Patterning of Inflorescences and Flowers by the F-Box Protein DOUBLE TOP and the LEAFY Homolog ABERRANT LEAF AND FLOWER of Petunia

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Angiosperms display a wide variety of inflorescence architectures differing in the positions where flowers or branches arise. The expression of floral meristem identity (FMI) genes determines when and where flowers are formed. In Arabidopsis thaliana, this is regulated via transcription of LEAFY (LFY), which encodes a transcription factor that promotes FMI. We found that this is regulated in petunia (Petunia hybrida) via transcription of a distinct gene, DOUBLE TOP (DOT), a homolog of UNUSUAL FLORAL ORGANS (UFO) from Arabidopsis. Mutation of DOT or its tomato (Solanum lycopersicum) homolog ANANTHA abolishes FMI. Ubiquitous expression of DOT or UFO in petunia causes very early flowering and transforms the inflorescence into a solitary flower and leaves into petals. Ectopic expression of DOT or UFO together with LFY or its homolog ABERRANT LEAF AND FLOWER (ALF) in petunia seedlings activates genes required for identity or outgrowth of organ primordia. DOT interacts physically with ALF, suggesting that it activates ALF by a posttranslational mechanism. Our findings suggest a wider role than previously thought for DOT and UFO in the patterning of flowers and indicate that the different roles of LFY and UFO homologs in the spatiotemporal control of floral identity in distinct species result from their divergent expression patterns.

INTRODUCTION

Flowering plants (angiosperms) display large variation in the time and the position that flowers are formed on the plant body. Distinct species show variation in both the season and the age at which the plant switches from vegetative growth to reproductive growth, and flowers may occur either solitarily at the end of a shoot or arranged along inflorescence branches in different patterns (Angenent et al., 2005; Benlloch et al., 2007). In racemes, such as the inflorescences of Arabidopsis thaliana and Antirrhinum majus, the apical shoot meristem is maintained and generates (lateral) floral meristems (FMs) at its periphery. In cymes, such as those formed by petunia (Petunia hybrida) and tomato (Solanum lycopersicum), the flower arises apically and growth continues from a lateral meristem that repeats this pattern. In panicles, both apical and lateral meristems develop into flowers.

In Arabidopsis, flowering is induced by a combination of endogenous and environmental cues (e.g., temperature and daylength), which ultimately activate a handful of genes (integrators), such as LEAFY (LFY) and APETALA1 (AP1), that promote the floral fate of meristems (Jack, 2004; Krizek and Fletcher, 2005; Parcy, 2005). LFY encodes a unique, plantspecific transcription factor that directly controls the transcription of AP1 and its partially redundant paralog CAULIFLOWER (CAL) (Wagner et al., 1999; William et al., 2004); these in turn can activate the transcription of LFY (Liljegren et al., 1999). In addition, UNUSUAL FLORAL ORGANS (UFO) has a weak effect on FM identity. That is, ufo mutants bear more cleft petal/yellow flowers, which are secondary inflorescences that derive, like flowers, from lateral meristems. This suggests that the ufo mutation partially reduces floral identity and transforms the first-arising flowers into inflorescences. In addition, ufo mutants have defects in the whorled pattern of floral organ primordia and the suppression of bracts (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995; Hepworth et al., 2006). UFO encodes the F-box protein component of an S-phase kinase–associated protein1/Cullin1/F-box protein (SCF) ubiquitin ligase complex (Ingram et al., 1995; Samach et al., 1999; Wang et al., 2003) and is thought to act in concert with LFY (Lee et al., 1997; Parcy et al., 1998).

Meristem identity genes control floral fate in part by activating distinct sets of homeotic genes, known as A, B, C, D, and E genes (Jack, 2004; Krizek and Fletcher, 2005), which in combination determine the identity of floral organs. During FM development, AP1 expression is confined to the outer two floral whorls and at this stage acts as an A gene needed for the development of sepals and petals (Mandel et al., 1992). LFY remains active in all organ primordia and activates in specific subdomains A-, B-, and C-class homeotic genes that determine the identity of sepals, petals, stamens, and carpels (Lohmann and Weigel, 2002; Jack, 2004; Krizek and Fletcher, 2005).

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been determined. Activation of the C gene AGAMOUS is confined to whorls 3 and 4, because it requires, besides LFY, the homeodomain protein WUSCHEL (WUS), which is expressed in the center of the flower (Lenhard et al., 2001; Lohmann et al., 2001). It is thought that UFO is the region-specific coactivator of LFY that confines the expression of B genes, which specify petal and stamen identity, to whorls 2 and 3. This is based on the expression of UFO in the presumptive whorls 2 and 3 of the young flowers and the observation that ufo compromises petal and stamen development and the activation of B genes (Levin and Meyerowitz, 1995; Ng and Yanofsky, 2001), whereas ubiquitous expression of UFO and LFY suffices to activate the B gene AP3 in virtually all tissues (Parcy et al., 1998).

Several findings suggest that alterations in the spatiotemporal expression pattern of LFY and AP1/CAL were important for the divergence of flowering time and inflorescence structure. In Arabidopsis, LFY and AP1 are activated in lateral (floral) meristems during the onset of flowering but are repressed in the apical meristem, while constitutive expression of LFY and/or AP1 results in precocious flowering and converts the racemose inflorescence into a solitary flower (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995; Liljegren et al., 1999). These data were incorporated in a computational model for the development and evolution of inflorescences, which proposes that distinct inflorescence types evolved by alterations in the spatiotemporal expression of vegetative or floral identity (Prusinkiewicz et al., 2007). This model reproduces the phenotype of Arabidopsis mutants, but its validity for other inflorescence types is unclear, because sufficient genetic data are not available. Moreover, the molecular basis of the proposed alterations in the spatial and temporal regulation of meristem identity remained unknown.

Petunia, like most other Solanaceae species, generates a cymose inflorescence in which the apical meristem terminates by forming a flower, while an inflorescence meristem (IM) emerges laterally that repeats this pattern (Souer et al., 1996, 1998) (Figures 1A and 1B). The LFY homolog ABERRANT LEAF AND FLOWER (ALF) is required for FM identity and is expressed in the apical (floral) meristem, rather than in lateral meristems, as LFY is in Arabidopsis (Souer et al., 1998). This is consistent with the theory that alterations in the spatiotemporal control of meristem identity resulted in distinct inflorescence architectures (Prusinkiewicz et al., 2007). In contrast with LFY, ALF is already strongly expressed during the early vegetative phase in emerging leaf primordia. Since alf mutants do not display defects during vegetative growth, the function of ALF, if any, during this stage of development is unclear (Souer et al., 1998).

Here, we show that the divergent mRNA expression patterns of LFY/ALF homologs can only partially account for the divergent inflorescence structures, as constitutive expression of LFY or ALF in petunia does not alter flowering time or inflorescence architecture. We show that in petunia, when and where flowers are formed are regulated via the transcription of another gene, DOUBLE TOP (DOT). DOT is the putative petunia ortholog of UFO and encodes an F-box protein that can bind to ALF and LFY and is required for FM identity and the expression of B-, C-, D-, and E-type organ identity genes. DOT and UFO as well as LFY and ALF encode functionally similar proteins, but they acquired distinct roles in the spatiotemporal control of FM identity through alterations in their expression patterns. Our results indicate an extensive rewiring of the transcriptional networks that control FM identity and flowering and identify a pathway that controls ALF/LFY activity by a post-translational mechanism that involves DOT/UFO.

