Loss-of-function mutants in either *AP3* or *PI* fail to form petals and stamens, and instead form a second whorl of sepals in place of petals, and additional carpels in place of stamens (Bowman *et al.*, 1989; Hill and Lord, 1989; Jack *et al.*, 1992). When the *AP3* and *PI* organ identity genes are expressed ectopically throughout the flower (with the *CaMV 35S* promoter) the class B domain is increased so that *35::AP3* flowers have staminoid carpels in the inner whorl (Jack *et al.*, 1994) and *35S::PI* flowers have petalloid sepals in the outer whorl (Krizek and Meyerowitz, 1996).

The organ identity genes themselves are regulated by floral initiation process (*FLIP*) genes which promote floral fate (Haughn *et al.*, 1995; Pidkowich *et al.*, 1999; Piñero and Coupland, 1998). Two *FLIP* genes which have aspects of their loss-of-function phenotypes in common and positively regulate class B function are *UNUSUAL FLORAL ORGANS* (*UFO*) and *LEAFY* (*LFY*). Loss-of-function mutations in *UFO* produce flowers lacking normal petals and stamens (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995) while ectopically expressing (gain-of-function) *UFO* plants produce flowers comprised of supernumerary petals and stamens (Lee *et al.*, 1997). Furthermore, *35S::AP3 and 35S::PI* transgenic constructs, when present in the same plant, are able to rescue class B organ identity defects in *ufo* mutants (Krizek and Meyerowitz, 1996). These data suggest a positive role for *UFO* in regulating class B. In addition to disrupting petal

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and stamen development, mutations in *UFO* result in a number of morphological abnormalities, including an increase in the number of sepals and carpels as well as the presence of filaments and empty flowers (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). Such defects suggest a role for the *UFO* gene in growth of the floral primordium. Lastly, indicative of meristem identity defects, *ufo* mutants show errors in their ability to switch from making paraclade meristems to making individual floral meristems (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995).

Loss-of-function mutations in *LFY* produce plants in which leafy shoots replace most flowers (implying a meristem identity defect) and the flowers produced by weak *lfy* alleles, like *ufo* mutant flowers, lack most petals and stamens (suggesting *LFY*'s influence over class B) (Huala and Sussex, 1992; Schultz and Haughn, 1991; Weigel and Meyerowitz, 1993; Weigel *et al*., 1992). *LFY* appears to function as a transcription factor and may bind directly to the promoters of some organ identity genes to activate their transcription (Parcy *et al*., 1998; Weigel and Meyerowitz, 1993; Weigel *et al*., 1992). Genetic evidence suggests that *LFY* and *UFO* act as co-regulators of class B floral organ identity (Lee *et al*., 1997; Parcy *et al*., 1998); however, little is known about the biochemical basis for this interaction.

Although the *UFO* gene has been cloned (Ingram *et al*., 1995; Lee *et al*., 1997), unlike *LFY* there have been few clues as to its molecular mode of action. The deduced amino-acid sequence of UFO has high similarity only to FIMBRIATA (FIM; Simon *et al*., 1994), the product of an orthologous gene from *Antirrhinum majus*. Both UFO and FIM proteins contain a newly characterized motif named the F-box (Bai *et al*., 1996; Ingram *et al*., 1997; Samach *et al*., 1996), a domain that has been shown to mediate interaction with the *SKP1* gene product in yeast (Bai *et al*., 1996; Connelly and Hieter, 1996). Furthermore, the FIM protein has been shown to interact with *Antirrhinum* SKP1-like proteins in a yeast two-hybrid system (Ingram *et al*., 1997).

In organisms as diverse as yeasts, plants and humans, an SCF complex of SKP1, cullin and an F-box protein acts as a ubiquitin E3 ligase that effects the targeted ubiquitination and subsequent proteolysis of various proteins (Feldman *et al*., 1997; Gray *et al*., 1999; Lyapina *et al*., 1998; Michel and Xiong, 1998; Patton *et al*., 1998a; Skowyra *et al*., 1997; Yu *et al*., 1998). The F-box proteins of different SCF complexes probably act as receptors to recruit specific protein targets for degradation (Kaiser *et al*., 1998; Skowyra *et al*., 1997). Although many F-box proteins studied to date appear to be involved in regulating cell division (Bai *et al*., 1996; Connelly and Hieter, 1996; Zhang *et al*., 1995), several, including the yeast proteins GRR1 and MET30, couple the regulation of cell division to specific aspects of metabolism (Jaquenoud *et al*., 1998; Kaiser *et al*., 1998; Li and Johnston, 1997), while others appear to target primarily transcription factors (Kumar and Paietta, 1998; for a review see Patton *et al*., 1998b).

To better understand how UFO regulates floral development we pursued several different strategies. Firstly, because facets of the *ufo* mutant phenotype appear to be due to changes in growth of the young floral primordium, we used genes expressed early in floral development as markers in order to examine differences in growth between wild-type and *ufo* mutants. Our results indicate that UFO is required for normal growth of the young floral primordium.

Secondly, previous studies have shown that normal levels and patterns of *AP3* and *PI* expression are dependent on UFO function (Hill *et al*., 1998; Lee *et al*., 1997; Levin and Meyerowitz, 1995; Parcy *et al*., 1998; Wilkinson and Haughn, 1995). However, as the AP3/PI heterodimeric protein is required for maintenance of expression of both *AP3* and *PI*, it has been unclear whether UFO itself directly regulates *AP3*, *PI* or both genes. To address this question, we employed several transgenic constructs that result in ectopic expression of class B organ identity genes during floral development. We show that UFO appears to be involved in maintaining the transcription of *AP3*, but not *PI*, and performs this regulation independently of its effect on the growth of early primordia.

Thirdly, in an effort to better understand the mechanistic role of *UFO* in floral development, we drew upon the yeast two-hybrid system to detect whether the UFO protein is able to interact with other *Arabidopsis* proteins and whether UFO functions as an F-box protein. We show here that UFO interacts both *in vivo* and *in vitro* with a class of SKP1-like proteins from *Arabidopsis*, and that the interaction in yeast is dependent on the F-box. Since *UFO* controls growth and *AP3* transcription independently during early floral development, as an F-box protein, UFO may target regulators of these processes in order to co-ordinate growth with expression of specific floral regulators to establish correct patterning.

