The evolutionary divergence of the genetic networks that control flowering in distinct species

Della Pina, S.

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Role of *DOUBLE TOP* in the activation of *ABERRANT LEAF AND FLOWER* during flowering time

Serena Della Pina, Bets Verbree, Ronald Breedveld, Erik Manders, Erik Souer and Ronald Koes

S.D.P., E.S. and R.K. designed experiments. S.D.P. carried out the majority of the experiments. S.D.P. and B.V. carried out experiments, analyzed and interpreted data. S.D.P. wrote the paper
Abstract

Flowering plants display a wide variety of inflorescence architectures due to the spatio-temporal expression patterns of two meristem identity genes that together specify the “floral fate” or “floral identity” of a meristem. In petunia *DOUBLE TOP (DOT)* is an F-box protein component of SCF complexes with E3 ubiquitin ligases function. *DOT* is essential to specify floral meristem identity and, trough interaction with *ABARRENT LEAF AND FLOWER (ALF)*, activates floral organ identity genes. Although *ALF* is expressed in the whole flower meristem (FM), *DOT* expression pattern hardly overlaps with that of ABC genes. Here we show that the DOT protein, after being synthesized in cells at the periphery of the FM in the sepals/petals boundary, migrates into the center of floral meristem. Moreover we showed that in order to activate ABC genes, DOT interacts with ALF, suggesting that it activates ALF by a posttranslational mechanism.
Introduction

The first steps in the development of a flower involve the activation of a handful of floral meristem identity (FMI) genes, which specify the floral fate of meristems. Hence, the spatial temporal regulation of FMI gene expression is a key factor that determines when (flowering time) and where (inflorescence architecture) flowers are formed, and where, within the flower, organs like sepals, petals, stamens and carpels are formed. LEAFY from *Arabidopsis* and homologs from a broad range of Angiosperms, such as *ABERRANT LEAF AND FLOWER* (*ALF*) from petunia, encode a unique transcription factor that is essential for FMI (Souer et al., 1998; Weigel and Nilsson, 1995). In petunia and tomato specification of FMI requires in addition an F-box protein, encoded by *DOUBLE TOP* and *ANANTHA* respectively, and mutations in *DOT* or *AN* cause a nearly complete transformation of flowers into inflorescence shoots which lack floral characteristics (Lippman et al., 2008; Souer et al., 2008). For unknown reasons mutations in the homologous genes from *Arabidopsis* (*UNUSUAL FLORAL ORGANS*, *UFO*), *Antirrhinum* (*FIMBRIATA*, *FIM*) and other species cause only a mild FMI defect (Samach et al., 1999; Simon et al., 1994; Taylor et al., 2001). In *Arabidopsis ufo* mutants, for example, the very first formed flowers are transformed into co-inflorescences, while later flowers do form but present defects in the development of petals and stamens (Hepworth et al., 2006; Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995).

In *Arabidopsis* LFY binds to and activates hundreds of downstream genes, including different sets of homeotic floral organ identity genes, known as type A, B, C, D and E genes, in specific subdomains of the floral meristem (Busch et al., 1999; Lohmann and Weigel, 2002; Parcy et al., 1998; William et al., 2004; Winter et al., 2011). Because LFY is expressed throughout the emerging floral meristem, it is thought to act in combination with distinct partners that are expressed in more restricted patterns, to activate downstream genes in different subdomains of the flower (Jack, 2004; Krizek and Fletcher, 2005). Transcription of the C-type organ identity gene *AGAMOUS* (*AG*), which specifies the identity of stamen and carpel primordia, for instance, is restricted to the center of the flower because that requires simultaneous binding of LFY and the homeodomain transcription factor *WUSCHEL* (*WUS*) to distinct sites in *AG* (Lohmann et al., 2001). It was proposed early on that UFO acts as a co-regulator of LFY to ensure that the B type organ identity genes *APETELA 3* (*AP3*) and *PISTILATA* (*PI*) are activated in whorls 2 and 3 (Chae et al., 2008; Samach et al., 1999). This was based on the findings that (i) *UFO* and B-gene expression patterns overlap, (ii) that *ufo* mutation reduces B-gene expression and that (iii) ectopic expression of *UFO* induces B gene expression outside their normal domain. Given that *UFO* is the F-box component of an SCF-
type ubiquitin ligase, it was assumed that UFO targets, via ubiquityination, an unknown inhibitory protein for degradation.