RESULTS

Ectopic Expression of ALF and LFY

To examine whether alterations in ALF expression could alter the arrangement of flowers, we constitutively expressed ALF from the cauliflower mosaic virus 35S promoter (35S:ALF) in Arabidopsis and petunia. In Arabidopsis, 35S:ALF caused precocious flowering, the conversion of secondary inflorescences into solitary flowers, and the formation of terminal flowers in the primary inflorescence (see Supplemental Figure 1 online), similar to 35S:LFY (Weigel and Nilsson, 1995). This confirms that LFY and ALF activate similar sets of target genes in Arabidopsis (Maizel et al., 2005). In petunia, however, neither 35S:ALF (16 lines) nor 35S:LFY (7 lines) caused phenotypic alterations, even though the transgenes were highly expressed and 35S:LFY partially complemented the alf mutant phenotype (see Supplemental Figure 1 online).

These findings suggested that the onset of flowering and the spatial expression of FM identity in petunia are not regulated via the transcription of ALF but are restricted by another gene.

Identification and Isolation of DOT

To identify other regulators of FM identity in petunia, we examined mutants with defects in floral identity. Among progeny of the petunia line W138, which contains frequently transposing dTPH1 elements, we found four independent alleles of a new meristem identity locus that we named DOT. In dot, most apical FMs are fully transformed into IMs that generate a new metamer containing two bracts and an apical and lateral meristem (Figures 1C and 1D). Some apical FMs, however, only form numerous bract- or sepal-like structures before they terminate by forming carpels, suggesting a partial transformation into an IM. The dot phenotype is virtually indistinguishable from the alf phenotype, and no additional defects were seen in alf dot double mutants (see Supplemental Figure 2 online). Furthermore, analysis of ~6000 mRNA fragments expressed in alf, dot, and alf dot inflorescence apices by cDNA-amplified fragment-length polymorphism analysis (Bachem et al., 1996) revealed no clear differences, underlining the similarity of the alf and dot phenotypes and strengthening the idea that ALF and DOT may act in one pathway (see Supplemental Figure 2 online).

To study the specification of floral identity, we isolated a petunia homolog of UFO by screening a cDNA library (see Methods). We identified mutants by screening 4000 petunia W138 plants by PCR for a dTPH1 insertion allele of the locus (Koes et al., 1995). To our surprise, one family of 25 plants cosegregated for a new dTPH1 insertion in the petunia UFO homolog and a new dot allele
The remaining plants of this population did not contain insertions in this UFO homolog or dot alleles. Subsequent PCR analysis showed that independently isolated dot\textsuperscript{X2586}, dot\textsuperscript{B3528}, and dot\textsuperscript{C3496} mutants also contained dTPH1 insertions in this gene, whereas these insertions were absent in closely related wild-type (DOT\textsuperscript{+/+}) progenitors and siblings of each of these mutants (Figure 1E; see Supplemental Figure 3 online). In dot\textsuperscript{B2413}, which arose in a different background, we found an insertion of an immobile dTPH7 transposon that lacked 4 bp on one end, whereas this insertion was absent in wild-type siblings. Together, these findings show that a new transposon insertion in the UFO homolog coincided with the occurrence of a new dot allele in the very same generation on five independent occasions. Thus, we concluded that the identified UFO homolog is DOT.

DOT displays high sequence similarity over the entire protein with UFO, FIMBRIATA (FIM) from Antirrhinum, STAMINA PISTILLOIDA (STP) from pea (Pisum sativum), and PROLIFERATING FLORAL ORGANS (PFO) from lotus (Lotus japonicus) (see Supplemental Figure 4 and Supplemental Data Set 1 online). This was surprising, because ufo, fim, stp, and pfo primarily affect the development of petals and stamens and have at most a subtle effect on FM identity (Ingram et al., 1997; Lee et al., 1997; Taylor et al., 2001; Zhang et al., 2003).

Among progeny of a DOT\textsuperscript{+/A2232} heterozygote, we identified a partial revertant allele (dot\textsuperscript{H2082}) in which dTPH1 excision created a 6-bp transposon footprint (Figure 1E; see Supplemental Figure 3 online). In dot\textsuperscript{H2082/A2232}, the formation of flowers and the cymose inflorescence structure are restored, but the petals

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**Figure 1.** Analysis of dot Mutants.

(A) Wild-type inflorescence. (B) Scanning electron micrograph of a wild-type inflorescence apex. Note that flowers at two developmental stages are seen. The youngest (bottom left) still lacks visible organ primordia, while the oldest (top) has generated primordia for sepals, petals (not visible), and stamens. (C) dot inflorescence. Note the proliferation of bracts and the absence of flowers. (D) Scanning electron micrograph of a dot inflorescence apex. (E) Map of DOT and mutant alleles. Yellow triangles indicate dTPH1 insertions; the green triangle indicates a dTPH7 insertion. All insertion alleles exhibit identical phenotypes. (F) and (G) Inflorescence (F) and flower (G) of the weak dot\textsuperscript{H2082/A2232} mutant. ap, apex; ax, axillary meristem; br, bract; f1 and f2, flowers 1 and 2; pe, petal; se, sepal; st, stamen. Bars = 100 μm.
contain streaks of sepal- or leaf-like tissue (Figures 1F and 1G). This indicates that DOT, like UFO, FIM, STP, and PFO, is required for the specification of petal identity.

**The DOT Homolog of Tomato Is Disrupted in an anantha Mutant**

To address whether the strong FM identity function of DOT is unique for petunia, we identified a DOT homolog, Sl DOT, in tomato. DOT and Sl DOT encode highly similar proteins and in addition share sequence similarity in the 3’ untranslated region of their mRNAs (see Supplemental Figures 4 and 5 online), suggesting that they may be orthologs. The wild-type tomato inflorescence is a cyme that, in contrast with petunia, lacks bracts (Figure 2A). In tomato anantha (an) mutants, FMs are converted into IMs (Allen and Sussex, 1996) (Figure 2B). In an-1 inflorescences, the amount of mRNA from the LFY homolog FALSIFLORA (FA; Molineros-Rosales et al., 1999) was strongly increased compared with the wild type, which may result from the overproliferation of meristem tissue, whereas the expression of Sl DOT RNA was abolished (Figure 2C). Molecular analysis indicated that the 5’ and 3’ ends of the Sl DOT coding sequence are intact in an-1 but are no longer contiguous (Figures 2D and 2E). This finding, together with DNA gel blot data (see Supplemental Figure 5 online), indicated that in an-1 mutants, Sl DOT is disrupted by a large genomic rearrangement, which could be an insertion or an inversion/translocation with a break point in Sl DOT.