Results

UFO is required for normal growth and patterning in the young floral meristem

The major role of UFO during floral development appears to be in controlling class B (petal and stamen) organ identity, but there are aspects of the *ufo* mutant phenotype which are dissimilar to those of *ap3* and *pi* mutants and seem to be related to growth, including changes in the numbers of sepals and carpels, changes in organ arrangement, and the replacement of flowers with filaments (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). Since

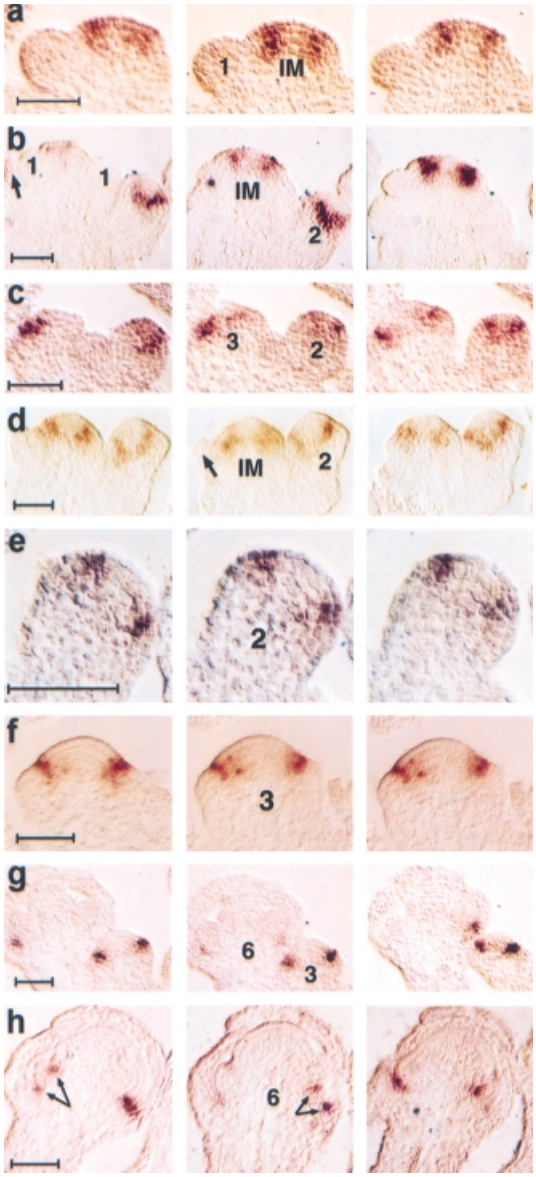

Figure 1. *In situ* hybridization of *UFO* probe to wild-type and *ufo-1* meristems.
Sequential longitudinal sections through wild-type (a, c, e, g) and *ufo-1* meristems (b, d, f, h); scale bar = 50 μm. The letters IM mark the inflorescence meristem and numbers indicate the stages of various primordia. (a) *UFO* is expressed in the wild-type inflorescence meristem but not in stage 1 primordia; (b) *UFO* is expressed broadly in the inflorescence meristem of *ufo-1*, but not in stage 1 primordia or bract-like organs (arrow). *UFO* is expressed in a shallow cup-like domain within stage 2 primordia. (c) *UFO* expression in the wild-type floral shoot begins in the centre of an early stage 2 meristem appearing in a cup-shaped domain and then, as the meristem continues to grow, *UFO* is expressed in a ring in late stage 2 and stage 3. (d) *UFO* is expressed more broadly in a *ufo-1* inflorescence meristem and a late stage 2 floral meristem. A bract-like outgrowth is present (arrow). (e) *UFO* is expressed in a ring in late wild-type stage 2 floral meristems. (f) *UFO* expression is seen in a ring in this broad *ufo-1* stage 3 meristem. (g) *UFO* is expressed at the abaxial base of developing petal primordia of stage 6 wild-type flowers and in a ring in stage 3 flowers. (h) The *UFO* expression domain is split (arrows) by the growth of second-whorl primordia of this stage 6 *ufo-1* flower.

it is unclear whether these defects arise before or after the onset of expression of organ identity genes, we examined the patterns of growth in the inflorescence apex of *ufo-1* more closely, using *in situ* hybridization with *UFO* and *PROLIFERA* (*PRL*) as probes (Figures 1 and 2). At least six apices each of *ufo-1* and wild type (Col-2) were serial sectioned in a longitudinal or transverse orientation.

UFO transcript is found in a subset of cells in all meristems showing distinct patterns in embryo, vegetative, inflorescence and floral meristems. *UFO* mRNA is first expressed in the apex of an embryo in the heart stage, and by the end of embryogenesis forms a cup-shaped domain

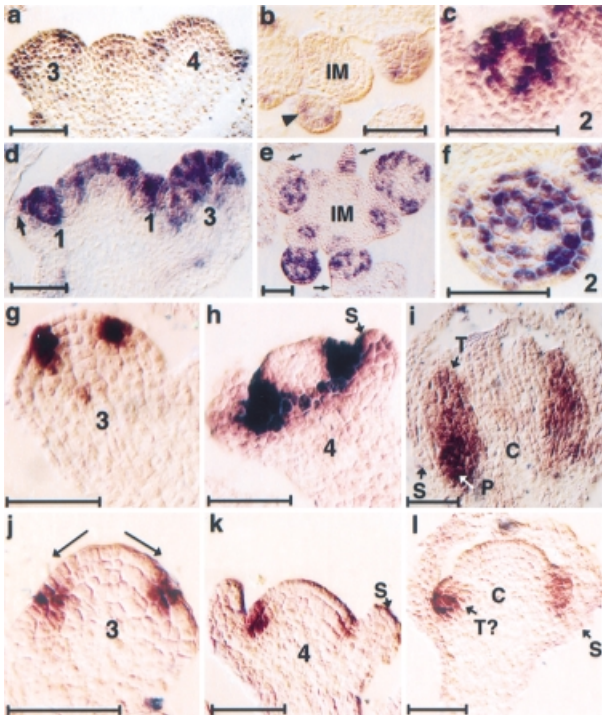

Figure 2. *In situ* hybridization of *PROLIFERA* and *AP3* probes to wild-type and *ufo-1* meristems.
RNA *in situ* hybridizations were performed on wild-type (a–c, g–i) and *ufo-1* (d–f, j–l) tissue sections. All sections are longitudinal through the inflorescence and floral meristems except b, c, e and f, which are transverse sections. Scale bars, 50 μm. *PROLIFERA* expression, which is observed in cells entering the S-phase of the cell cycle, is used as a marker for dividing cells (a–f). In the wild-type stage 2 flower *PROLIFERA* is expressed as a ring of cells surrounding the central portion of the meristem (b, arrow; c). In stage 2 in *ufo-1* mutant flowers, *PROLIFERA* expression appears to be distributed more randomly than wild type (e, f). The architecture of the inflorescence meristem is changed in the *ufo-1* mutant with an increase in size of the inflorescence meristem (compare b with e) and more early stage primordia are initiated at any one time compared to the wild type (compare b with e). Also, *ufo-1* initiates some bract-like outgrowths (d,e, arrows). Expression of the class B gene *AP3* differs between wild type and *ufo-1* (g–l) starting at the onset of expression in stage 3 (compare g with j), where it appears in *ufo-1* that additional cells distance the *AP3* expression domain from the centre of the primordium (j, arrows). In later stages *AP3* is expressed in a smaller subset of *ufo-1* cells than in the wild type (compare k with h and l with i). Developing floral organs are identified by the following letters: sepals, S; petals, P; stamens, T; carpels, C.

surrounding the central region of the shoot apical meristem (Long and Barton, 1998). In vegetative and inflorescence meristems, *UFO* is expressed in a ring-like pattern surrounding the central zone (Figure 1a) (Ingram *et al.*, 1995; Lee *et al.*, 1997), although an asymmetric pattern in some sections suggests that the ring may have one or more gaps. In flowers, *UFO* transcript is first detected in the centre and then as a cup-shaped domain in stage 2 meristems (Figure 1c) (Ingram *et al.*, 1995; Lee *et al.*, 1997) analogous to the pattern seen in embryos. As the floral meristem grows further, cells expressing *UFO* are gradually positioned further from the centre (compare stage 2 flower in Figure 1c with stage 3 flower in Figure 1g), forming first a ring and finally four clusters lying on the abaxial side of the petal primordia (stage 6 flower, Figure 1g). In a *ufo-1* mutant (nonsense mutation) the *UFO* expression domain in the inflorescence meristem forms a ring as in the wild type (Figure 1b,d), but the ring is broader. Stage 2 mutant flowers, like the wild type, exhibit a cup-shaped *UFO* expression domain, but the shape of the cup is more shallow and irregular and the width of the domain is greater than in the wild type (compare Figure 1c,e with b,d), suggesting that *UFO* activity limits growth in the stage 2 floral meristem. These differences are apparent prior to the initiation of class B expression and before any signs of organ differentiation. A similar effect on *UFO* expression is seen in *lfy* loss-of-function mutations (Lee *et al.*, 1997), most probably because UFO activity requires functional LFY protein (Lee *et al.*, 1997; Parcy *et al.*, 1998). At later stages in *ufo-1* mutant floral development, early growth of second whorl primordia can split the large *UFO* expression pattern into two (Figure 1h). As for the wild type, *UFO* transcript was not observed in *ufo-1* flowers prior to stage 2.

