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

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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, plant-specific 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 cofluorescences, 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,

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2004; Krizek and Fletcher, 2005). Whether LFY also activates D genes, which determine the identity of ovules, and E genes, which are required for the identity of all floral organs, has not 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 growth 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

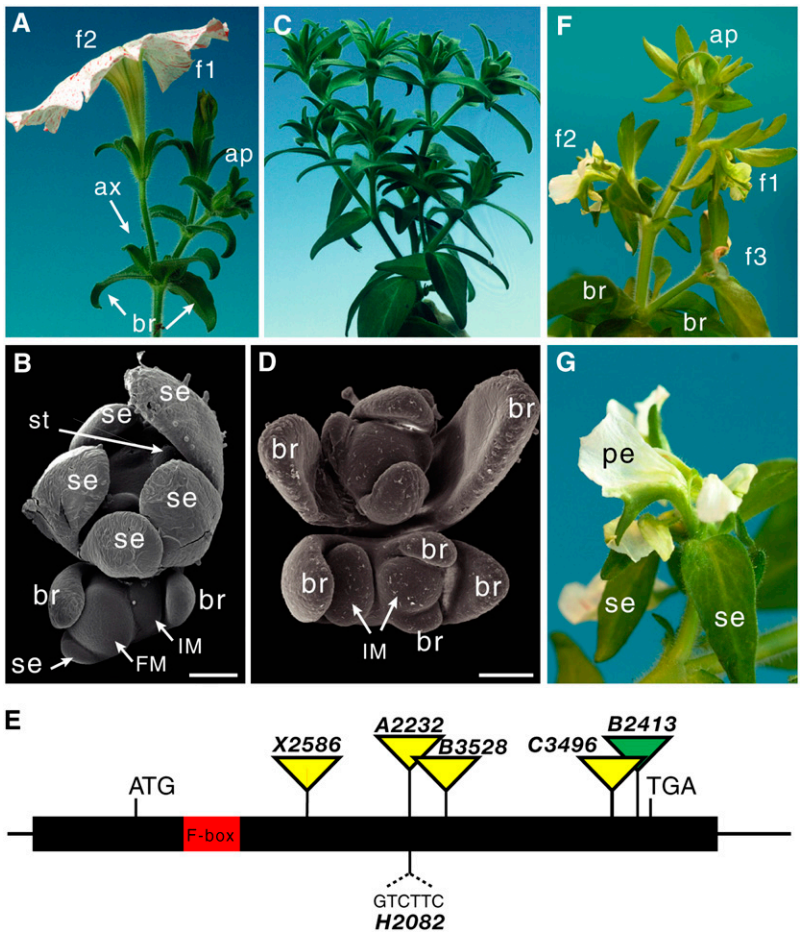

Figure 1. Analysis of *dot* Mutants.

(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 $dot^{H2082/A2232}$ mutant.
ap, apex; ax, axillary meristem; br, bract; f1 and f2, flowers 1 and 2; pe, petal; se, sepal; st, stamen. Bars = 100 μm.

(dot^{A2232}). 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^{X2586}, dot^{B3528}, and dot^{C3496} mutants also contained *dTPH1* insertions in this gene, whereas these insertions were absent in closely related wild-type ($DOT^{+/+}$) progenitors and siblings of each of these mutants (Figure 1E; see Supplemental Figure 3 online). In dot^{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 $DOT^{+/A2232}$ heterozygote, we identified a partial revertant allele (dot^{H2082}) in which *dTPH1* excision created a 6-bp transposon footprint (Figure 1E; see Supplemental Figure 3 online). In $dot^{H2082/A2232}$, the formation of flowers and the cymose inflorescence structure are restored, but the petals

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*; Molinero-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*.