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**Figure 2. Molecular Analysis of Tomato an-1 and an-3 Mutants.**

(A) Inflorescence from wild-type tomato.
(B) Inflorescence from an-1 tomato. Note that no flowers are formed but instead the inflorescence only generates new IMs, leading to a cauliflower-like structure.
(C) RT-PCR analysis of mRNAs from wild-type (+) and an (−/−) tomato inflorescences.
(D) PCR analysis of DNA from two homozygous wild-type (+) and two an-1 (−/−) tomato plants, showing the genomic disruption in the an-1 allele. PCR used primers complementary to FA (primer pair A) or distinct regions of Sl DOT (primer pairs B to H; shown in [E]).
(E) Map of Sl DOT/Sp DOT showing the position of the mutations in an-1 and an-3. Solid lines indicate fragments that can be amplified from an-1 with the indicated primer pair; dotted lines denote fragments that cannot be amplified from an-1 (see Supplemental Table 1 online for primer sequences).
(F) PCR analysis using primer pair I (see [E]) of a homozygous wild-type plant, the an-3 mutant, and a heterozygous plant cosegregating for the an phenotype (+ for the wild type and − for the mutant).
We used primers complementary to Sl DOT to amplify part of the homologous gene from the closely related species Solanum pimpinellifolium (Sp DOT; see Supplemental Figure 5 online). In the S. pimpinellifolium an-3 mutant, which phenocopies tomato an-1 (Stubbe, 1971), we found a 39-bp deletion in the 3’ end of the Sp DOT coding sequence that cosegregated with an-3 (Figure 2F; see Supplemental Figure 5 online).

Together, these data show that AN is a homolog of DOT and imply that the important role of DOT homologs for the specification of FM identity is conserved among distinct Solanaceae species.

Expression Pattern of DOT

In situ hybridization revealed that the expression pattern of DOT differs in several important respects from that of UFO and other homologs. In Arabidopsis, UFO is first expressed in the apical meristem during embryogenesis (Long and Barton, 1998) and remains active throughout the vegetative and flowering phases (Lee et al., 1997). In petunia, however, we could not detect DOT mRNA in the apex of vegetative plants (Figure 3A).

**Figure 3.** In Situ Localization of DOT mRNA in Petunia.

(A) to (D) Expression of DOT mRNA in a vegetative apex (A) and inflorescence apices and young FMs of different stages ([B] to [D]).

(E) ALF expression in the section adjacent to that in (D).

(F) and (G) Expression of DOT (F) and ALF (G) in adjacent sections through a young FM.

(H) DOT expression in a late FM. DOT expression ceases shortly after.

(I) and (J) DOT expression in a dotA2232 (I) and an alf (J) inflorescence.

(K) ALF expression in a dot inflorescence.

br, bract; ca, carpel; ct, cotyledon; “FM,” FM that is homeotically transformed into an IM; lp, leaf primordium; pe, petal; se, sepal; st, stamen. Bars = 100 μm.
In the inflorescence, DOT mRNA first becomes visible in a wedge-shaped domain within the future FM anlagen shortly before the IM and FM separate. At this stage, DOT mRNA is expressed within the ALF expression domain (Figures 3B to 3E). In the young FM, DOT mRNA is initially expressed as a stripe at the base of the first sepal primordium (Figure 3B). At this stage, FIM and UFO are expressed in the center of the FM (Simon et al., 1994; Lee et al., 1997). Slightly later, when all sepal primordia are formed, DOT mRNA has expanded into a ring overlapping the presumptive whorls 1 and 2 (Figures 3C) and subsequently moves outward to the sepal/petal boundary (Figures 3D, 3F, and 3H) to cease by the time the carpels fuse. At these later stages, DOT and ALF mRNA no longer overlap (Figures 3D to 3G).

We examined whether DOT and ALF act in a transcriptional hierarchy and found that DOT mRNA is expressed in a normal intensity and pattern (i.e., in the anlagen of one of the two meristems) in dotA2232 and alf inflorescences (Figures 3I and 3J). ALF is normally expressed in dot (Figure 3K). Thus, the transcription of ALF and DOT is not mutually dependent.

Ectopic Expression of DOT and UFO

In order to further unravel the function of DOT during plant development, we ectopically expressed DOT in petunia, using the constitutive cauliflower mosaic virus 3Ss promoter (3Ss:DOT). We obtained three independent lines that expressed the transgene. These lines displayed the same dramatic phenotype, which remained constant over four generations. First, 3Ss:DOT transformants flower within 25 d after germination after forming 4 true leaves (4 ± 0.9; n = 31), whereas wild-type plants flower after ~45 d and the formation of 15 leaves (15.2 ± 0.8; n = 19) (Figure 4A). Second, 3Ss:DOT reduces the cymose inflorescence to a solitary flower (Figure 4B). Scanning electron microscopy revealed that the lateral IM is absent and that the FM generates seven to eight petal primordia and six to seven stamen primordia, instead of five, as in

![Figure 4. Phenotype of 3Ss:DOT Petunia Transformants.](image)

(A) Wild-type and 3Ss:DOT plants making their first flowers. The wild-type plant is about twice as old as 3Ss:DOT (45 versus 25 d, respectively).
(B) The 3Ss:DOT inflorescence is a solitary flower.
(C) and (D) Scanning electron micrographs of a 3Ss:DOT inflorescence apex. Note that the lateral IM is missing and that the flower contains supernumerary petals and stamen primordia. Sepals, petals, and stamens are indicated by green, red, and yellow asterisks, respectively.
(E) and (F) Carpel–stamen chimeras formed in whorl 4 of some 3Ss:DOT flowers. In (E), anthers develop within the ovary. The carpel in (F) is almost completely converted into a stamen with some ovules along the filament.
(G) Sepals of a 3Ss:DOT transformant in a background that specifies red coloration of petal limbs.
(H) Leaves of wild-type and 3Ss:DOT plants in a background specifying pale red coloration of petal limbs.
(I) Scanning electron micrograph of a 3Ss:DOT leaf, showing a region with leaf epidermal cells and trichomes (right side of image) and a region with epidermal petal cells and lacking trichomes (left side of image; cf. [J] and [K]).
(J) and (K) Epidermal cells from a wild-type petal limb (J) and leaf (K).
(L) Flower from the wild type (bottom) and a 3Ss:DOT Arabidopsis ecotype Columbia line.
ca, carpel; ov, ovules; se, sepal. Bars = 100 μm.
the wild type (Figures 4C and 4D). This suggests that if the lateral IM (anlagen) acquires floral identity very early, the entire apical dome, including the lateral IM anlagen, turns into a single (enlarged) flower. Third, 35S:DOT induces homeotic transformations of floral organs and leaves. 35S:DOT flowers contain in the center either normal carpels or carpel–stamen chimeras (Figures 4E and 4F). Furthermore, 35S:DOT leaves, bracts, and sepals contain patches of petal-like tissue (Figures 4G and 4H; see Supplemental Figure 6 online). The epidermis of these patches consists of cone-shaped cells and lacks trichomes, similar to the adaxial epidermis of petal limbs (Figures 4I to 4K). The transformable petunia line (W115), into which 35S:DOT was introduced, has white petal limbs due to a mutation in ANTHOCYANIN2 (AN2; Quattrocchio et al., 1999). In this an2 background, the petal patches in 35S:DOT leaves, bracts, and sepals have a white color. However, when we crossed 35S:DOT into an AN2+ background, these patches of petal tissue became magenta colored, like the petal limb, confirming that these cells have petal limb identity (Figures 4G and 4H).