Because our *UFO in situ* analysis indicated that the *UFO* expression domain was altered in *ufo-1* mutants, we decided to investigate whether this alteration was due to a change in growth. The *PRL* gene, transcribed in cells entering the S-phase of the cell cycle, provides a useful probe for cells undergoing division (Springer *et al.*, 1995). As expected in the wild type, *PRL* transcript was found in individual or small clusters of cells in meristems or young primordia (Figure 2a–e). In stage 2 wild-type flowers, cells expressing high levels of *PRL* transcript form a cylinder-like pattern (Figure 2b,c). In stage 2 floral meristems the *PRL* expression pattern is roughly complementary to that of *UFO*, with *PRL* transcript located more to the periphery (Figure 2b,c) and *UFO* transcript more to the centre when viewed in transverse section (data not shown). In a *ufo-1* background, *PRL* expression in flowers of the same stage (Figure 2e,f) is broader and less regular than observed in the wild type (reflecting the *ufo-1* mutant floral phenotype). Importantly, the cylinder-like *PRL* expression pattern observed in wild-type flowers was never observed in *ufo-1* mutant flowers. These findings suggest that UFO activity suppresses cell division in the central regions of stage 2 flowers.

Several morphological defects were observed in *ufo-1* inflorescence apices. Firstly, the *ufo-1* inflorescence meristems were broader (compare Figure 1b with a, and Figure 2e with b). More early-stage primordia were present at a given time than in wild-type inflorescences (compare Figure 2e with b) and *ufo* flowers of a specific developmental age were positioned lower on the inflorescence relative to the inflorescence meristem than in the wild type (stage 2 flowers, compare Figure 1c with b). In addition, unlike the wild type, *ufo1-1* inflorescence meristems often initiated bract-like outgrowths in place of or subtending

Figure 3. Models of class B regulation by *UFO* and tests of these models with *35S::AP3, 35S::PI* and *DEF*.
The left-hand column postulates how *UFO* would affect the class B (petal and stamen) domain if *UFO*'s role was restricted to controlling cell-division patterns within the developing flower to effect proper overlap between the *AP3* and *PI* expression domains. In the right-hand column we predict the pattern of class B expression if *UFO* controls transcription of *AP3*, but not *PI*. For each genotype shown, the patterns of organ identity (ABC) genes are illustrated as they would appear in a longitudinal section through a floral primordium. The initial stage 3 class B (*PI* and *AP3*) expression patterns are shown as boxes with diagonal lines. Below this, the later class B expression pattern, derived from the region of *PI*/*AP3* overlap, is shown as cross-hatched boxes overlapping the appropriate regions of classes A and C. At the top of both columns the wild-type pattern of organ identity genes is illustrated: C genes (gray) are expressed in the centre of the flower (carpels and stamens); A genes (white) are to the outside (sepal and petal whorls), and B genes (cross-hatched) are expressed in a ring overlapping part of both A and C domains (petal and stamen whorls). Within the flowering shoot initial *PI* expression is found in the centre of the flower in a domain that extends to the edge of the presumptive petal whorl. Initial *AP3* transcripts are expressed in a ring that encompasses the future petals and stamens as well as part of the sepal whorl. As shown by cross-hatched boxes below, later-stage expression of both these class B genes is limited to the cells in which their transcription overlaps (petals and stamens).The overlap model (left column) considers that in *ufo-1* mutants there may be more cell divisions in the centre of the flower, so that the *AP3* domain is pushed farther to the outside of the primordium (Figure 2j). This would produce little overlap between the *AP3* and *PI* expression domains, resulting in a very small class B domain so that few petals and stamens would develop as occurs in *ufo-1* mutants. Ectopic *AP3* expression with the *35S::AP3* transgene in *ufo-1* mutants should rescue the normal class B function in the second and third whorls and, in addition, provide class B function in the centre of the flower. Ectopic *PI* (*35S::PI*) expression would cause class B gene function in the outer whorls of the flower. If *DEF*, an *AP3* orthologue from *Antirrhinum*, was supplied to *ufo-1* mutants we would predict class B to be rescued, as it was with *35S::AP3*. Except for *35S::AP3 ufo-1*, this first model does not fit our data (Table 1). When we postulate that *UFO* controls transcription of *AP3* alone (right-hand column), initial *AP3* expression is illustrated as a very minimal (thin) box in *ufo-1* mutants. As *AP3* and *PI* must overlap to form class B, *ufo-1* mutants lack any real class B domain. Ectopic *AP3* supplied by *35S::AP3* could result in a large class B domain that would encompass the centre of the flower. Ectopic *PI* (*35S::PI*) would not cause formation of the class B domain because *AP3* would still be lacking. If *UFO* controls transcription of the *AP3* orthologue *DEF*, then supplying *DEF* to *ufo-1* mutants would not rescue class B. This model best fits our observed data (Table 1).

floral primordia (arrows, Figure 1b,d and Figure 2d,e). These *ufo-1* outgrowths usually arrested before they grew beyond the height of a stage 3 primordium.

Effect of the ufo-1 mutation on early expression of AP3

Genetic analysis has indicated that *UFO* is a positive regulator of class B organ identity gene function (Hill *et al.*, 1998; Lee *et al.*, 1997; Levin and Meyerowitz, 1995; Parcy *et al.*, 1998; Wilkinson and Haughn, 1995). Loss-of-function mutations in *UFO* result in homeotic changes in the second and third floral whorls. In addition, the expression domains of both class B organ identity genes *AP3* and *PI* are substantially reduced by the time of initiation of second- and third-whorl primordia of *ufo* mutant flowers. In wild-type stage 3 flowers, *AP3* and *PI* are initially expressed in non-identical but overlapping domains. Only those cells in which both *AP3* and *PI* are expressed establish the class B domain, as both AP3 and PI proteins are required for the normal expression and function of *AP3* and *PI* genes beyond stage 3 (Goto and Meyerowitz, 1994; Jack *et al.*, 1992; 1994; Krizek and Meyerowitz, 1996). Our *in situ* hybridization data have shown that the expression domain of *PI* in stage 3 *ufo-1* flowers is similar in time of