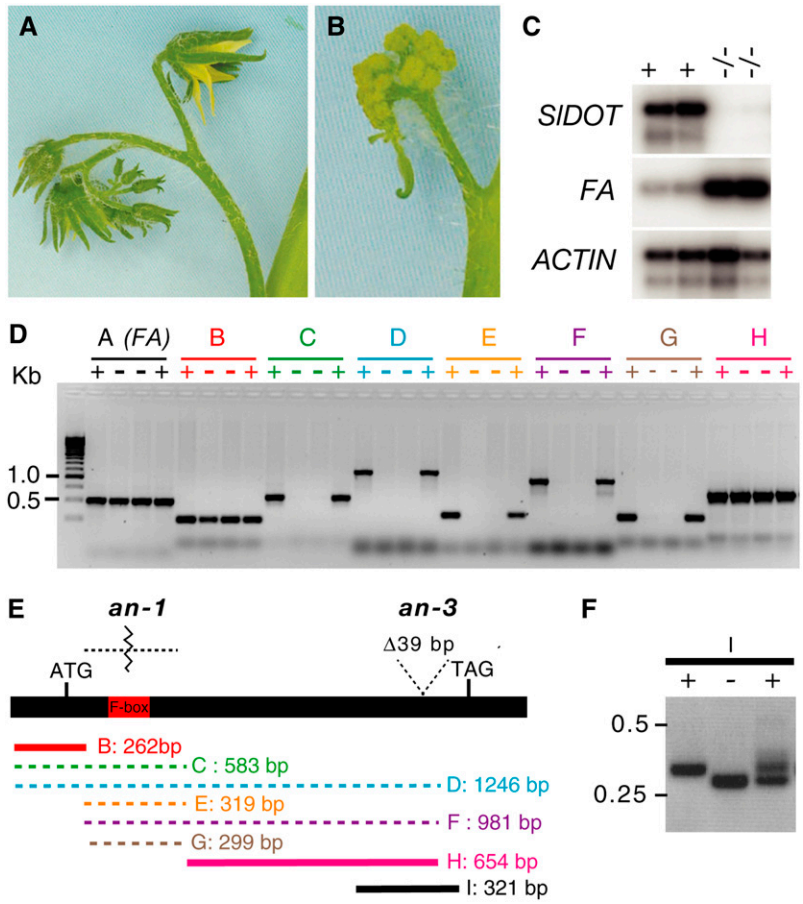

Figure 2. Molecular Analysis of Tomato *an-1* and *an-3* Mutants.

(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).

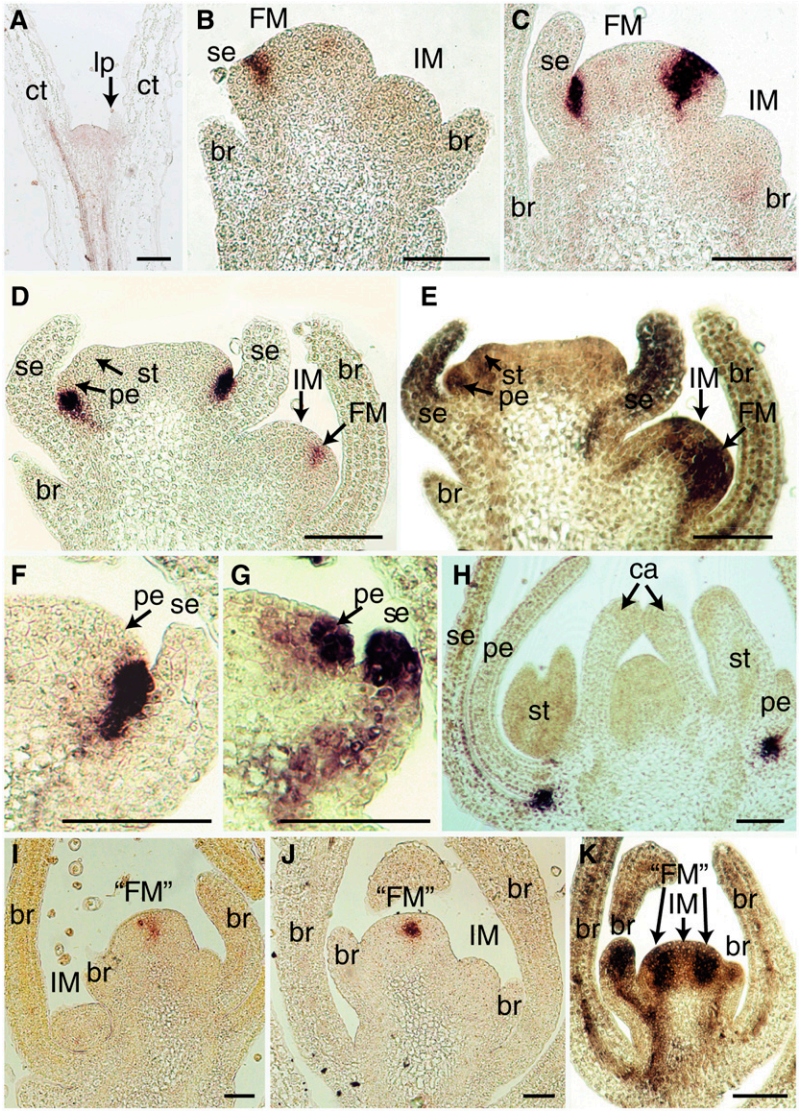

Figure 3. In Situ Localization of *DOT* mRNA in Petunia.