A range of subsequent findings in petunia and Antirrhinum were incompatible with this early model, and suggested a slightly different function for UFO and homologs (Simon et al., 1994; Souer et al., 2008). First, it was found that UFO and the petunia homolog DOT directly interact with LFY and ALF, and that UFO can bind, via LFY, to the PI promoter (Souer et al., 2008; Chae et al., 2008). This suggested that the role of UFO/DOT is to promote the transcription activation potential of ALF/LFY rather than triggering the degradation of an unknown (intermediate) inhibitor. Second, strong dot alleles cause, unlike ufo, a (near) complete transformation of flowers into shoots, and constitutive co-expression of DOT (or UFO) and ALF (or LFY) activated in petunia seedlings a broad set of organ identity genes of the B, C and E class (A-type genes were not tested), indicating that DOT/UFO is involved in the activation of many more LFY-regulated genes than B-type organ identity genes. Third, within the developing flower DOT mRNA is restricted to narrow stripe of cells at the boundary of whorls 1 (sepals) and 2 (petals) and, unlike UFO, does hardly or not overlap with the (future) expression domain of mRNAs of ALF or the downstream B and C genes. To reconcile these findings we proposed a refined version of the model, which stated that (i) the DOT protein may travel between cells from the whorl 1/2 boundary toward the center of the floral meristem into the region where B and C genes are activated, potentially forming a gradient, and (ii) that the role of DOT and UFO is to boost the transcription activation potential of ALF/LFY by a post-translational mechanism that might be similar to the way some yeast transcription factors get activated by the ubiquitin proteasome system.

To assess the validity of this refined model we have put the two major underlying premises to a test. Here we show that ALF can activate the promoters of AG, a C-type organ identity gene, and AP1, a gene with FMI and A-type organ identity function in yeast, but only if it is co-expressed with DOT and that in planta a genetic fusion of Ubiquitin to ALF can rescue the FMI and organ identity defects in dot mutants. Furthermore we provide evidence that GFP-tagged DOT protein is mobile and it can travel from the sepal/petal boundary to the center of the floral meristem.

Results

Role of DOT in posttranslational activation of ALF

Although LFY can bind to specific binding sites in subordinate floral organ identity genes on its own, additional factors are required to activate transcription. For example, transcriptional activation of AGAMOUS in Arabidopsis or in yeast requires co-expression of LFY with the
transcription factor *WUS* (Lenhard *et al.*, 2001; Lohmann *et al.*, 2001). In petunia seedlings, *ALF* can ectopically activate a wide array of floral organ identity genes, but only when co-expressed with *DOT* (Souer *et al.*, 2008). In yeast and *Arabidopsis* cells the requirement of additional proteins for activation of *AGAMOUS* promoter (pAG) can be bypassed by fusion of an heterologous transcription activation domain, such as VP16 for example, to LFY (Lohmann *et al.*, 2001; Parcy *et al.*, 1998). We hypothesized that UFO/DOT may be the co-factor that is required in order to activate B and C genes. In order to test this idea, we analyzed in a yeast one-hybrid assay whether ALF and DOT can activate regulatory sequences of AG, which is a C gene of *Arabidopsis*, using the same pAG:LacZ reporter construct previously used by Parcy et al. We found that either ALF or DOT alone are incapable of promoting strong pAG:lacZ expression in yeast, whereas co-expression of ALF and DOT strongly induced pAG:lacZ expression (Figure. 1A). We next quantified the activity of the LacZ reporters fused to either AG enhancers or a promoter element of the A-type gene *AP1* (Parcy *et al.*, 1998) by measuring hydrolysis of the chromogenic substrate o-nitrophenyl-β-D-galactoside (ONPG) (Miller, 1972) (Fig. 1B). When we expressed both ALF and DOT we observed a strong activation of the pAG:LacZ reporter, while expression of β-galactosidase in the presence or absence of ALF alone the signal was barely detectable. Also, the activity of AP1:LacZ was boosted when by ALF and DOT, but in this case the basal activity of the pAP1:LacZ construct appeared slightly higher.