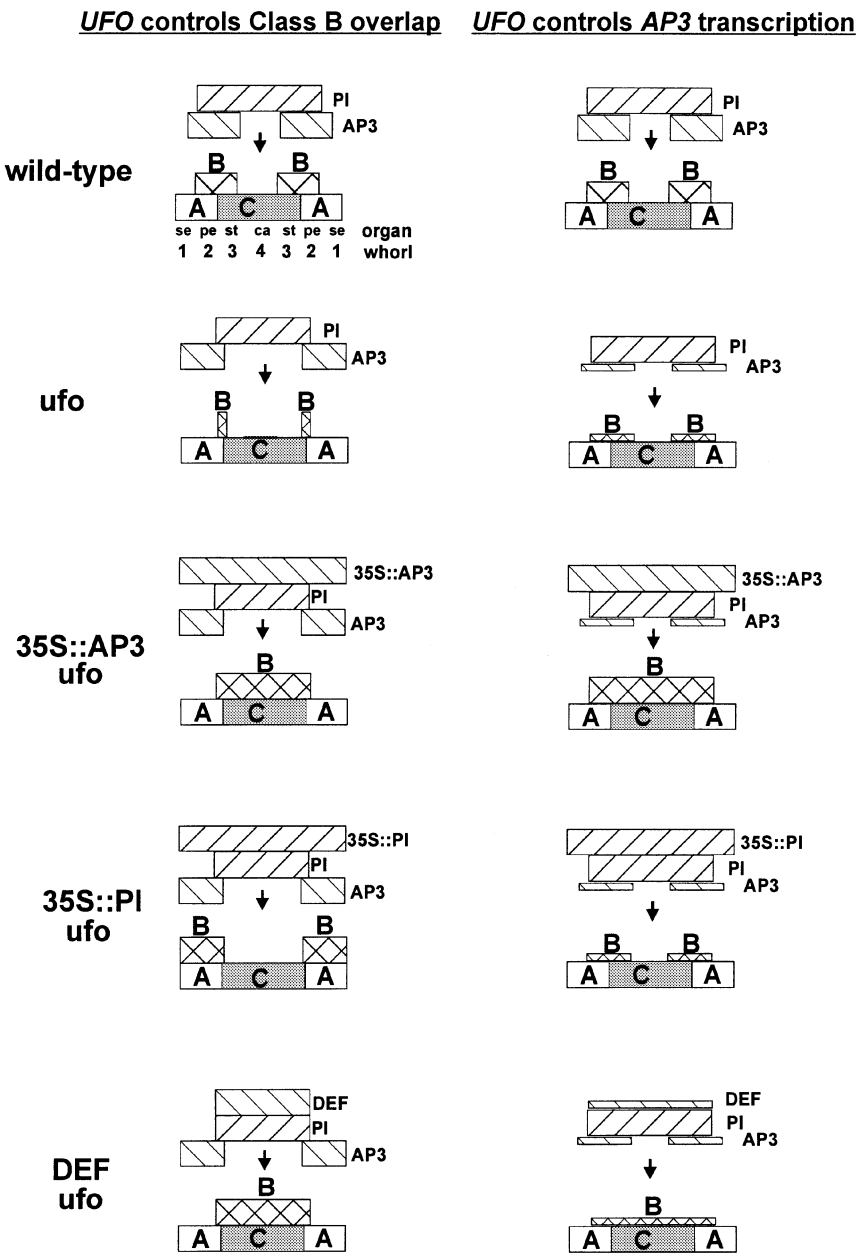

Table 1. Rescue of *ufo-1* by class B transgenic loci

	No transgenic locus				*35S::AP3*			*35S::PI*			*DEF*		
Plant parts	wt $n=60$	ufo-1 $n=52$	ap3-3 $n=43$	pi-1 $n=33$	wt $n=34$	ufo-1 $n=68$	ap3-3 $n=32$	wt $n=118$	ufo-1 $n=83$	pi-1 $n=24$	wt $n=35$	ufo-1 $n=108$	ap3-3 $n=44$
1st whorl													
sepals	4[a]	4.02	4.02	4	3.94	4.01	4	0	4	0	4	4	4
sepal-petals	0	0	0	0	0.09	0	0	4	0.02	4	0	0	0
2nd whorl													
sepals	0	0.37	3.98	4	0	0.14	0.09	0	0.4	0	0	2.19	1.66
sepal-petals	0	1.35	0	0	0	0.99	3.88	0	1.05	0	0	0.43	2.34
petals	4	0.5	0.05	0	4.03	0.35	0.03	4	0.67	4	3.94	0.06	0
2nd–3rd whorl													
petal-stamens	0	0.31	0	0	0	2.23	0	0	0	0	0.14	0.15	0
filaments	0	5.44	3.23	3.21	0	0.92	0	0	4.19	0	0	1.71	2.02
stamens	5.95	0.13	0	0	7.74	5.13	5.38	5.97	0.48	1.54	4.94	0.21	0.2
3rd–4th whorl													
stamen-carpels	0	0.1	0	0	1.44	2.18	2.13	0	0.65	3.58	1.03	0.29	3.32
carpels	2	2.96	2.82	2.26	1.53	2.77	2.47	2	3.44	2.71	2.4	2.96	2.43

Flowers from nodes 1–20 were used in the above data set.
[a]Each number represents the average number of an organ type observed for each genotype.

appearance, size and position (centre of the meristem) to that of the wild type (Wilkinson and Haughn, 1995; M.D. Wilkinson and G.W. Haughn, unpublished results). We examined the expression of the *AP3* gene in wild-type and *ufo-1* flowers, with emphasis on its initial patterns of expression (Figure 2g–l). Like *PI*, *AP3* is initially expressed in *ufo-1* stage 3 flowers in a domain equivalent in size to that of the wild type (compare Figure 2g with j). Relative to stage 3, the number of cells expressing *AP3* in the stage 4 floral primordium increases in the wild type, but stays the same in the *ufo-1* mutant (compare Figure 2g,h with j,k, respectively). In later stages the *AP3* expression domain, like that of *PI*, encompasses all cells of developing second- and third-whorl organs in wild-type (Figure 2i) but not in *ufo-1* mutant flowers (Figure 2l). These results suggest that *UFO* function is required for the maintenance, but not initiation, of *AP3* and *PI* expression, although we cannot eliminate the possibility that residual UFO activity in the *ufo-1* mutant accounts for the *AP3* expression observed.

UFO is required for AP3 but not PI expression

AP3 and PI form a heterodimeric protein required for the maintenance of *AP3* and *PI* expression (Jack *et al.*, 1994; Krizek and Meyerowitz, 1996; Riechmann and Meyerowitz, 1997). UFO could be directly required for *AP3* and *PI* expression as a positive regulator of *AP3*, *PI* or both (Figure 3, right column, wild type). Alternatively, the requirement for UFO for class B expression may be an indirect consequence of the requirement for UFO for normal growth of the stage 2 floral primordium (Figure 3, left column, wild type). As discussed above, mutations in *UFO* result in increased growth of the floral primordium prior to the onset of class B gene expression. This could cause less overlap in the initial expression patterns of *PI* and *AP3* compared to the wild type, thereby decreasing the size of the subsequent class B domain (Figure 3, left column, *ufo-1*). To distinguish between these hypotheses we constructed three different lines, each homozygous for the *ufo-1* mutation but carrying a different transgenic class B gene. Two of the lines contained either a *PI* or *AP3* cDNA under the control of the *CaMV 35S* promoter, which promotes transcription throughout the floral primordium (Jack *et al.*, 1992; Krizek and Meyerowitz, 1996). The third line contained the *Antirrhinum majus* class B gene *DEF* under control of its own promoter. The *DEF* gene encodes an AP3-like activity which is expressed in a domain similar to *PI* (Samach *et al.*, 1997; Schwarz-Sommer *et al.*, 1992). If the *ufo-1* mutant simply reduces overlap in expression of *AP3* and *PI*, then all three transgenic constructs should be able to rescue the *ufo-1* class B organ identity defects. Table 1 shows that while *35S::AP3* rescues the *ufo-1* organ identity defect, *35S::PI* and *DEF* do not. These data indicate that the overlap hypothesis alone cannot account for the loss of class B function in *ufo-1* flowers (Figure 3, left column). Further, the fact that the *ufo-1* mutation can be rescued by *35S::AP3* but not *35S::PI* suggests that UFO is directly required for *AP3*, but not *PI* expression (Figure 3, right column).