In Arabidopsis, the identity of petals is specified by a combination of MADS box proteins encoded by an A gene (AP1), two B genes (AP3 and PISTILLATA), and an E gene (a member of the SEPELLATA family), which are thought to act in a higher order complex (a floral quartet) (Honma and Goto, 2001). When ectopically expressed, these factors are necessary and sufficient to convert Arabidopsis leaves into petals (Honma and Goto, 2001; Pelaz et al., 2001). In 35S:DOT petunia leaves, the expression of the B genes petunia DEFICIENS (Ph DEF; also known as GREENPETALS) and petunia GLOBOSA1 (Vandenbussche et al., 2004) and the E genes FLORAL BINDING PROTEIN9 (FBP9) and FBP23 is strongly upregulated compared with the wild type, whereas expression of the C genes FBP6 and FBP14 was unaltered (see Supplemental Figure 6B online). The activation of Ph DEF is essential for the formation of ectopic petal tissue, as this was eliminated when 35S:DOT was crossed into a Ph def background (see Supplemental Figure 6C online). Since a true AP1 ortholog has not been identified in petunia, it is unclear whether it is needed for petal identity or is expressed in 35S:DOT leaves. Except for this uncertainty, the upregulation of E and both B genes in 35S:DOT leaves is consistent with and can account for the transformation to petals.

We crossed the 35S:DOT transgene into a dot background and found that this complements the mutant phenotype only partially (see Supplemental Figure 7 online). 35S:DOT dot plants flowered precociously, but most of them generated leafy flowers, which lacked petals and stamens and contained sepal-like organs in a whorled arrangement. These leafy flowers occurred usually solitary at the end of a branch, but in few plants they were arranged in a cymose inflorescence. As observed by in situ hybridization, the DOT mRNA signals in developing flowers are extremely strong (stronger than for any other gene that we have analyzed so far), indicating that DOT expression in these cells is very high. We suggest that 35S:DOT cannot fully complement the dot mutant phenotype because the 35S promoter activity in these cells is not adequate to locally drive expression at similar levels as the DOT promoter.

The strong 35S:DOT phenotype in petunia was remarkable, because it was not observed in 35S:UFO Arabidopsis lines (Lee et al., 1997). To find the reason for this disparity, we swapped the transgenes between both species. In Arabidopsis, 35S:DOT caused the occasional formation of supernumerary petals but had no obvious effect on flowering time, inflorescence architecture, or organ identities (Figure 4L), similar to 35S:UFO lines (Lee et al., 1997). In petunia, however, all four independent 35S:UFO lines that expressed the transgene gave a strong phenotype similar to 35S:DOT (see Supplemental Figure 8 online). This indicates that UFO and DOT proteins have very similar activities, which are more restricted in Arabidopsis compared with petunia.

Physical Interaction of DOT with SKP1 Homologs and ALF

To identify interacting proteins that might restrict the activity of ALF/LFY in petunia and DOT/UFO in Arabidopsis, we used yeast two-hybrid analysis. Fusions of LFY and ALF to the DNA binding domain of GAL4 (GAL4BD) resulted in low activation of the GAL4-responsive reporter genes, which was lost in truncated versions of these proteins lacking the N termini (Figure 5A). A yeast two-hybrid screen of an inflorescence cDNA library with truncated ALF (ALF151–412) as bait yielded no interacting proteins.

A DOT-GAL4BD fusion containing the full DOT sequence did not activate the GAL4-responsive reporters and was used as bait to screen the inflorescence library. We identified 71 clones that strongly activated the LacZ reporter when coexpressed with DOT-GAL4BD, but not when they were coexpressed with GAL4BD. Seventy clones with high similarity to yeast S-phase kinase–associated protein1 (SKP1) and a subclass of Arabidopsis homologs encoded four distinct petunia proteins (named PSK1 [for petunia SKP] to PSK4) (see Supplemental Figure 9 and Supplemental Data Set 2 online). PSK1 proteins are core components of SCF complexes, which are ubiquitin ligases (Patton et al., 1998). UFO and FIM also interact with SKP1 homologs in yeast (Ingram et al., 1997; Samach et al., 1999). To our surprise, we found that the remaining clone encoded ALF.

To roughly map the interaction domains, we examined ALF and DOT deletions (Figure 5B). Deleting the 93 N-terminal amino acids from DOT, including the F-box, abolished the interaction with PSK1 but not with ALF. A larger deletion that removed 189 amino acids from the N terminus also abolished the interaction with ALF. Thus, DOT interacts with PSK1 and ALF through distinct domains. The N-terminal 150 amino acids of ALF are sufficient for the interaction with DOT. UFO and LFY also interacted in these assays and were exchangeable with DOT and ALF (Figure 5C).

To examine whether DOT can interact with ALF and PSK1 in vivo, we determined their intracellular localizations. Therefore, we introduced genes expressing green fluorescent protein (GFP) fusions in petunia leaves and petals by agroinfection. We observed that GFP-PSK1 accumulates in different cell types at about equal levels in the cytoplasm and the nucleus (see Supplemental Figure 10 online). ALF-GFP and DOT-GFP are accumulated primarily, but not exclusively, in the nucleus, and this pattern was not altered when putative interactors were coexpressed. Thus, ALF, DOT, and PSK1 coexist in the same compartments.

To study whether DOT can interact with PSK1 and ALF in plant cells, we used bimolecular fluorescence complementation (BIFC; split yellow fluorescent protein [YFP]) (Hu et al., 2002). We expressed DOT fused to an N-terminal fragment of YFP...
(DOT-YFP³) and ALF or PSK1 to a C-terminal YFP fragment (ALF-YFPC or PSK1-YFPC) in petals using agroinfection. Coinfection with constructs expressing DOT-YFPN and ALF-YFPC or PSK1-YFPC resulted in many brightly fluorescing cells, indicating reconstitution of YFP (Figure 5D). This fluorescence depended on the interaction of DOT with ALF or PSK1, because it was not seen when DOT-YFPN was replaced with YFPN or ALF-YFPC or when PSK1-YFPC was replaced with YFPC.

Genetic Interactions between DOT/UFO and ALF/LFY

SCF complexes are ubiquitin ligases that bind their substrates via the F-box protein and usually target their substrate for degradation by the proteasome (Patton et al., 1998). To determine the consequences of the interaction between ALF and DOT, we examined how alterations in ALF expression affected the activity of 35S:DOT in petunia. When we crossed 35S:DOT into an alf background, the 35S:DOT phenotype disappeared completely (Figure 6A). This fluorescence depended on the interaction of DOT with ALF or PSK1, because it was not seen when DOT-YFP³ was replaced with YFPN or ALF-YFPC or when PSK1-YFPC was replaced with YFPC.

Next, we crossed 35S:ALF and 35S:LFY with either 35S:DOT or 35S:UFO and found that petunia seedlings containing both transgenes were growth-arrested (Figure 6B), similar to 35S:LFY 35S:UFO Arabidopsis seedlings (Parcy et al., 1998). After initiating two or three leaf primordia with a normal appearance, further growth stopped and the cotyledons did not unfold.