(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 dot^{A2232} **(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 *dot*^{A2232} 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 *35S* promoter (*35S:DOT*). We obtained three independent lines that expressed the transgene. These lines displayed the same dramatic phenotype, which remained constant over four generations. First, *35S: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, *35S: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

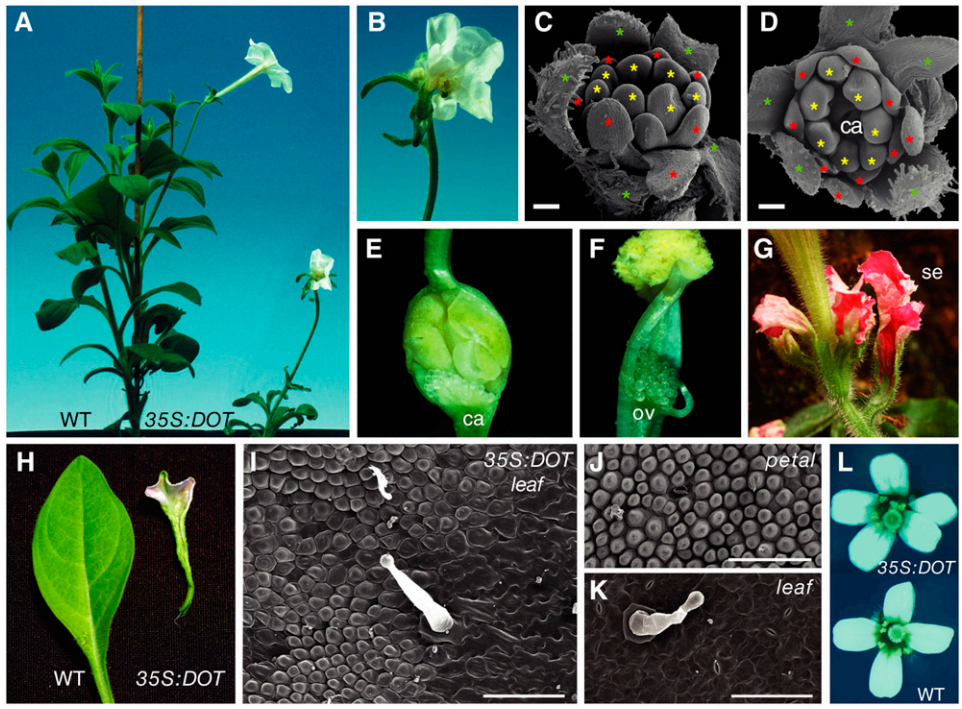

Figure 4. Phenotype of *35S:DOT* Petunia Transformants.

(A) Wild-type and *35S:DOT* plants making their first flowers. The wild-type plant is about twice as old as *35S:DOT* (45 versus 25 d, respectively).
(B) The *35S:DOT* inflorescence is a solitary flower.
(C) and **(D)** Scanning electron micrographs of a *35S: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 *35S: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 *35S:DOT* transformant in a background that specifies red coloration of petal limbs.
(H) Leaves of wild-type and *35S:DOT* plants in a background specifying pale red coloration of petal limbs.
(I) Scanning electron micrograph of a *35S: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 *35S: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 SEPALLATA 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 (GAL4^{BD}) 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 (ALF$^{151\text{-}412}$) as bait yielded no interacting proteins.