It is interesting to note that *35S::AP3 and 35S::PI* fail to completely complement *ap3-3* and *pi-1* mutations, respectively (Table 1; Jack *et al.*, 1994; Krizek and Meyerowitz, 1996). *35S::AP3* rescues stamens more successfully than petals, and *35S::PI* rescues petals more successfully than

Figure 4. Alignment of deduced amino-acid sequences for the *Arabidopsis* genes *ASK1* and *ASK2* to Human SKP1.
Identity of the two *Arabidopsis* sequences to SKP1 is 53.4 and 55.2%, respectively, while identity between them is 72.1%. Identity (|), conservative (:) and neutral (.) changes are depicted. Asterisks correspond to the amino terminus of the shortest interacting clone characterized.

stamens. These data suggest that the *35S* promoter must provide gene expression that is quantitatively or qualitatively different from the native promoter, as suggested previously (Irish and Yamamoto, 1995). *35S::AP3 35S::PI* completely complements *ap3-3* (Krizek and Meyerowitz, 1996) indicating that the deficiency in expression with *35S::AP3* alone can be corrected by an increase in *PI* expression. Given that AP3 and PI function together as a heterodimer that is required for autoregulation of both *AP3* and *PI* genes, it is not surprising that the relative concentration of the two proteins is critical for development. We suggest that endogenous *PI* is greater in the stamen than in the petal, and *vice versa* for *AP3*. The low level of one of the two partners makes the plant sensitive to fluctuations in the level of the other. For example, in the petal anlagen of wild-type flowers the low concentration of *PI* is compensated for by the higher *AP3* dose, thereby ensuring that there is enough heterodimeric complex to drive petal development.

UFO specifically interacts with SKP1-like Arabidopsis *proteins*

To gain insight into the biochemical role of the UFO protein we used a yeast two-hybrid screen (Kohalmi *et al.*, 1997) to detect *Arabidopsis* proteins with which UFO can interact. A pBI-770-*UFO* bait construct was transformed into the two-hybrid host strain YPB2 (Bartel *et al.*, 1993) and the recombinant strain used to screen an *Arabidopsis* two-hybrid cDNA expression library representing all the above-ground tissues of the plant. This screen identified over 300 cDNAs which represent just two genes verified by direct sequencing and Southern analysis. Both genes have deduced amino-acid sequences with a high degree of sequence similarity to each other and to the product of

Table 2. β-galactosidase assays of yeast strains carrying UFO/ASK yeast two-hybrid bait and prey plasmids

	Bait construct		
Prey construct	pBI-770:UFO	pBI-770:ASK1	pBI-770:ASK2
Null	0.15 + 0.01	0.08 + 0.01	0.12 + 0.05
pBI-771	0.23 + 0.01	0.08 + 0.01	0.03 + 0.02
pBI-771:UFO	0.11 + 0.02	4.60 + 0.05	33.2 + 0.26
pBI-771:ASK1	66.8 + 0.25	0.11 + 0.07	0.06 + 0.02
pBI-771:ASK2	74.6 + 1.11	0.09 + 0.02	0.03 + 0.02
pBI-771:CRUCIFERIN	0.08 + 0.02	0.07 + 0.01	0.04 + 0.01

Values are given in Miller units, as defined in the methods.
This assay was performed at 30°C.

SKP1, a previously identified component of the cell-cycle regulatory complex in human cells and yeast (Bai *et al.*, 1996; Connelly and Hieter, 1996; Zhang *et al.*, 1995) (Figure 4). The complete sequence and expression of one of the genes has been reported recently (Porat *et al.*, 1998), but the name used (AtSkp1) does not conform with *Arabidopsis* genetic nomenclature (Meinke and Koornneef, 1997; Meinke, 1995). We therefore designate these genes as *ASK1* (*ARABIDOPSIS SKP1-LIKE 1*, formerly AtSkp1) and *ASK2* (*ARABIDOPSIS SKP1-LIKE 2*)

Quantitative assays of the reporter β-galactosidase were carried out for yeast strains carrying various combinations of *UFO*, *ASK1*, *ASK2* and *CRUCIFERIN* (negative control) as two-hybrid bait and/or prey. The results (Table 2) support the conclusion that UFO specifically interacts with ASK1 and ASK2 in yeast.

SKP1 interacts with proteins carrying an F-box domain. UFO includes a domain with high sequence similarity to an F-box (Bai *et al.*, 1996). To test whether the UFO F-box was required for interaction with ASK, two *UFO* bait constructs carrying deletions in the F-box were constructed (UFO–ΔF). Both UFO–ΔF constructs were unable to interact with ASK1 or ASK2 in a two-hybrid assay (Table 3), and no interactors have been detected as yet when these UFO–ΔF constructs have been used as bait in a partial screen of the *Arabidopsis* two-hybrid prey library (1/20 of the total library). These results are consistent with the hypothesis that the UFO F-box is required for interaction with ASK.

UFO and ASK1 proteins interact in vitro

Our yeast two-hybrid results indicate that UFO and *Arabidopsis* SKP1-like proteins directly interact. We took an independent approach to validate the two-hybrid results by analyzing the ability of recombinant UFO and ASK1 proteins to interact *in vitro*. Affinity resin containing ASK1 or an unrelated control protein (*Arabidopsis* ALS-C3) were made by incubating His_6-tagged ASK1 and ALS-C3 proteins, respectively, with Ni^+-NTA beads.

Table 3. β-galactosidase assays of yeast strains carrying UFO-$_{\Delta}$F/ASK yeast two-hybrid bait and prey plasmids

Prey construct	Bait construct pBI-770:UFO	pBI-770:UFO-$_{\Delta}F_{1–87}$	pBI-770:UFO-$_{\Delta}F_{50–62}$
pBI-771:ASK1	63.8 + 7.40	0.42 + 0.06	0.35 + 0.46
pBI-771:ASK2	62.6 + 21.1	0.52 + 0.11	0.58 + 0.14
pBI-771:CRUCIFERIN	0.27 + 0.02	nd	nd

This experiment differs from that in Table 2 in that it was performed at 20°C and the pBI-771:ASK1 and ASK2 constructs had a slightly larger 5′ UTR.
When the bait pBI-770:GAL4 alone was tested in this assay it resulted in 312.97 + 21.91 Miller units.