Analysis of mRNAs extracted from entire seedlings showed that 35S:ALF does not activate any of the B, C, D, or E genes examined, whereas 35S:DOT alone is sufficient to induce B and E genes (Figure 7). Given that ALF is required for the activity of 35S:DOT (Figure 6A), the expression of B and E genes in 35S:DOT seedlings is probably restricted to regions in the emerging leaves where ALF is expressed. Coexpression of 35S:DOT with 35S:ALF enhanced B and E expression, presumably by activating them in a wider domain, and activated an additional set of genes that includes the C- and D-class organ identity genes PETUNIA FLOWERING GENE and FBP26, which encode AP1-like MADS box proteins (Angenent et al., 2005), and FLOOZY, which is the homolog of YUCCA genes from Arabidopsis and is required for the outgrowth of floral organ primordia (Tobéña-Santamaría et al., 2002). 35S:LFY and 35S:UFO had a similar
regulate it by classical ubiquitin-mediated proteolysis. The latter requires both LFY and WUS, which is expressed in the center of the shoot apical meristem. This suggests that besides ALF/LFY and TER/WUS, one or more additional factors are required to activate C genes. The strong expression of C genes in 35S:ALF 35S:DOT and 35S:LFY 35S:UFO seedlings raised the possibility that DOT/UFO is a third factor required for C gene expression (Figure 7A). To distinguish whether DOT activates the C gene FBP14 in concert with WUS (and LFY) or simply bypasses the requirement of WUS, we used in situ hybridization to determine the expression pattern of FBP14 and, as a control, of the B gene Ph GLO. We observed that the expression of FBP14 in 35S:ALF 35S:DOT petunia seedlings is confined to the center of the meristem, where TER is expressed (Stuurman et al., 2002), whereas Ph GLO is activated in a much wider domain, which includes the entire meristem and emerging leaf primordia, which is consistent with the idea that B genes are activated independently from WUS/TER (Figures 7B and 7C). This indicates that DOT/UFO is the third factor that is required in conjunction with ALF/LFY and WUS/TER for the activation of C genes.

To further study the activation of B (Ph GLO), C (FBP14), and E (FBP5) genes by DOT in developing flowers, we compared their expression patterns using double label in situ hybridization. Strikingly, we observed that the DOT mRNA pattern overlaps poorly or not at all with the organ identity genes examined (Figures 7D to 7F). Expression of Ph GLO, FBP14, and FBP5 first became detectable at the time that DOT was expressed in a narrow ring-shaped domain bordering whorls 1 and 2. At this developmental stage, cells in the very center of the flower (presumptive whorl 4), which express FBP14 and FBP5, are up to 100 μm away from those expressing DOT, being separated by some 20 cells that do not express DOT. This suggests that DOT activates these genes in a non-cell-autonomous manner (see Discussion).

**DISCUSSION**

**Role of DOT and UFO in Posttranslational Activation of ALF and LFY**

Genetic and biochemical evidence indicates that UFO operates as part of an SCF complex that is associated with the COP9 signalosome (Zhao et al., 2001; Wang et al., 2003; Ni et al., 2004). SCF complexes are ubiquitin ligases that bind their substrate via F-box proteins (Patton et al., 1998). Because (poly-)ubiquitination often targets a protein for degradation by the proteasome, it was generally assumed that the substrate of SCF^{UFO} is an unknown inhibitor of FM/organ identity (Loehmann and Weigel, 2002). Given that DOT or UFO can activate target genes in virtually all tissues of petunia and *Arabidopsis* (Parcy et al., 1998), this inhibitor should be ubiquitously expressed and well conserved. Our data suggest an entirely different model and indicate that the major target for DOT is ALF. First, two-hybrid screens with DOT bait yielded, besides ALF and the expected SKP1 homologs, no other strong interacting proteins, indicating that the ALF–DOT interaction is specific. Second, in BiFC assays, we observed strong fluorescence signals, indicating that the interaction between ALF and DOT is direct and occurs in planta. Third, the genetic data show that ALF/LFY and DOT/UFO are not clearly altered by coexpression of DOT or chemical inhibition with the inhibitor MG132 (see Supplemental Figure 11 online).

Spatial Expression of DOT and Target Organ Identity Genes

We examined the activation of B- and C-type organ identity genes in 35S:ALF 35S:DOT seedlings in more detail by in situ hybridization. In *Arabidopsis*, the activation of the C gene AG requires both LFY and WUS, which is expressed in the center of the FM. Ectopic expression of WUS is sufficient to ectopically activate AG in outer floral whorls (Lenhard et al., 2001; Lohmann et al., 2001). However, *Arabidopsis* 35S:LFY seedlings or petunia 35S:LFY or 35S:ALF seedlings (Parcy et al., 1998) (Figure 7A) do not express C genes, despite coexpression of LFY/ALF with WUS or its petunia homolog TERMINATOR (TE;

Stuurman et al., 2002) in the center of the shoot apical meristem. This suggests that besides ALF/LFY and TER/WUS, one or more additional factors are required to activate C genes. The strong expression of C genes in 35S:ALF 35S:DOT and 35S:LFY 35S:UFO seedlings raised the possibility that DOT/UFO is a third factor required for C gene expression (Figure 7A). To distinguish whether DOT activates the C gene FBP14 in concert with WUS (and LFY) or simply bypasses the requirement of WUS, we used in situ hybridization to determine the expression pattern of FBP14 and, as a control, of the B gene Ph GLO. We observed that the expression of FBP14 in 35S:ALF 35S:DOT petunia seedlings is confined to the center of the meristem, where TER is expressed (Stuurman et al., 2002), whereas Ph GLO is activated in a much wider domain, which includes the entire meristem and emerging leaf primordia, which is consistent with the idea that B genes are activated independently from WUS/TER (Figures 7B and 7C). This indicates that DOT/UFO is the third factor that is required in conjunction with ALF/LFY and WUS/TER for the activation of C genes.

To further study the activation of B (Ph GLO), C (FBP14), and E (FBP5) genes by DOT in developing flowers, we compared their expression patterns using double label in situ hybridization. Strikingly, we observed that the DOT mRNA pattern overlaps poorly or not at all with the organ identity genes examined (Figures 7D to 7F). Expression of Ph GLO, FBP14, and FBP5 first became detectable at the time that DOT was expressed in a narrow ring-shaped domain bordering whorls 1 and 2. At this developmental stage, cells in the very center of the flower (presumptive whorl 4), which express FBP14 and FBP5, are up to 100 μm away from those expressing DOT, being separated by some 20 cells that do not express DOT. This suggests that DOT activates these genes in a non-cell-autonomous manner (see Discussion).

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fully interdependent for activity, which provides a simple explanation for the very different gain-of-function phenotypes of these genes in *Arabidopsis* and petunia (see below). Together, these data suggest that DOT promotes ALF activity posttranslationally, rather than inhibiting ALF by tagging it for classical proteasome-mediated destruction. As we did not observe any effects of ectopic ALF or LFY expression on phenotype or expression of downstream genes, it appears that, at least in petunia, both ALF and LFY have little or no activity on their own and are fully dependent on DOT or UFO.