A DOT-GAL4^{BD} 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-GAL4^{BD}, but not when they were coexpressed with GAL4^{BD}. 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). SKP1 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

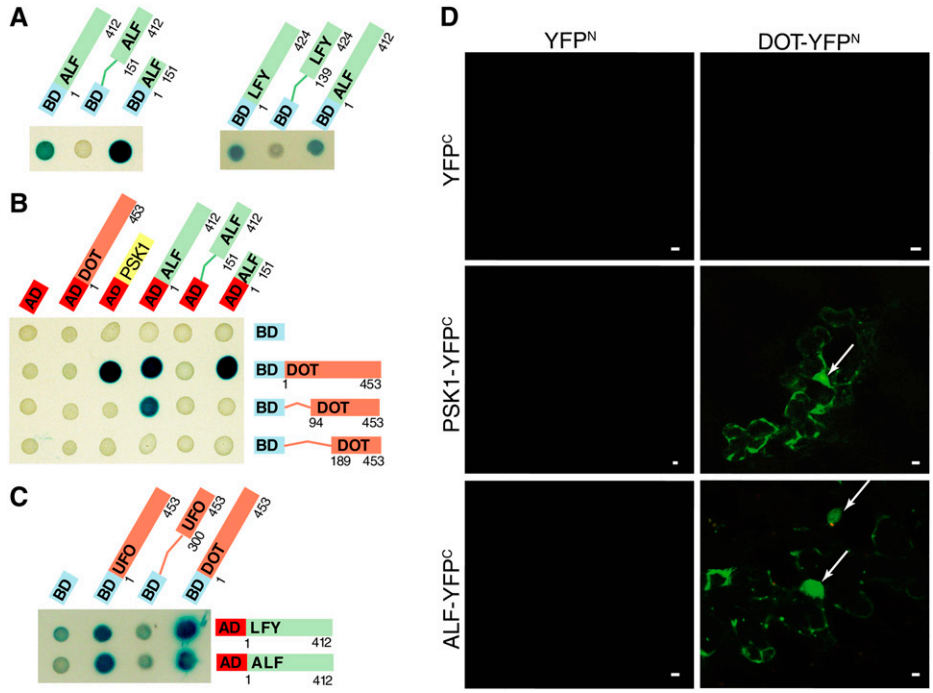

Figure 5. DOT and UFO Interact with ALF and LFY.

(A) Activation of a GAL4-responsive *LacZ* reporter gene in yeast strain expression fusions of $GAL4^{BD}$ to ALF and LFY or fragments thereof. Numbering indicates amino acid residues.

(B) and **(C)** Activation of a GAL4-responsive *LacZ* gene in yeast strains expressing different $GAL4^{BD}$ and $GAL4^{AD}$ fusions.

(D) Confocal images of subepidermal petal cells after coinfection with constructs expressing fusions of DOT, ALF, and PSK1 and the N-terminal or C-terminal part of YFP (YFP^N or YFP^C) or, as a negative control, YFP^N and YFP^C alone. The arrows mark strongly fluorescent nuclei. Bars = 10 μm.

(DOT-YFP^N) and ALF or PSK1 to a C-terminal YFP fragment (ALF-YFP^C or PSK1-YFP^C) in petals using agroinfection. Coinfection with constructs expressing DOT-YFP^N and ALF-YFP^C or PSK1-YFP^C 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-YFP^N was replaced with YFP^N or ALF-YFP^C or when PSK1-YFP^C was replaced with YFP^C.

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). That is, leaves of *35S:DOT alf* were similar to those of *alf* and the wild type. Moreover, *35S:DOT alf* plants switched from vegetative to reproductive growth at the same time as wild-type plants, but they produced an abnormal inflorescence similar to *alf*. In fact, we could not distinguish *35S:DOT alf* from *alf* siblings by phenotype. Hence, even though *ALF* expression during the vegetative phase has no apparent function in the wild type (Souer et al., 1998), it is essential in *35S:DOT* for precocious flowering and the transformation of leaves into petals. These observations support our yeast two-hybrid results and indicate that *ALF* is the major target of *DOT*.

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 (Tobeña-Santamaria et al., 2002). *35S:LFY* and *35S:UFO* had a similar