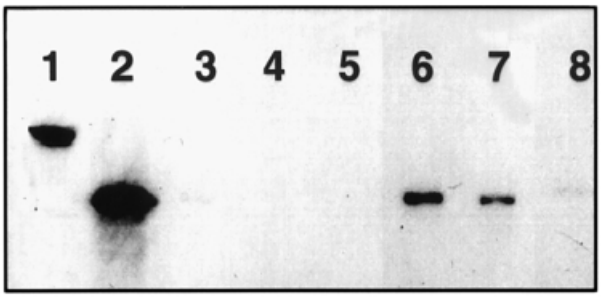

Figure 5. ASK1 *in vitro* interaction with UFO.
SDS–PAGE of ^{35}S-labeled UFO binding to ASK1-Ni^{+} agarose beads. 5 μl ^{35}S-labeled Luciferase or UFO proteins are shown in lanes 1 and 2, respectively. ^{35}S-labeled UFO (5 or 2 μl input) was bound by 4 μg ASK1-Ni^{+} agarose beads (lanes 6 and 7, respectively), while ^{35}S-labeled Luciferase (5 or 2 μl input) was not (lanes 4 and 5, respectively). UFO was retained specifically by ASK1 protein, as evidenced by no binding of UFO to beads alone (lane 3), or beads carrying the unrelated ALS-C3 protein (lane 8).

^{35S}-Met labeled UFO and, as a control, firefly Luciferase proteins were expressed *in vitro* and tested for their ability to bind ASK1 or ALS-C3 protein immobilized to the affinity matrix. The results of these experiments are shown in Figure 5. UFO protein was bound specifically by the ASK1-containing matrix (compare lanes 6 and 7 with lanes 3 and 8) while Luciferase-containing and control beads were not (lanes 4 and 5), indicating a specific interaction between UFO and ASK proteins *in vitro*.

We used *in situ* hybridization to investigate the expression pattern of *ASK1*. The *ASK1* transcript was found in and restricted to cells of tissues maintaining the ability to divide (meristematic) including floral apical meristems, inflorescence apical meristems, developing floral organs, developing ovules, and root apical meristems (A. Samach and G.W. Haughn, data not shown). These results are in agreement with *ASK1* expression studies reported previously (Porat *et al.*, 1998). Because of the high sequence similarity shared between the current 13-member *Arabidopsis ASK* gene family (Figure 4, Gray *et al.*, 1999; E. Risseeuw and W.L. Crosby, unpublished results), we cannot eliminate the possibility that the *ASK1* probe detects transcripts from *ASK2* and other members of the *Arabidopsis ASK* gene family. Significantly, the *ASK* expression domain overlaps that of *UFO* in inflorescences and flowers (Figure 1; Ingram *et al.*, 1995; Lee *et al.*, 1997), allowing for the possibility that the UFO–ASK1 interaction characterized in recombinant yeast and *in vitro* may also occur *in planta*.

Discussion

UFO is required for normal growth of the floral meristem

Our *in situ* hybridization data using *UFO* and *PRL* as probes suggest that *UFO* activity is required to correctly regulate growth in stage 2 floral meristems by limiting the number of cell divisions in the central region. This conclusion is consistent with other aspects of the *ufo* loss-of-function phenotype described previously (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). Mutant *ufo* flowers exhibit an increased number of carpels relative to the wild type, and the indeterminate floral meristems of *ag-1 ufo-1* double mutants progressively enlarge and fasciate to form a large fan-shaped structure. These aspects of the *ufo* phenotype are reminiscent of those resulting from loss-of-function mutations in *CLV1*, a gene that negatively regulates meristematic growth (Clark, 1997). The growth defects in *ufo* stage 2 mutants are correlated temporally with the expression of *UFO* transcripts in the flower, suggesting a function for UFO analogous to that of select F-box proteins in other systems, which act to regulate cell division. However, we cannot exclude the possibility that *UFO* expression earlier in the inflorescence meristem could influence the growth pattern of the stage 2 floral primordium. Regardless, it seems reasonable to speculate that *UFO* plays a role in establishing domains of differential cell division necessary for proper patterning in the young floral meristem.

The structural changes observed in the *ufo* inflorescence apex, such as the change in inflorescence meristem size, the increase in the number of stage 1 floral primordia, and the appearance of bract-like outgrowths, all occur prior to expression of *UFO* in stage 2 flowers. Thus these effects must be attributed to *UFO* expression in inflorescence

meristems rather than stage 2 flowers. More detailed analysis of the *ufo* inflorescence apex suggests that these early defects relate to the role of *UFO* in regulating meristem identity (J. Klenz and G.W. Haughn, unpublished results).

Role of UFO in floral organ identity

In addition to its effects on growth of the floral primordium, *UFO* is required for floral organ identity in the second and third whorls. Both loss- and gain-of-function *ufo* phenotypes (Hill *et al.*, 1998; Lee *et al.*, 1997; Levin and Meyerowitz, 1995; Parcy *et al.*, 1998; Wilkinson and Haughn, 1995) indicate that *UFO* plays a role in positively regulating the class B organ identity genes *PISTILLATA* (*PI*) and *APETALA3* (*AP3*) required to specify petal and stamen development. Indeed, it has been shown that expression of both *AP3* and *PI* under the control of the constitutive *35S* promoter rescues the floral organ identity defects of *ufo* loss-of-function mutants (Krizek and Meyerowitz, 1996).

We have shown that a *35S::AP3*, but not a *35S::PI* transgene alone can rescue the floral organ identity defect of *ufo* nonsense mutation. These results suggest that UFO acts as a direct, positive regulator of *AP3* expression. In contrast, the control of *PI* expression by UFO must be indirect. Since *AP3* function is required for maintenance of *PI* transcription (Jack *et al.*, 1994), the effects of *UFO* on *PI* expression are probably a consequence of UFO regulation of *AP3* expression.

The mechanism by which UFO, an F-box protein, positively regulates *AP3* is unclear. If UFO functions like other known F-box proteins, then it must lead to the degradation of a negative regulator of *AP3*. The initial (stage 3) spatial and temporal expression of *AP3* does not seem to be dependent on *UFO* function, although *UFO* is required for normal *AP3* expression at later stages. Therefore *UFO* may be necessary to degrade a repressor modulating the level or maintenance of *AP3* transcription. A gene such as *WLC*, in which loss of function causes both early flowering and ectopic *AP3* expression (Levy and Dean, 1998), might be an interesting candidate.

The *LFY* gene encodes a transcription factor that positively activates the organ identity genes, including *AP3* (Parcy *et al.*, 1998). LFY and UFO are believed to function together to positively regulate *AP3* and *PI* (Lee *et al.*, 1997; Parcy *et al.*, 1998), although the mechanism of this co-regulation has been unclear. One simple hypothesis consistent with the available data is that *AP3* expression requires the binding of LFY to the *AP3* promoter and the degradation of an *AP3* repressor by UFO.

The *Antirrhinum DEF* gene encodes an *AP3*-like protein (Irish and Yamamoto, 1995), but is expressed in a domain similar to *PI* (Samach *et al.*, 1997). Curiously, a transgenic *DEF* gene under the control of its own promoter is able to rescue *ap3,* but not *ufo* loss-of-function mutations (Samach *et al.*, 1997; this study). These data suggest that the *DEF* gene, like *AP3* but unlike *PI*, requires UFO for its expression. Thus although *DEF* is expressed in a pattern similar to *PI*, with respect to its regulation by UFO *DEF* is more similar to *AP3*.