Recently, Chae et al. (2008) reported largely complementary data, which indicate that UFO is recruited by LFY to the promoter of the B gene *AP3* to promote *AP3* transcription. Our yeast two-hybrid results differ on several points with those of Chae et al. (2008). In our assays, which were dedicated to a library screen, we detected a weak transcription activation activity in the N terminus of ALF and LFY that was not detected by Chae et al. (2008), possibly due to a lower sensitivity of their quantitative β-galactosidase assay. Second, we mapped the ALF domain interacting with full DOT in the N terminus (ALF1-152), whereas Chae et al. (2008) mapped the interaction of a truncated UFO protein to a C-terminal part (LFY142-420). The reasons for this discrepancy are unknown, but they might be due to the different break points of the ALF and LFY deletions, the opposite orientations of prey and bait, or the use of the entire DOT protein versus a truncated UFO.

The ubiquitin–proteasome system has been shown to stimulate the activity of several transcription factors in yeast (for reviews, see Conaway et al., 2002; Muratani and Tansey, 2003). In some cases, activation involves ubiquitin and proteasome-dependent cleavage of inhibitory domains that block nuclear entry or interaction with other proteins (Conaway et al., 2002). For another and possibly larger set of these proteins, the activity of their transcription activation domain is dependent on SCF complexes and ubiquitination (Salghetti et al., 2001; Lipford et al., 2005; Muratani et al., 2005). Although several models have been proposed to explain this surprising link between transcription activation and the ubiquitin–proteasome system, the underlying mechanisms are still poorly understood.

Because most ALF-GFP localizes in the nucleus in tissues that lack DOT (e.g., leaves), it is unlikely that DOT is required for nuclear entry of ALF. On immunoblots, Chae et al. (2008) observed a smear of 150- to 220-kD polyubiquitinated isoforms of LFY and a 155-kD species that does not react with anti-ubiquitin.
both of which are reduced in the ufo-2 background. However, we could not detect such isoforms in petunia seedlings expressing 35S:LFY together with either 35S:UFO or 35S:DOT (see Supplemental Figure 11 online). Instead, we observed a minor band of ~55-kD that cross-reacted with anti-LFY. Because this species was also seen in seedlings expressing 35S:DOT alone, albeit at somewhat lower abundance, it might represent a mixture of modified LFY and ALF. However, because the anti-LFY serum detected a protein of similar size in petal extracts, we cannot rule out that the 55-kD band represents an unrelated petal-specific protein that reacts to the anti-LFY serum. Thus, it remains to be established whether DOT induces transcription by ubiquitination of ALF or via another protein in the transcription complex. Transcriptional activation has been associated with the rapid recruitment of a 19S signalosome subcomplex to the promoter (Gonzalez et al., 2002). As SCF\textsubscript{UFO} is associated with the structurally related COP9 signalosome complex (Wang et al., 2003), it is also conceivable that the role of DOT/UFO is to recruit the COP9 signalosome complex to the chromatin.

The strong phenotype of petunia dot and tomato an mutants indicates that ALF and FA have little or no activity in the absence of DOT and AN. By contrast, ufo, fim, and stp primarily affect the development of floral organs, especially petals and stamens, and FM identity defects are more subtle, suggesting that the activity of their LFY-like partners is not completely abolished (Ingram et al., 1995; Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995; Taylor et al., 2001; Zhang et al., 2003). We and others (Maizel et al., 2005) did not observe clear functional differences between ALF and LFY proteins, which may account for the different strengths of the ufo and dot phenotypes. Since the phenotype of the weak dot\textsuperscript{R2082} allele is similar to that of ufo and fim mutants, it seems likely that the function of UFO in Arabidopsis and other species is partially redundant. Consistent with this idea, Arabidopsis lines expressing a dominant-negative fusion of UFO, by addition of a transcription repression domain, display a strong FM identity phenotype (Chae et al., 2008) that is similar to that of dot.

Role of UFO and DOT in Flower Development

LFY induces a cascade of events that promote the formation of a flower, including the activation of floral organ identity genes in specific subdomains of the FM. LFY activates A (AP1), B (AP3), and C (AG) genes directly by binding to cis-regulatory elements (Lohmann et al., 2001; William et al., 2004; Chae et al., 2008). Because LFY is expressed in all four floral whorls, it is thought that the expression of A-, B-, and C-type genes is confined to specific subdomains by region-specific coregulators that act in conjunction with LFY (Lohmann and Weigel, 2002). The weak ufo and 35S:UFO phenotypes together with the UFO mRNA expression pattern suggested that UFO is the coregulator that restricts the expression of B genes to whorls 2 and 3 (Lee et al., 1997; Parcy et al., 1998). Our results, however, suggest a wider and more complex role for DOT and UFO in the specification of organ identity.

We found that constitutive expression of ALF/LFY and DOT/UFO in petunia is sufficient to activate B, C, D, and E genes in seedlings and results in arrested growth. Because 35S:LFY 35S:UFO Arabidopsis seedlings display the same phenotype, it is likely that they also ectopically express B, C, D, and E genes (Parcy et al., 1998). For the B gene AP3, this has been confirmed, but C, D, and E genes were not examined (Parcy et al., 1998). These gain-of-function data are consistent with loss-of-function phenotypes. The similarity of the dot, alf, and dot alf phenotypes and transcriptomes indicates that DOT is required for the activation of (nearly) all ALF targets, including most, if not all, organ identity genes. We consider it unlikely that the downregulation of organ identity genes in dot is an indirect effect of the loss of FM identity, because gain-of-function mutants show that they can be expressed in nonfloral tissues in both Arabidopsis and petunia (Parcy et al., 1998) (Figure 7; see Supplemental Figure 6 online).

In Arabidopsis, WUS is the region-specific activator that acts in conjunction with LFY and restricts expression of the C gene AG to the center of the flower (Lenhard et al., 2001; Lohmann et al., 2001). Although 35S:LFY Arabidopsis lines coexpress LFY and WUS in vegetative meristems, this does not lead to the activation of AG (Parcy et al., 1998). Also in petunia 35S:LFY or 35S:ALF seedlings, C (and D) genes are not activated despite coexpression of ALF or LFY with the WUS homolog TDE in the center of the shoot meristem (Stuurman et al., 2002). This indicates that besides WUS/TER and ALF/LFY, at least one other factor is required for C and D gene activation. Our results suggest that this factor is DOT/UFO, as ubiquitous expression of ALF or LFY and DOT or UFO in seedlings efficiently induces C genes, but only in the center of the meristem, where TER is expressed. This role of DOT in C gene activation appears to be conserved, because C gene expression is reduced and delayed in Antirrhinum fim flowers (Ingram et al., 1997). ufo flowers do not display defects in C gene expression (Levin and Meyerowitz, 1995), although UFO activates C genes in petunia seedlings as efficiently as DOT. UFO also might play a role in activating C gene expression in Arabidopsis, but this role might be obscured by a redundant factor in the ufo mutant background.