These experiments indicate that *DOT* is necessary and sufficient to induce the transcription activation potential of ALF and can promote the expression the A and C type floral organ identity genes *AP1* and *AG*. 


Figure 1: DOT can provide transcriptional activation activity to ALF.
(A) Expression of pAG::LacZ in yeast cells expressing ALF or DOT or both (ALF+DOT) visualized by a Xgal-based color assay. (B) β-galactosidase activity of yeast cells the containing the reporter gene pAG::LacZ (violet bars), pAP1::LacZ (grey bars) or none (white bars) and effector genes expressing ALF, DOT or both.

**p35S:UB-ALF partially rescues the dot mutant phenotype.**

In recent years it was shown for several transcription factors that in addition to DNA binding per se, the ubiquitin-proteasome system is required for transcription activation, either by preventing that the transcription factor is stripped from the chromatin, or by enhancing the activity of the transcription activation domain (Archer et al., 2008; Salghetti et al., 2001). To examine whether DOT or the SCF^{DOT} complex acts to enhance the transcription activation domain of ALF by ubiquitinylation, we asked whether the expression of a genetic fusion of Ubiquitin to ALF in petunia could bypass the necessity of DOT. To this end, we generated a translational fusion between ALF and a single UBIQUITIN and tested its ability to complement the defects seen in dot mutants. Constitutive expression of a native ALF protein (p35S:ALF) in dot mutant was unable to rectify the inflorescence architecture or the flower identity defects (Fig. 2C). This result is consistent with earlier data showing that (i) ALF mRNA remains normally expressed in dot meristems and that alf dot double mutants have the same defects as either single mutant and (ii) that ALF expression in vegetative tissues, expressed from the endogene or a 35S:ALF transgene, has no effect on development, unless DOT is ectopically expressed (Souer et al., 2008).
Figure 2: Expression of *UB-ALF* restores the development of flowers and floral organs in *dot* mutants.
(A-B) Wild type W115 and *dot* mutant inflorescences. (C) Constitutive expression of *p35S:ALF* in a *dot* mutant does not rescue floral meristem or floral organ identity. (D-G) Rescue of the *dot* phenotype by the *p35S:UB-ALF* transgene. (D-F) Both inflorescence structure and flowers are restored almost as a wild type. (G) Detail of a flower on a strong *p35S:UB-ALF* expressor. Flowers appear identical to those on wild type inflorescences. (H) cDNA and gDNA amplification from a *p35S:UB-ALF* transformant, *dot* mutant and a wild-type plant showing B type gene expression in a *dot* mutant background and the presence of the transposon in native *DOT* gene.

When we ectopically expressed the ubiquitinylated form of ALF (*p35S:UB-ALF*), in six out of twenty independently transformed *dot* mutants the mutant phenotype was complemented to various degrees. Two showed complementation of the inflorescence architecture in some branches with partially fertile flowers (Fig. 2D). Three transformants showed near complete complementation with a stronger restoration of the flower defects and the inflorescence architecture was restored in more branches (Fig. 2E-G) and one plant was showing occasional petal tissue along *dot* mutant branches (Fig. 2F). It was also possible to spot petal tissue appearing around the main vein of some bracts, which is a typical feature of plants that ectopically express DOT. We then checked whether the
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Complementation was really due to the presence of the UB-ALF transgene and not to excision of the transposon from the endogenous DOT gene, although we never saw any somatic reversion in the dot^{A2232} allele (used in this experiments); only among progeny DOT^{+}\text{A2232} heterozygote, was identify a partial revertant allele (dot^{H2082}) where flowers and the cymose inflorescence structure were restored, but the petals showed stripes of sepal- or leaf-like tissue (Souer et al., 2008). As shown in Fig. 2H, the transposon was still present in the DOT coding sequence. We also showed the activation of the B-type floral organ identity gene TOMATO BOX 6 (TM6) in plants expressing the UB-ALF transgene. These results showed that when the protein of ALF is artificially ubiquitined, and so able to activate the floral organ identity genes, the function of DOT is no longer necessary.