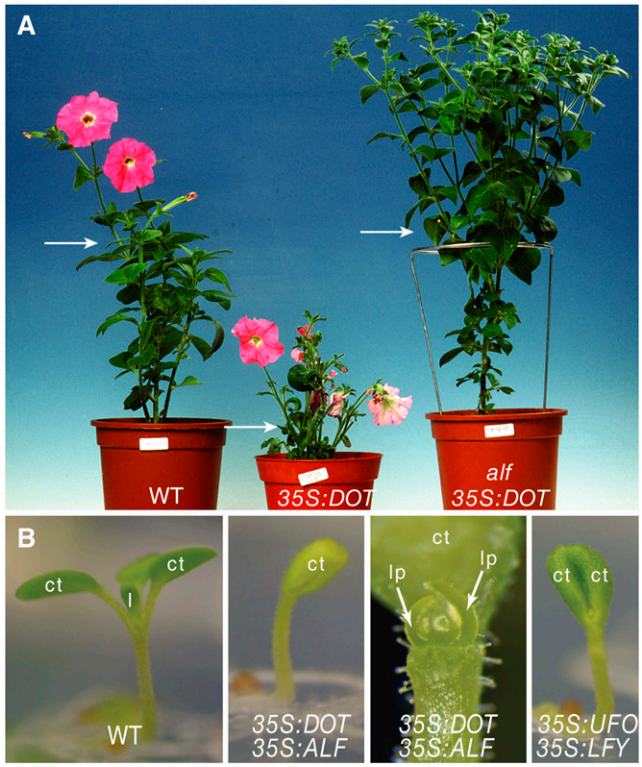

Figure 6. Genetic Interaction of *ALF* and *DOT*.

(A) Comparison of wild-type, *35S:DOT*, and *35S:DOT alf* phenotypes. The arrows indicate the stage when the plants switched from vegetative to reproductive growth.

(B) Phenotypes of wild-type (WT) and growth-arrested *35S:DOT 35S:ALF* and *35S:UFO 35S:LFY* petunia seedlings. ct, cotyledon; l, leaf; lp, leaf primordium.

effect, which again shows that ALF and LFY as well as DOT and UFO proteins are functionally similar (Figure 7A).

Together, these findings show that DOT/UFO is required to promote ALF/LFY-mediated transcription rather than to down-regulate it by classical ubiquitin-mediated proteolysis. The latter is further substantiated by the finding that LFY protein levels are not clearly altered by coexpression of DOT or chemical inhibition of proteasome activity 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 coexpresssion of *LFY/ALF* with *WUS* or its petunia homolog *TERMINATOR* (*TER*; 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).

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 (Lohmann 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

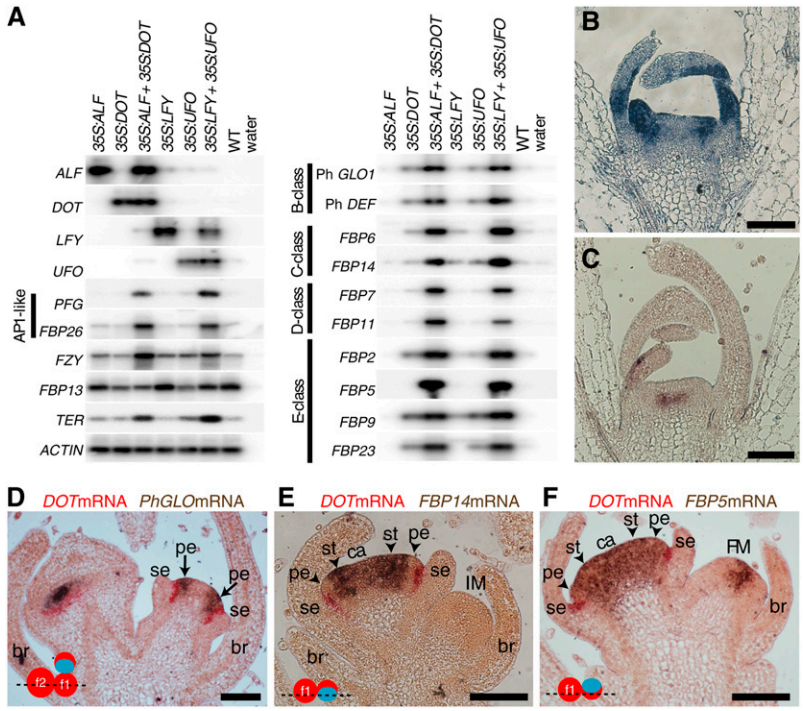

Figure 7. Regulation of Organ Identity Genes by DOT.