Paradoxically, the mRNA expression patterns of DOT and B, C, and E genes in the FM show little or no overlap. The same holds for the expression of FlM and B and C genes in Antirrhinum flowers, which can be explained by the cell nonautonomy of FIM activity (Schultz et al., 2001). Because the interactions between DOT/UFO and ALF/LFY and the target promoters are direct, cell nonautonomy probably results from the intercellular movement of DOT/UFO rather than that of a downstream factor. Given that DOT is not fully targeted to an intracellular compartment, it is likely to move between cells by passive diffusion, similar to LFY (Sessions et al., 2000; Wu et al., 2003), which would result in a DOT gradient within the flower (Figure 8). Weak fim and dot alleles primarily affect petal development (Ingram et al., 1997) (Figure 1), suggesting that the activation of B genes requires higher DOT and FIM activity than the activation of C genes. This may explain why a defect in petal development is the common denominator of ufo/dot mutants in distinct species. Interestingly, fusion of LFY to the strong transcription activation domain VP16, which also requires activation by an F-box protein (Salgheetti et al., 2001), overcomes the requirement of UFO (and WUS) for activation of the C gene AG but not for the B gene AP3 (Parcy et al., 1998). Thus, the expression of B and C genes (and possibly D and E genes) has a different requirement for DOT/UFO, for reasons that are currently unclear. However, this does imply that the simple absence or
The spatiotemporal expression of floral identity determines when ALF/LFY expression in the absence of DOT results in inactive protein, as indicated by the unshaded portions.

presence of DOT or UFO protein cannot explain why B gene expression is confined to whorls 2 and 3, suggesting that a refinement of the model of Parcy et al. (1998) is required. Although the current data are suggestive, they are insufficient to conclude whether DOT and UFO act like morphogens and activate distinct genes in a concentration-dependent manner.

**Evolution and Development of Distinct Inflorescences**

The spatiotemporal expression of floral identity determines when (i.e., flowering time) and where (inflorescence architecture) flowers are formed. We found that in petunia, both flowering time and inflorescence architecture are primarily regulated via transcriptional regulation of DOT. This contrasts with Arabidopsis, in which the onset of flowering and the architecture of its racemose inflorescence are primarily regulated via transcription of LFY (Weigel and Nilsson, 1995). This functional divergence of LFY and DOT/UFO homologs is largely due to divergence of their expression patterns, rather than to the encoded proteins (Figure 8B).

Computer modeling suggested that the evolution of distinct inflorescence structures requires changes in the spatiotemporal regulation of the identity of apical and lateral meristems. The shift in FM identity from lateral meristems in racemes to apical meristems in cymes is in part due to the altered expression patterns of LFY homologs. In Arabidopsis and Antirrhinum, these are expressed in lateral meristems and are excluded from the apical IM, whereas in the cymose inflorescences of petunia, tobacco (Nicotiana tabacum), and tomato, LFY homologs are expressed in the apical meristem.

To generate a cyme, it is essential that the FM identity of the lateral meristem is transiently repressed (Prusinkiewicz et al., 2007). Consistent with this notion, ALF mRNA expression is delayed in the lateral IM compared with the apical FM (Souré et al., 1998). However, this is apparently insufficient to establish a cyme, because ectopic expression of DOT disrupts the development of the lateral IM anlagen, presumably because it acquires floral fate precociously. Because this effect of DOT requires ALF (Figure 8A), it appears that ALF expression in the apical FM can alter the identity of the lateral IM in a non-cell-autonomous manner. It was previously shown that the cell nonautonomy of LFY activity is associated with the movement of the protein between meristem cells (Sessions et al., 2000; Wu et al., 2003). Thus, it is the transcription pattern of DOT in the apex, rather than that of ALF, that restricts floral identity to the apical meristem and specifies the cymose architecture.

Because ectopic expression of FM identity throughout the apex can convert both a raceme (Weigel and Nilsson, 1995) and a cyme into a solitary flower, albeit by ectopic expression of distinct meristem identity genes, this supports the hypothesis that a simple genetic path, based on alterations in meristem identity gene expression, accounts for their divergence (Prusinkiewicz et al., 2007).

Interestingly, ALF/LFY and DOT/UFO display additional differences in their expression during vegetative growth, which account for their distinct roles in the onset of flowering (Figure 8B). In Arabidopsis, UFO mRNA is expressed in the apical meristem from embryogenesis on and throughout the vegetative phase (Lee et al., 1997; Long and Barton, 1998), while LFY is activated at the end of the vegetative phase (Blazquez et al., 1997; Hempel et al., 1997). Thus, constitutive transcription of LFY is sufficient to trigger the precocious formation of (terminal) flowers. In petunia, however, ALF is expressed during the vegetative phase and DOT is inactive. Hence, in petunia, the transcriptional activation of DOT is necessary and sufficient to induce flowering. The finding that constitutive expression of both ALF/LFY and DOT/UFO causes a similar phenotype in Arabidopsis and petunia supports the idea that the distinct Arabidopsis and petunia gain-of-function phenotypes are largely due to different expression patterns of the endogenous genes.

At this stage, it is difficult to see why selection would favor the regulation of flowering via the transcription of ALF/LFY or of DOT/UFO homologs, as it essentially results in a shift from transcriptional to posttranslational regulation of ALF/LFY without altering the ultimate output (i.e., active ALF/LFY). Since many plant species express their LFY homologs during vegetative growth (Benlloch et al., 2007), the regulation of flowering time via their UFO homologs may be widespread. Because Antirrhinum has a racemose inflorescence and expresses neither FIM nor its LFY homolog FLORICAULA during the vegetative phase, the
divergence of the expression patterns in vegetative tissues and inflorescences is not necessarily linked. In some legumes, LFY and UFO homologs are involved in the specification of compound leaves (Taylor et al., 2001). Thus, it is possible that the expression of meristem identity genes in vegetative tissues of species with simple leaves, like Arabidopsis and petunia, is a relic of evolution that lost its function and may disappear in time. It is possible that comparative analyses of a wider set of more closely related species could shed light on this important point.

METHODS

Plant Material
The alleles dot\textsuperscript{2508}, dot\textsuperscript{2232}, dot\textsuperscript{93528}, and dot\textsuperscript{C3496} arose among progeny of petunia (Petunia hybrida) line W138 (Koes et al., 1995), and dot\textsuperscript{B2413} arose in the population of a breeder (Syngenta). The weak dot\textsuperscript{V2006} allele was identified among progeny of a self-fertilized DOT\textsuperscript{+}/A2232 heterozygote. The radiation-induced tomato (Solanum lycopersicum) an-1 allele in a Condine Red genetic background was obtained from the Tomato Resource Center at the University of California Davis (accession number LA 0536). Wild-type and an-3 Solanum pimpinellifolium (accession numbers LYC 1231 and MLP 10, respectively) were obtained from the Leibniz Institute of Plant Genetics and Crop Plant Research.

Plants were grown under normal greenhouse conditions. Because seasonal changes in daylength and/or light intensity might influence plant development and flowering time, care was taken to grow different genotypes side by side under the same conditions for comparison.