**DOT protein migrates from the adaxial sepal boundary to the floral meristem center**

The expression domain of DOT mRNA hardly overlaps with the expression domain of the target ABC genes and therefore the activation of these target genes seems to occur in a non-cell-autonomous manner (Souer et al., 2008). To test the possibility of cell-to-cell movement of DOT, we generated a chimeric DOT protein by fusing the GFP coding sequence at the C-terminus of the DOT ORF. This fusion construct was expressed from the native DOT promoter, pDOT:DOT-GFP:tDOT (Fig. 3A). Previous experiments (Chapter 2) showed that these regulatory regions are sufficient to fully complement the dot defects when fused to a DOT coding sequence.

First we characterized the DOT-GFP fusion by constitutively expressing it from the 35S promoter in wild type petunia (Fig. S1). We found that the p35S:DOT-GFP transformants flowered precociously compared to wild-type plants, that the cymose inflorescence was reduced to a solitary flower (Fig. S1B-C) and that leaves, bracts, and sepals contained patches of petal-like tissue (Fig. S1D), similar to what was observed with p35S:DOT (Souer et al., 2008). These results indicate that the GFP tag does not affect the DOT function. Furthermore, we confirmed by immunoblot analysis that the DOT-GFP fusion protein remains intact and is not cleaved (Fig. 3B). These We then expressed DOT-GFP using a 4.6 kb 5′-flanking sequence (pDOT\textsuperscript{4.6}) and a 3′ flanking sequence of DOT (pDOT\textsuperscript{4.6}:DOT-GFP:tDOT construct) in a dot mutant background. We previously demonstrated that a GUS transgene driven by these regulatory (pDOT\textsuperscript{4.6}:GUS:tDOT) accurately reproduced the DOT expression pattern and, when fused to DOT could fully rectify the phenotype of dot mutants (Chapter 2). We found that a once again that the protein fusion showed correct functional activity, since all the defects caused by dot were completely rescued. The above results showed that the pDOT\textsuperscript{4.6}:DOT-GFP:tDOT construct expresses an intact and functional DOT-GFP protein at the correct time and place in development.
Figure 3: The pDOT:DOT-GFP:tDOT construct expresses an intact and functional DOT-GFP protein at the correct time and place in development.

(A) Diagram of pDOT:DOT-GFP:tDOT. The GFP protein is fused to the C-terminus of the DOT protein (green). (B) DOT-GFP fusion proteins detected with anti-GFP in meristems of p35S:DOT-GFP showing intact protein fusion. A 35S:GFP transformant as well as a untransformed plant are used as controls.

(C-D) A fully developed wild type flower (C) compared to a flower from a dot mutant expressing pDOT:DOT-GFP:tDOT. (E) SEM analysis of reproductive wild type apex showing a terminal flower (f3) and a sympodial meristem (i). (F) Confocal image of an apex on a pDOT:DOT-GFP:tDOT transformant showing a reproductive apex at approximately the same stage as in E. (G) SEM analysis of reproductive wild type apex slightly later in development, showing a further developed terminal flower (f3) and a sympodial meristem (i). (H) Confocal image of pDOT:DOT-GFP:tDOT expressors showing a floral meristem at a similar stage as in G. Note that the GFP signal seems to extend towards the center of the floral meristem. Scale bar, 50 µm