UFO is an F-box protein

Genetic analysis has implicated the *UFO* gene as a regulator of floral meristem and floral organ identity (Lee *et al.*, 1997; Levin and Meyerowitz, 1995; Parcy *et al.*, 1998; Wilkinson and Haughn, 1995). Despite the fact that the *UFO* gene is cloned, its molecular mode of action has been unclear. Several lines of evidence strongly suggest that UFO acts as an F-box protein. The deduced sequence of both the UFO protein and its orthologous counterpart *FIM* in *Antirrhinum* contain an F-box motif (Bai *et al.*, 1996; Ingram *et al.*, 1997; Pyke, 1997), a domain that was first shown to mediate interaction with the *SKP1* gene product in yeast (Bai *et al.*, 1996; Connelly and Hieter, 1996). As we have shown both in yeast and *in vitro*, the UFO protein interacts with *Arabidopsis* SKP1-like proteins (ASK) with a high degree of specificity, and deletion of the F-box eliminates this interaction in yeast. That this interaction may occur *in planta* is supported by the finding that the *UFO* and *ASK* transcript expression domains overlap. *In planta* interaction between UFO and ASK is further suggested by an *ask1-1* loss-of-function mutation which causes defects in floral morphology analogous to *ufo* (Zhao *et al.*, 1999). Finally, similar interactions between FIM protein and *Antirrhinum* SKP1-like proteins (FAP) in yeast have been shown (Ingram *et al.*, 1997).

In organisms as diverse as fungi and humans, SKP1, cullin-like and F-box containing polypeptides form an SCF complex that functions as a ubiquitin E3 ligase, targeting specific proteins for degradation (Patton *et al.*, 1998b). F-box proteins are required for a large variety of cellular and developmental processes, and may carry out different regulatory roles within SCF complexes by acting as the receptors which recruit specific proteins for targeted degradation (Kaiser *et al.*, 1998; Skowyra *et al.*, 1997). The protein targets of specific F-box-containing SCF complexes include cell-cycle regulators (Bai *et al.*, 1996; Connelly and Hieter, 1996; Feldman *et al.*, 1997; Lyapina *et al.*, 1998; Michel and Xiong, 1998; Patton *et al.*, 1998a; Skowyra *et al.*, 1997; Yu *et al.*, 1998; Zhang *et al.*, 1995), transcription factors (Kumar and Paietta, 1998) or both (Jaquenoud *et al.*, 1998; Kaiser *et al.*, 1998; Li and Johnston, 1997). Assuming that F-box proteins have similar roles in plants, UFO may also target specific proteins for degradation. A clear mechanistic understanding of UFO function would

therefore require identification of proteins targeted for turnover by a putative UFO-containing plant SCF complex, and such investigations are in progress.

F-box proteins are believed to interact directly with their targets through protein–protein interactions (Bai *et al.*, 1996). However, only ASK proteins were recovered from a yeast two-hybrid screen where UFO, either with or without the F-box, was used as bait. It is possible that conditions not present in the yeast two-hybrid screen must be met before UFO can interact with its target. For example, at least some targets must be phosphorylated before they are recognized by the SCF complex (Verma *et al.*, 1997). Alternatively, UFO–target interactions may depend on an intact plant SCF complex.

SKP1 and cullin-like proteins are thought to serve as a scaffold that recruits different F-box proteins to the SCF complex (Patton *et al.*, 1998b). Thus both proteins should be required for all SCF-mediated functions, including the degradation of cell-cycle regulators. Indeed, yeast *SKP1* function is required for progression through the cell cycle (Bai *et al.*, 1996; Connelly and Hieter, 1996; Zhang *et al.*, 1995) and in plants assists auxin-mediated growth (Gray *et al.*, 1999). Although the function(s) of the ASK proteins themselves remain unknown, the finding that *ASK* transcript is present throughout meristematic tissues is consistent with a role in cell division and/or patterning.

UFO has multiple roles in floral development

UFO appears to independently regulate both transcription of a specific gene (*AP3*) and growth during the early stages of floral development. It has also been suggested that FIM–FAP complexes play two roles in *Antirrhinum* flower development, regulating organ identity and establishing morphological boundaries (Ingram *et al.*, 1997). Another F-box protein, the yeast GRR1 protein, has been shown to regulate two distinct processes independently: cell division and hexose transport. GRR1 targets for degradation the transcription factor RGT1 (a negative regulator of genes involved in hexose transport) as well as G_1 cyclins (Barral *et al.*, 1995; Li and Johnston, 1997), thus co-ordinating cell division and metabolism. How might such a dual regulation of organ identity and growth operate in a floral primordium? One possible scenario is that UFO could initially suppress cell division until the presumptive class B domain is established. If, once established, class B organ identity activity then promotes cell division, as has been suggested previously (Jack *et al.*, 1992), UFO may serve to limit division of cells immediately adjacent to the class B domain. In this way, UFO would co-ordinate cell division with promotion of class B expression to establish the correct class B floral organ identity functional domain.

Experimental procedures

Plant lines

Wild-type seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0) used for library construction were obtained from Lehle Seeds (Round Rock, TX, USA; Cat No. WT-2). Homozygous *ufo-1* mutant (Wilkinson and Haughn, 1995) and wild-type *A. thaliana* plants (ecotype Col-2) were used as sources of tissue for *in situ* hybridization. The *ufo-1 dis2-1* double mutant line (Wilkinson and Haughn, 1995) was used for line construction. Transgenic lines carrying *CaMV 35S::AP3* (ecotype Ler, Jack *et al.*, 1994) and *CaMV 35S::PI* (ecotype Ler, Krizek and Meyerowitz, 1996) and a line homozygous for the *ap3-3* allele (ecotype Ler, Jack *et al.*, 1992) were gifts from Dr E. Meyerowitz. The *pi-1* mutant was a gift of Dr M. Koornneef. The transgenic line carrying *DEF* (*TDEF1–1*) has been described previously (Samach *et al.*, 1997).

Strain construction

To create the lines *35S::AP3 ap3-3, 35S::AP3 ufo-1, 35S::PI pi-1, 35S::PI ufo-1* and *TDEF1-1 ap3-3,* the single homozygous transgenic lines containing *35S::AP3, 35S::PI* and *TDEF1-1* constructs were used as male parents in crosses. Single mutant lines homozygous for *ap3-3* and *pi-1* were used as female parents, as was the double mutant homozygous for *ufo-1* and *dis2-1*. In the *ufo-1 dis2-1* line, the mutant allele of *dis2–1* (*DISTORTED TRICHOMES2*) is tightly linked to *ufo-1* (Wilkinson and Haughn, 1995) and was used as a morphological marker to identify plants homozygous for *ufo-1*. Putative double mutants were confirmed by segregation in subsequent generations after a cross to wild type (ecotype Col-2).

To create double mutants of *TDEF1-1 ufo-1*, *ufo-1 dis2-1* plants (female parent) were crossed to homozygous *ap3-3* plants carrying *TDEF1-1* (male parent). The *dis2* phenotype allowed identification of F_2 plants homozygous for *ufo-1*. The presence of *TDEF1-1* in some of these plants was suggested by carpel defects and was confirmed by segregation in the next generation after a cross to wild type. The absence of *ap3-3* in the *TDEF1-1 ufo-1* double mutants studied was also determined by segregation analysis after a cross to wild type.