DNA Methodology
The DOT cDNA was isolated by screening of a petunia R27 inflorescence cDNA library with a FIM cDNA probe. The 5’ end of the DOT cDNA was identified in a tomato EST collection (http://ted.bti.cornell.edu/digital/interface/blast.html). The entire SI DOT cDNA was amplified from cDNA obtained from tomato inflorescences using a primer complementary to the 5’ untranslated region and an oligo(dT) primer that was extended with mRNA from tomato inflorescences using a primer complementary to the 5’ untranslated region and an oligo(dT) primer that was extended with poly(A+) RNA and to construct a cDNA–DNA library.

Expression Analysis
RT-PCR analysis was performed as described (Quattrocchio et al., 2006) using gene-specific primers (see Supplemental Table 2 online) and a limited number of cycles (22 cycles for Ph ACTIN and DOT, UFO, ALF, and LFY expressed from 3SS promoters; 28 cycles for FBP7 and FBP11; and 26 cycles for all other transcripts). PCR amplification products were blotted on nylon membranes and hybridized with gene-specific probes. Signals were read using a phosphorimager analyzer (GE Healthcare). All experiments were performed at least twice, and typical results are shown.

BiFC and GFP Fusion Constructs
A 35S:GFP:NOS expression cassette (Chiu et al., 1996) was cloned as an EcoRi/XhoI fragment into the binary T-DNA vector Bin19 to create RM7. The open reading frames of ALF and DOT without the stop codon were amplified from cDNAs using primers extended with a BamHI site and ligated as BamHI fragments into RM7 to create 3SS:ALF-GFP and 3SS:DOT-GFP. The same BamHI fragments were cloned into pSPYCE-3SSKAN and pSPYNE-3SSKAN (Walter et al., 2004) to create 3SS:ALF-YFP\textsuperscript{C} and 3SS:DOT-YFP\textsuperscript{C}, respectively. The coding sequence of PSK1 without the stop codon was amplified from inflorescence cDNA using primers extended with a BamHI site and cloned into pSPYCE-3SSKAN to create 3SS:PSK1-YFP\textsuperscript{C}. 3SS:GFP-PSK1 was made by recombining the PSK1 coding region into pK7WGF2.0 (Karimi et al., 2002).

Regulation of Flowering by DOT and UFO

Plant Transformation
For 35S:ALF, the ALF coding sequence was amplified from a cDNA clone with primers flo11 (5’-GCTCTAGACCTAGGACCAATGCTG-3’) and flo9 (5’-GGGATCCATGATGATGATGATGATG-3’) and ligated as an XbaI/BamHI fragment into pGreen2K (Bellens et al., 2000). For 35S:DOT, the full DOT cDNA was ligated as a filled-in (Klenow polymerase) Smal/Xhol fragment in the Smal site of pGreen2K. 35S:LYF (DW229) constructs and Arabidopsis thaliana lines were described before (Weigel and Nilsson, 1995; Lee et al., 1997). Constructs in binary vectors were transformed to Agrobacterium tumefaciens AGL0 and used to transform petunia via leaf disc transformation or Arabidopsis by the floral dip method (Horsch et al., 1985; Clough and Bent, 1998). Data shown are representative phenotypes based on the analysis of multiple independent transplants.
50 mM MES, pH 5.7, 0.5% glucose, 2 mM NaH₂PO₄, and 100 μM acetylsyringone (Apollo Scientific) to an O₆₀₀ of 0.3. The bacterial suspension was infiltrated into the abaxial side of petunia W115 leaves or the upper layer of W115 or M1xV30 petals using a 1-mL syringe without a needle. Leaves and flowers were kept at 25°C for 36 to 48 h after infiltration. For coexpression of two transgenes, Agrobacterium suspensions were mixed 1:1 prior to infiltration.

Microscopy

GFP and YFP (BiFC) were visualized using a confocal laser scanning microscope (Bio-Rad Radiance 2000 laser) with an argon laser. Scanning electron microscopy analysis was performed as described previously (Suer et al., 1998).

Phylogenetic Analysis

Multiple sequence alignments of full protein sequences of SKP1 homologs were produced with a Web-based version of ClustalX (http://bips.u-strasbg.fr/fr/Documentation/ClustalX) using default settings. The phylogenetic tree was calculated using the neighbor-joining method and bootstrap analysis (1000 replicates) with PAUP 4.0b10 (Swofford, 2003) and visualized with Treeview version 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: S71192 (FIM), NM102834 (UFO), AF004843 (STP), EU352681 (DOT), EU352683 (PSK1), EU352684 (PSK2), EU352685 (PSK3), EU352686 (PSK4), EU352682 (SI DOT/AN), EU489138 (Sp DOT/AN), NP565123 (ASK1), NP566603 (ASK2), NP565604 (ASK3), NP564105 (ASK4), NP567091 (ASK5), NP566978 (ASK6), NP566693 (ASK7), NP566692 (ASK8), NP566694 (ASK9), NP566695 (ASK10), NP567659 (ASK11), NP567697 (ASK12), NP567609 (ASK13), NP565296 (ASK14), NP566773 (ASK15), NP566297 (ASK16), NP565467 (ASK17), NP563864 (ASK18), NP565295 (ASK19), and NP010615 (SKP1).

Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Phenotypes of 35S:LFY and 35S:ALF in Petunia and Arabidopsis.

**Supplemental Figure 2.** cDNA-Amplified Fragment-Length Polymorphism Analysis of the alf, dot, and alf dot Inflorescences.

**Supplemental Figure 3.** Sequence Alterations in dot Alleles.

**Supplemental Figure 4.** Alignment of DOT and Homologous F-Box Proteins from tomato (Sl DOT), Antirrhinum majus (FIM), Arabidopsis (UFO), and Pea (STP).

**Supplemental Figure 5.** Analysis of Sl DOT and Sp DOT in an-1 and an-3 Mutants.

**Supplemental Figure 6.** 35S:DOT Activates B- and E-Class Organ Identity Genes in Green Tissues.

**Supplemental Figure 7.** Phenotypes of dot, 35S:DOT, and 35S:DOT dot Progeny.

**Supplemental Figure 8.** Phenotype of 35S:UFO in Petunia.

**Supplemental Figure 9.** Analysis of Petunia SKP1 Homologs (PSK1 to PSK4) isolated by Yeast Two-Hybrid Screening with DOT Bait.

**Supplemental Figure 10.** Intracellular Localization of SKP1, ALF, and DOT.

**Supplemental Figure 11.** Immunodetection of LFY Protein Expressed in Petunia Seedlings.

**Supplemental Table 1.** PCR Primers Used for Analysis of Sl FALSIFLORA, Sl DOT, and Sp DOT.

**Supplemental Table 2.** Primers Used for RT-PCR Analysis.

**Supplemental Data Set 1.** Text File of Alignment Corresponding to Supplemental Figure 4 online.

**Supplemental Data Set 2.** Text File of Alignment Corresponding to Supplemental Figure 9 online.

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Recruitment of a 19S proteasome subcomplex to an activated pro-


Patterning of Inflorescences and Flowers by the F-Box Protein DOUBLE TOP and the LEAFY Homolog ABERRANT LEAF AND FLOWER of Petunia
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