We then analyzed the localization of the DOT-GFP protein fusion in the apical meristem by laser scanning confocal microscopy and observed a bright fluorescence signal within the emerging FM at the site where DOT is normally expressed (Fig. 3F and 3G). In the young FM, GFP signal was restricted to a stripe of cells on the adaxial side of sepal primordia (Fig. 3F). When all sepal primordia were formed, GFP expression had expanded into a ring marking the boundary between whorls 1 and 2. This localization overlaps with the expression pattern of DOT mRNA. A detailed comparison of the DOT mRNA and the DOT-GFP expression pattern revealed that the DOT protein moves laterally from its site of synthesis to the center of the
floral meristem by at least two cell layers (Fig. S2). At slightly later developmental stages, GFP signal was visible in the entire FM dome forming a gradient of intensity: in the source area, where the DOT gene is transcribed, the signal was very strong and faded towards the FM center (Fig. 3G-H). Since by in situ hybridization we never detected neither the DOT mRNA nor GUS mRNA of pDOT^{5':3':GUS} in the FM center (Fig 4 in Chapter 2), we inferred that the GFP signal here was due to DOT protein moving from the cells of the sepal/petal boundary, where it is synthesized, to adjacent cells in the FM center.

**Figure 4: DOT protein moves towards the center of floral meristems.**

(A–E) FRAP in the flower meristem of pDOT:DOT-GFP:tDOT cells. After photobleaching GFP signal is restored in the proximal and distal region of DOT expression after 150 seconds. (A) Longitudinal floral meristem section showing the situation before (A), immediately after (B) and 150s (C) after photobleaching. Scale bars, 50 μm. (D) FRAP measured after photobleaching of the marked colored Regions Of Interest (ROIs) in three different positions on the pDOT:DOT-GFP:tDOT meristem. 150 s after photobleaching almost 100% of the GFP signal was restored. (E) Detail of the quantification of green and blue regions.
FRAP reveals DOT protein movement in the FM center

To obtain further evidence that DOT-GFP moves between cells, we performed fluorescence recovery after photobleaching (FRAP) in several regions of the FM dome (Fig. 4). FRAP is a conventional technique for studying GFP diffusion in biological systems (Axelrod et al., 1976). We chose three different spots in the flower meristem to quantify GFP recovery (Fig 4A): close to the expression site (red square), just outside the expression site (green square) and in the FM center (blue square). Bleaching with maximal laser intensity (10s) was followed by 150 seconds tracking of fluorescence recovery with imaging every 10 seconds (Fig. 4B-C). The FRAP experiments showed that the signal within the regions of interest (ROI) recovered by ~45% after 20 seconds and almost completely after 30 seconds or longer in all the ROIs (Fig. 4D). When we focused on the areas outside of DOT transcription region (green and blue ROIs) (Fig. 4E), we could clearly see a negative relationship between the velocity of GFP recovery and the distance from the expression site. Moreover, in the center of the meristem GFP signal was much lower compared to the other ROIs, which caused the scattering in GFP signal (“noise”) that resulted in the “zig-zag” quantification of the GFP (Fig. 4E). This fast recovery, especially in the green and blue regions, could be explained only assuming that the protein can move between cell-to-cell.

Discussion

Although the UFO gene, and its Antirrhinum homologs FIMBRIATA were discovered two decade ago, their mode of action is still poorly understood. Here we report data on the petunia homologs, DOT, indicating that (i) DOT can boost the transcription activation potential of ALF and (ii) that DOT proteins travels between cells in a floral meristem and, hence, is involved in the transcriptional activation of a broad set of ALF regulated genes. Even though LFY can bind functional cis-regulatory elements in downstream floral organ identity genes, it cannot activate transcription of reporter genes containing these sites in yeast cells, suggesting that in vivo additional plant proteins are required. The fusion of heterologous transcription activation domain to LFY can by-pass their absence (Parcy et al., 1998). Our finding that ALF can activate the same reporters if co-expressed with DOT has multiple implications. First, it suggests that DOT is one of the long-sought proteins whose role can be by-passed in yeast, and possibly also in planta, with the VP16 fusion. Interestingly, the transcription activation capacity of VP16 in yeast fully depends on an endogenous F-box protein (Met30) that binds to VP16 itself (Salghetti et al., 2001). This implies that VP16 fusion provides to ALF/LFY, in the absence of DOT/UFO, an alternative way to recruit an SCF-complex and the related ubiquitin proteasome machinery. Second, it
proves that the binding of ALF/LFY to UFO/DOT, seen in yeast two hybrid assays and bimolecular fluorescence complementation (“split YFP”), is functionally relevant. Third, it confirms that DOT/UFO can activate besides B-type genes, also A and C-type genes in a direct manner.