(A) RT-PCR analysis of RNA extracted from entire seedlings with different genotypes (see Supplemental Table 2 online for primer sequences).
(B) In situ hybridization of the Ph *GLO* mRNA in the apex of a *35S:ALF 35S:DOT* seedling.
(C) In situ hybridization of *FBP14* mRNA in the apex of a *35S:ALF 35S:DOT* seedling.
(D) to **(F)** Double label in situ hybridization of *DOT* mRNA (**[D]** to **[F]**) and mRNA from the B gene Ph *GLO* **(D)**, the C gene *FBP14* **(E)**, and the E gene *FBP5* **(F)**. *DOT* mRNA is seen as a red signal, and RNAs from Ph *GLO*, *FBP14*, and *FBP5* are seen as brown signal. The inset diagrams depict top views of the plane and position (black line) of the section on the inflorescence, as deduced from examination of a complete series of sections. The red circles indicate the FM and older flowers (f1), and the blue circle represents the IM.
br, bract; ca, carpel; pe, petal; se, sepal; st, stamen. Bars = 100 μm.

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 (ALF^{1-152}), whereas Chae et al. (2008) mapped the interaction of a truncated UFO protein to a C-terminal part ($LFY^{142-420}$). 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^{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^{H2082} 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 *TER* 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 *FIM* 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 (Salghetti 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

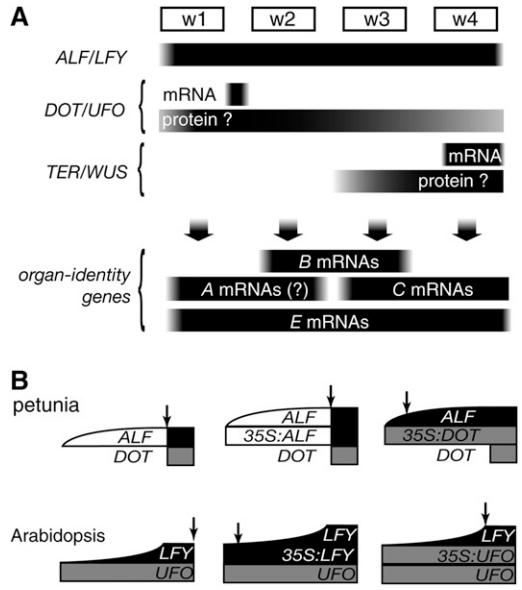

Figure 8. Model Explaining the Role of DOT in the Onset of Flowering and the Patterning of Flowers.

(A) Model for the role of DOT in the activation of distinct classes of organ identity genes. The white blocks at the top indicate organ primordia in whorls 1 to 4 (w1 to w4). Expression patterns of various mRNAs and proteins are indicated by shaded bars. Black color denotes a high concentration, and lighter (gray) color indicates a lower concentrations. The distribution patterns of DOT/UFO and TER/WUS proteins are hypothetical, as indicated by the question marks. A-type organ identity genes similar to *AP1* have not been identified in petunia, as indicated by the question marks.

(B) Model explaining the disparate phenotypes of homologous petunia and *Arabidopsis* mutants. The graphs depict gene expression (vertical axis) during plant development (horizontal axis). The arrows indicate the onset of flowering. ALF 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 (Souer 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 6A), 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*^{X2586}, *dot*^{A2232}, *dot* ^{B3528}, and *dot*^{C3496} arose among progeny of petunia (*Petunia hybrida*) line W138 (Koes et al., 1995), and *dot*^{B2413} arose in the population of a breeder (Syngenta). The weak *dot*^{H2082} allele was identified among progeny of a self-fertilized *DOT*$^{+/A2232}$ 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 Sl *DOT* was identified in a tomato EST collection (http://ted.bti.cornell.edu/digital/interface/blast.html). The entire Sl *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 multiple restriction sites. Primers used for PCR analysis of *an* mutants are shown in Supplemental Table 1 online. DNA isolation and DNA gel blot analysis were done as described (Koes et al., 1995) using high-stringency posthybridization washes (0.1× SSC [1× SSC is 0.15 M NaCl and 0.015 M sodium citrate], 65°C).

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 35S 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 phosphorimage analyzer (GE Healthcare). All experiments were performed at least twice, and typical results are shown. In situ hybridization was performed as described (Souer et al., 1998) using digoxygenin-labeled RNA probes and Western Blue stabilized substrate for alkaline phosphatase (Promega). Posthybridization washes included an RNase treatment, which essentially eliminates cross-hybridization to related mRNAs and reduces background resulting from nonspecific probe binding. *ALF* and *DOT* probes were prepared from full-size cDNA. Probes for Ph *GLO*, *FBP5*, and *FBP14* were prepared from N-terminally truncated cDNA, which lacked the region encoding the MADS box. For double label in situ hybridization, a fluorescein-labeled *DOT* probe was synthesized with T7 RNA polymerase using a fluorescein labeling kit (Roche). Fluorescein- and digoxigenin-labeled probes were hybridized simultaneously. The fluorescein-labeled *DOT* probe was detected by anti-fluorescein antibodies linked to alkaline phosphatase and staining for 40 h using Fast Red tablets as recommended by the supplier (Roche), resulting in a red precipitate. After photography, the slides were incubated for 2 h in 2× SSC at 65°C to inactivate the alkaline phosphatase. Subsequently, the digoxigenin-labeled probe was detected as above and slides were photographed again.