Plasmid construction

Two plasmids were constructed with deletions of the *UFO* F-box. To delete the first 87 amino acids of *UFO*, a *Xho*I–*Not*I fragment from pBI770-*UFO* carrying the *UFO* open reading frame lacking the first 87 codons was cloned into the *Sal*I/*Not*I sites of pBI990. Plasmid pBI990 was derived from pBI770 bait vector (Kohalmi *et al.*, 1997), except the *Sal*I site which defines an alternative open reading frame (GTC–GAC). For construction of mutant *UFO* derivatives deleted for amino acids 50–62, the bait vector pBI770 was modified by site-directed mutagenesis using the QuikChange™ Kit from Stratagene (La Jolla, CA, USA) and the primers

5′-GGA AAA ACG CCG GAG GTG GCT TGC TCC AAA TCC GAC AG-3′

5′-CTG TCG GAT TTG GAG CAA GCC ACC TCC GGC GTT TTT CC-3′

The antisense *PROLIFERA* (*PRL*) probe for *in situ* hybridization was generated by first amplifying 1.8 kb of *PRL* genomic DNA from a cloned template (provided by P. Springer; Springer *et al.*,

1995) using primers 5′-ACGAGTTGTTACCCGAAC and 3′-CATGAATAGCAGTACGATC. This 1.8 kb fragment containing within it 1121 base pairs of *PRL* exon sequence was cloned into the *Hin*cII site of the PT7T318 U vector (Pharmacia Biotech, Baie d' Urfé, QC, Canada) to create plasmid pAS3.

Two-hybrid library construction and screening

Seeds were sown in soil and tissues harvested from shoots at four different stages of development, approximately 2 weeks apart, from early rosette stage through to plants bearing siliques and showing early signs of senescence. An *Arabidopsis* two-hybrid cDNA expression library was constructed in the *CEN–ARSH* yeast–*E. coli* shuttle vector pBI-771 (Kohalmi *et al.*, 1997) using polyA$^+$ mRNA isolated from equal mass portions of shoot tissues harvested at each stage of development. The final assembled library contained 2×10^7 independent clones and was about 96% recombinant. The cDNAs encoding UFO-interacting proteins were identified using a modified *GAL4*-based yeast two-hybrid system (Fields and Song, 1989; Kohalmi *et al.*, 1997). A total of 1.8×10^7 yeast transformants were screened for cDNAs encoding interacting proteins using procedures described elsewhere (Kohalmi *et al.*, 1997). A total of 311 positives were retrieved, all of which co-activated both two-hybrid marker genes, for a combined frequency in the library of 1.7×10^{-5}. Plasmids from positive clones were transformed into *E. coli* and their recombinant inserts sequenced. By direct DNA sequence or cross-hybridization criteria, 299 independent clones represented one gene designated *ASK1*, plus a further six independent clones representing a second gene designated *ASK2*. The sequences of *ASK1* and *ASK2* have been allocated GenBank Accession numbers U97020 and U97021, respectively.

β-*Galactosidase assays*

A 3 ml overnight yeast culture was grown at 22°C in SD medium supplemented with appropriate amino acids, diluted to OD_{600} 0.35 and grown for an additional 4 h, after which 4 ml culture was harvested by centrifugation. Crude cell extracts were prepared by resuspension in 0.2 ml yeast lytic enzyme (YLE) solution (0.1 M KPO_4 pH 7.5, 1.07 M sorbitol, 0.8 mg ml^{-1} YLE; ICN Pharmaceuticals, Montreal, QC, Canada, Cat # 360942), incubating at 37°C for 1 h, adding 2 ml Z-buffer (0.1 M $NaPO_4$ pH 7.5, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol) followed by incubation at room temperature for 30 min. Lysed cells (0.25 ml) were added to each of four tubes with 0.5 ml Z-buffer. Reactions were started with the addition of 0.15 ml *o*-nitrophenyl-β-D-galactoside solution (4 mg ml^{-1} in Z-buffer), incubated at 30°C and stopped at different times by adding 0.366 ml 1.5 M Na_2CO_3. Following centrifugation the supernatant absorbance was measured at 420 and 550 nm. Miller activity units were calculated as $[1000 \times (OD_{420} - (OD550 \times 1.75)/\text{time(min)} \times \text{vol(ml)} \times OD_{600}) \times 8.8]$. Mean mass-specific activity values and time-course linearity were determined from the four reactions. Assays were repeated three times each for each two-hybrid interaction strain, and the means and SD were calculated.

Protein binding assays

^{35S}-Met labeled UFO and firefly Luciferase proteins were expressed from T7 promoter constructs (pSPORT1, BRL Life Technologies, Burlington, ON, Canada) using an *in vitro* coupled rabbit reticulocyte transcription/translation system (TNT, Promega, Madison, WI, USA). N-terminal His_6 epitope-tagged ASK1 protein was cloned into a modification of pRSET-B (Invitrogen, Carlsbad, CA, USA) and expressed in *E. coli*. Ni$^+$-NTA beads (Qiagen, Mississauga, ON, Canada) were equilibrated and blocked in NETN buffer (Bai *et al.*, 1996) lacking EDTA (NTN), and supplemented with 2 mg ml^{-1} BSA. 50 µl equilibrated beads were incubated with 2–3 µg unlabeled His_6-ASK1 in 1 ml of NTN buffer for 2 h with tumbling at 10°C followed by washing with 2×1 ml NTN buffer. Binding experiments were carried out in a total volume of 100 µl NTN containing 10 µl beads, plus 2–5 µl (0.6–1.5 pg) ^{35}S[Met]-labeled UFO or Luciferase protein. Binding reactions were incubated at 10°C for 2 h, followed by washing with 3×500 µl NTN. Washed beads were eluted with 10 µl SDS-containing denaturation buffer at 100°C for 5 min, and bound ^{35}S-labeled proteins analysed by SDS–PAGE. Gels were imbibed with a fluorography enhancer (Amplify, Amersham, Toronto, ON, Canada) prior to drying and exposure to X-ray film. *Arabidopsis ALS-C3*, used in this study as a control, was cloned on the basis of its interaction in the yeast two-hybrid system with the product of the *CSR1* locus from *Arabidopsis* (acetolactate synthase catalytic subunit; Haughn and Somerville, 1986; W.D.R. Pokatong, S.E. Kohalmi and W.L. Crosby, unpublished results).

In situ *analysis*

Tissues from wild-type (ecotype Col-2) or mutant *Arabidopsis* plants were fixed and sectioned for RNA *in situ* hybridization as described (Samach *et al.*, 1997). Sections were photographed under differential interference contrast optics, where the presence of transcript is visible as dark brown- or blue-stained regions.

Numbers represent stage of floral development (Smyth *et al.*, 1990). The following digoxigenin-labeled antisense RNA probes were used: *APETALA3* (E. Meyerowitz, Caltech, CA, USA, used as described by Wilkinson and Haughn, 1995), *PROLIFERA* (this study), *UFO* (Ingram *et al.*, 1995) and *ASK1* (this study). All micrographs presented were digitized and manipulated using PHOTOSHOP (Adobe Systems Inc., San Jose, CA, USA).

UFO antisense probe was prepared as described previously (Wilkinson and Haughn, 1995). *PRL* probes were generated by digesting pAS3 with *Bam*HI (for antisense probe) or *Xho*I (for sense probe), and transcribed *in vitro* in the presence of digoxigenin-labeled nucleotides using T7 RNA polymerase (for antisense probe) or T3 RNA polymerase (for sense probe).

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