Over the past years ubiquitinylation has arisen as an additional post-translational regulation mechanism. The consequence of poly-ubiquitinylation usually is destruction by the 26S proteasome pathway. However, ubiquitinylation may have different consequences for the substrate protein (Haglund and Dikic, 2005; Kanayama et al., 2004; Miranda and Sorkin, 2007; Weake and Workman, 2008). Activation of a transcription factor appears difficult to reconcile with degradation via the ubiquitinylation/proteasome pathway. However, a range of findings led to the conclusion that ubiquitinylation and proteolysis of transcription activators is essential to activate gene transcription (Lipford et al., 2005; Muratani et al., 2005; Muratani and Tansey, 2003; Salghetti et al., 2001), although a general way in which ubiquitin acts has not been described yet. Ubiquitin might promote cleavage of inhibitory domains that block nuclear entry or interaction with other proteins in a proteasome-dependent manner (Conaway et al., 2002). In our case, however, it is unlikely that DOT is required for nuclear entry of ALF since it was shown that ALF reaches the nucleus in cells that lack DOT (Souer et al., 2008). Recently it has been proposed that monoubiquitylation protects an activator from the “stripping” activity of the proteasomal ATPases (Ferdous et al., 2007). In this view the coupling between activator mono-ubiquitylation and the ability to resist the destabilization process maintain the activator-DNA binding allowing promoter escape and elongation. Although ALF is already present in both floral and inflorescence meristems, there is no expression of floral homeotic genes until also DOT is transcribed. Thus mono-ubiquitylation performed by DOT on ALF might be necessary to activate/protect ALF function from promoter stripping.

The finding that a mono-ubiquitinated form of ALF was able to partially rescue the dot phenotype, strongly suggests that ubiquitinylation of ALF performed by SCF\textsuperscript{DOT} complex is the key process for flowers development in petunia. The fact that the p35S:UB-ALF was not able to fully complement all the dot defects was probably because in the genetic fusion ubiquitin was attached (via a normal peptide bond) to the N-terminal methionine, whereas ubiquitinylation normally occurs on internal lysine residues. Although genetic fusion of ubiquitin to ALF was sufficient to mimic the DOT function and so to activate the B and C genes (Fig 2), it remains to be established whether ALF-Ubiquitin conjugates also form in vivo. Previous reports suggested the presence of high molecular weight LFY isoforms in Arabidopsis plants, which were reduced or abolished in backgrounds lacking UFO (Chae et al., 2008), but we have been unable to produce these results (Souer et al., 2008). In petunia seedlings that constitutively expressed ALF and or DOT we detected a protein that was
slightly larger than native ALF, which may represent a mono-ubiquitinated ALF isoform or a petal-specific protein that binds a-specifically to anti-LFY antibodies (co-expression of ALF and DOT triggers ectopic petal identity). Hence the existence of ubiquitin ALF conjugates, which may be rare short-lived proteins formed only within the chromatin, remains to be proven.

Plant meristems integrate several signals and they are important growth sites of plants, initiating new organs at specific developmental stage. They are composed of central cells, which are undifferentiated and maintain the cell population, and a peripheral zone that form the different types of organ primordia. After cell division plant cells remain fixed at a given position due to the presence of a rigid cell wall, so a precise communication between adjacent cells ensures a correct spatial development of primordia and what their identity will be. This communication can be achieved through transcription factors movement between cells. Here we have showed that DOT can move between cells, as already shown for LFY (Sessions et al., 2000; Wu et al., 2003), resulting in a DOT gradient within the flower. As expected, the higher signal from the DOT-GFP fusion was observed in the sepal/petal boundary (Fig 3F), which is the region where DOT is expressed. Previous studies showed that the expression level reached by pDOT is extremely high, demonstrated by the fact that pDOT4.6:GUS resulted in a ectopic coloration of the flower meristem, whereas with a shorter promoter GUS signal was only visible in the sepal/petal boundary (Chapter 2). This would explain why petal development is the first processes affected by weak dot (Souer et al., 2008) and fim alleles (Ingram et al., 1997). As the flower development proceeds, DOT-GFP signal is observed in the whole flower dome, creating a gradient toward the center of flower meristem (Fig 3H). This behavior resembles a special category of signalling molecules called morphogens that act in a concentration depended matter diffusing away from their original location. Although the current data are promising, they are insufficient to conclude whether DOT act like morphogens.