Immunoblot analysis followed standard procedures using an anti-LFY antiserum (a kind gift of Detlef Weigel). For MG132 treatment, samples were incubated with or without 50 μM MG132 for 16 h.

Plant Transformation

For *35S:ALF*, the *ALF* coding sequence was amplified from a cDNA clone with primers flo11 (5′-GCTCTAGAACATGGACCCAGAG-3′) and flo9 (5′-CGGGATCCTTAGAATGACAACCTAA-3′) and ligated as an *Xba*I/*Bam*HI fragment into pGreen2K (Hellens et al., 2000). For *35S:DOT*, the full *DOT* cDNA was ligated as a filled-in (Klenow polymerase) *Sma*I/*Xho*I fragment in the *Sma*I site of pGreen2K. *35S:LFY* (DW151) and *35S:UFO* (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 transformants.

Yeast Two-Hybrid Analysis

Inflorescence apices (line W138) similar to those in Figure 1B were dissected and used to isolate poly(A$^+$) RNA and to construct a cDNA–DNA library in λ hybri-ZAP (Stratagene) according to the instructions. Library screening was performed as described (Quattrocchio et al., 2006). To assay yeast two-hybrid interactions between specific proteins, the corresponding coding sequences were amplified from cDNA clones, ligated in pAD-GAL4 and pBD-GAL4, and tested for the activation of GAL4-responsive *HIS*, *ADE*, and *LacZ* reporters as described (Quattrocchio et al., 2006). Each combination of bait and prey was transformed in yeast two times independently. Cultures used to assay reporter gene activity were checked by PCR to ensure that they contained the appropriate bait–prey combinations.

BiFC and GFP Fusion Constructs

A *35S:GFP:NOS* expression cassette (Chiu et al., 1996) was cloned as an *Eco*RI/*Xho*I fragment into the binary T-DNA vector Bin19 to create RAM7. The open reading frames of *ALF* and *DOT* without the stop codon were amplified from cDNAs using primers extended with a *Bam*HI site and ligated as *Bam*HI fragments into RAM7 to create *35S:ALF-GFP* and *35S:DOT-GFP*. The same *Bam*HI fragments were cloned into pSPYCE-35SKAN and pSPYNE-35SKAN (Walter et al., 2004) to create *35S:ALF-YFP*^{C} and *35S:DOT-YFP*^{N}, respectively. The coding sequence of *PSK1* without the stop codon was amplified from inflorescence cDNA using primers extended with a *Bam*HI site and cloned into pSPYCE-35SKAN to create *35S:PSK1-YFP*^{C}. *35S:GFP-PSK1* was made by recombining the *PSK1* coding region into pK7WGF2.0 (Karimi et al., 2002).

Agroinfiltration of Petals and Leaves

Agrobacterium GV3101 cells containing an appropriate construct were grown at 30°C in Luria-Bertani (LB) broth containing 150 mg/L kanamycin and 100 mg/L rifampicin, harvested by centrifugation, and suspended in

50 mM MES, pH 5.7, 0.5% glucose, 2 mM NaH_2PO_4, and 100 μM acetosyringone (Apollo Scientific) to an OD_{600} 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 (Souer 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 (Sl *DOT*/*AN*), EU849138 (Sp *DOT*/*AN*), NP565123 (*ASK1*), NP568603 (*ASK2*), NP565604 (*ASK3*), NP564105 (*ASK4*), NP567091 (*ASK5*), NP566978 (*ASK6*), NP566693 (*ASK7*), NP566692 (*ASK8*), NP566694 (*ASK9*), NP566695 (*ASK10*), NP567959 (*ASK11*), NP567967 (*ASK12*), NP567090 (*ASK13*), NP565296 (*ASK14*), NP566773 (*ASK15*), NP565297 (*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 PSK1, 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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