The movement and non-cell-autonomous activity in plants is predicted to occur for 17–29% of transcription factors (Rim et al., 2011). So far, it is poorly understood what make a transcription factors able to move, if a specific sequence is sufficient to make mobile an immobile protein. Generally transcription factors that localized in the cytoplasm are thought to be able to move, either by non-targeted passive diffusion or active targeted movement. So far it is still unclear whether DOT movement is passively by diffusion or facilitated by bound to a carrier.

Taken together, our findings suggest that DOT protein moves from the region were is expressed toward the center of the flower meristem dome forming a protein gradient (Fig 5). Since the transcription factor ALF is the target of DOT, the consequence of the DOT
gradient is the generation of an ubiquitinated ALF gradient. Once ALF is in the active form, it can activate the downstream organ identity genes (Fig 5). Whether this ALF-DOT-UB interaction take place before or after DOT movement it still remain to be proved.

**Figure 5: Model for the role of DOT in the activation of distinct classes of organ identity genes.**

Scanning electron microscopy of a mature flower meristem of petunia *W138*, colors represent the different organ tissues: green for sepals, violet for sepals, yellow for stamens and red for carpels. The blocks at the top indicate organ primordia in whorls 1 to 4 (W1 to W4). Bars indicate expression patterns of various mRNAs and/or proteins. Red color denotes a high concentration and yellow lower concentrations of proteins. The expression pattern of TER/WUS proteins is hypothetical, as indicated by the question marks. To date A-type organ identity genes similar to AP1 have still to be identified in petunia.
Materials and methods

Construction of transgenes and plant transformation

The p35S:UB-ALF was created by ligating an XbaI/BamHI digested PCR fragment spanning the ALF coding sequence, which we generated from a petunia cDNA library with the primers listed in table 1, and of UB in between the CaMV35S promoter and the CaMV 35S terminator of the binary vector pGreen7K (Hellens et al., 2000). The p35S:DOT:GFP and pDOT:DOT-GFP:tDOT constructs were generated using the primers listed in table 1. The p35S and t35S were amplified from the pK2GW7.0 destination vectors. The DOT:GFP fragment was generated from a previous construct used in Souer et al., 2008. The promoter and terminator region of DOT were amplified from W115 gDNA. Each PCR fragments were recombined into the GATEWAY pDONR221 vector (Invitrogen) and then transferred in the desired destination vectors using the Gateway multisite recombination system (Karimi et al., 2002).

All the constructs were amplified with Phusion High-Fidelity DNA Polymerase (Finzymes) and sequenced with Big Dye terminator technology (Perkin elmer) before they were transformed into petunia plants by Agrobacterium tumefaciens mediated leaf discs transformation (Spelt et al., 2000).

Yeast one-hybrid assay

The yeast one-hybrid experiments were performed with the full length ALF and DOT coding sequence into the yeast expression vector pDS848. AP1 and AG regulatory sequence were fused in front at the β-GAL (constructs provided by Detlef Weigel (Busch et al., 1999; Parcy et al., 1998) ). Yeast strain PJ69-4A/α were transformed with the ALF or DOT (A) and AP1 or AG (α) constructs using the lithium acetate method (Gietz et al., 1992). Colonies were grown for a few days on drop-out plates lacking leucine, tryptophan and histidine (-LTH). LacZ reporter activation was assayed by a semi-quantitative overlay assay. Yeast spotted and grown on -LT selective plates was permeabilized by chloroform, and subsequently covered with X-Gal containing top-agar (1% low melting point agar in 0.1M KPO4 buffer pH 7.0, 10 mg X-Gal, at 42°C). Pictures were taken after an incubation period of 1 to 6 hours at 37°C.

Positive colonies from the previous experiment were further used for the β-galactosidase activity assay that was measured at 28°C and pH 7 by using o-nitrophenyl-β-D-galactopyranoside (ONPG) according to previously published methods (Miller, 1972).

Western blotting

Stable transformed petunia leaves were used for protein extraction. The protein samples were heated at 100 °C for 2 min and size-separated on polyacrylamide-SDS protein gel.
Proteins were transferred from the gel to a nitrocellulose membrane using the Wet/Tank Blotting Systems (Bio-Rad) according to the manufacturer’s protocols. The primary antibodies used for western blotting was anti-GFP (Santa Cruz Biotechnology). Final concentrations of the antibody preparations were optimized experimentally. The secondary antibodies was goat anti-mouse IgG–HRP (Santa Cruz Biotechnology).

**In situ hybridization**

In situ hybridization was performed as described, (Souer et al., 1996; Rebocho et al., 2008) for double label hybridizations. Antisense fluorescein (DOT) and digoxigenin (FBP1) labeled RNA were synthetized from full coding sequences in vitro using the T7 polymerase (Roche). Post-hybridization washes included a 30 minute RNaseA treatment to eliminate non-specifically bound probe as well as cross-hybridization to related mRNAs. Digoxigenin-labeled probes were detected with an antidigoxigenin antibody conjugated to alkaline phosphatase (Roche Applied Science) and Western Blue stabilized alkaline phosphatase substrate (Promega). This results in a brownish signal, which turns dark blue after dehydration in an ethanol series. Fluorescein-labeled probes were detected as a red signal using an anti-fluorescein antibody conjugated with alkaline phosphatase and Fast Red tablets (Roche).

**Confocal laser scanning microscopy**

Petunia inflorescences were dissected from stable transformed W115 with pDOT:DOT-GFP:tDOT using a scalpel and foreceps, through a binocular microscope. Samples were immerged in water for confocal microscopy and imaged using a Nikon A1 with appropriate filter sets for GFP detection (excitation 488 nm, emission 505-550 nm) and 40x water immersion objective. For FRAP experiments, a rectangular region of interest (ROI) of 40x40 pixel was applied interactively at the transversal FMs section. Bleaching with maximal laser intensity was followed by ≈170 seconds tracking of fluorescence recovery with imaging every 10 seconds.

**Plant photography**

Pictures of plants were taken with a Sony Cyber-shot DSC-RX100. The background was blacked out using Adobe Photoshop Software.

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Supplementary Information

Figure S1: Overexpression of p35S:DOT-GFP cause the same phenotypic variation as p35S:DOT. (A) Wild type petunia W115 showing a developing inflorescence. (B) Ectopic expression of DOT causes the conversion of a cymose inflorescence into a solitary flower. (C) p35S:DOT-GFP also reduce the cymose inflorescence into a solitary flower. (D) As p35S:DOT, strong p35S:DOT-GFP overexpressors show patches of petal tissues in leaves.

Figure S2: DOT protein movement is required for its function.
(A) Double localization in longitudinal section of a flower meristem of DOT mRNA (red arrows) and FBP1 mRNA (black arrows). (B) Side view of flower meristem showing DOT-GFP protein extending from the DOT expression site (red arrow) toward the center of FM. (C) GUS staining of pDOT<sup>4.6</sup>:GUS in petunia inflorescence. Scale bars, 50 µm, * inflorescence meristem, dashed line indicates the meristem’s center.

**Figure S3: Quantification of GFP signal after photobleaching GFP in the proximal and distal region of DOT expression.**

(A–E) FRAP in the flower meristem pDOT:DOT-GFP:tDOT cells. (A) Longitudinal floral meristem section showing the situation before (A), immediately after (B) and 70s after the photobleaching (C). Scale bars, 50 µm. (D) FRAP measured in three different position of the pDOT:DOT-GFP:tDOT meristem. 70s after photobleaching 90% of the GFP signal was restored. (E) Detailed of the quantification of green and blue regions.
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References


