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Changes in cis-regulatory elements of a key floral regulator are associated with divergence of inflorescence architectures

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Link to publication

UFO and DOT are interchangeable F-Box protein components of an SCF ubiquitin-ligase complex that bind to LFY and ALF to promote the transcription of downstream genes (Wang et al., 2003; Chae et al., 2008; Souer et al., 2008). Inactivation of *DOT* or *AN* leads to complete loss of floral identity, whereas *ufo* and *fim* have more subtle floral meristem identity defects (Levin and Meyerowitz, 1995; Ingram et al., 1997; Hepworth et al., 2006). Expression of *DOT* and *AN* strictly coincides with the development of flowers. Both genes become first expressed upon the onset of flowering young FMs, initially as a stripe at the adaxial side of the first emerging sepal primordium that subsequently expands into a ring as the other sepal primordia initiate (Lippman et al., 2008; Souer et al., 2008; R. Castel, PhD Thesis, VU University Amsterdam, 2009) (supplementary material Fig. S1). Constitutive expression of *DOT* (or *UFO*) in petunia causes the precocious formation of flowers in ectopic positions, apparently via the post-translational activation of ALF in vegetative tissues (Souer et al., 2008). In *Arabidopsis*, however, *UFO* is not limiting for flower formation, as it is expressed from embryogenesis onwards in virtually all aerial meristems (Lee et al., 1997; Long and Barton, 1998), and ectopic UFO expression does not alter flowering time or inflorescence architecture (Lee et al., 1997). Souer et al. (2008) postulated that the time and place of simultaneous expression of *LFY* and *UFO* or their homologs determine the flowering time and position of flowers, and that alterations in the expression of both *LFY* and *UFO* homologs were involved in the divergence of flowering time and inflorescence architecture (Souer et al., 2008).

Curiously, the expression pattern of *UFO*, *FIM* and *DOT* within the flowers also diverged (supplementary material Fig. S1), even though they are thought to have a similar role in the activation of organ-identity genes (Lee et al., 1997; Schultz et al., 2001; Souer et al., 2008). In young FMs, *UFO* and *FIM* mRNA are expressed throughout the meristem dome and in later stages become confined to the petal/sepal boundary (Simon et al., 1994; Lee et al., 1997). *DOT* and *AN* mRNA, however, are never expressed in the FM center (Lippman et al., 2008; Souer et al., 2008).

What caused the changes in the expression patterns of these FMI genes, however, remains unknown. To address whether the FMI gene expression patterns were altered by *cis*-regulatory mutations and/or by changes in the upstream *trans*-regulatory network, we compared the activity of homologous FMI gene promoters from *Arabidopsis*, *Antirrhinum*, tomato and petunia by swapping them between *Arabidopsis* and petunia. It appeared that the divergent expression of *LFY* homologs is caused by alterations in the upstream *trans*-regulatory network. Conversely, the divergent expression of *UFO* homologs is due to cis-regulatory differences, which make these genes responsive to distinct sets of transcription factors that appear largely conserved between species.

RESULTS
Promoter regions sufficient for correct spatio-temporal expression
To study the genetic basis of the different expression patterns of homologous FMI genes in petunia and *Arabidopsis*, we compared the activity of their promoters. We isolated 5′ non-coding regions of *ALF* and *DOT* by PCR-based methods, fused the 2.8 kb *ALF* promoter (*pALF*) and 3.1 kb *DOT* promoter (*pDOT$^{3.1}$*) to the *ALF* and *DOT* cDNAs, and introduced the *pALF:ALF* and *pDOT$^{3.1}$:DOT* genes (supplementary material Fig. S2) in petunia *alf^{W2167}* and *dot^{A2232}* null mutants.

In *alf* and *dot* mutants, apical FMs develop as a SIM, which forms another sympodial unit instead of a flower (Souer et al., 1998, 2008).

The reiteration of this process results in a green bushy structure lacking flowers (Fig. 1A,B; supplementary material Fig. S3A,B). In two out of 19 independent transgenic lines, *pALF:ALF* fully complemented the *alf* phenotype (supplementary material Fig. S3B), whereas eight lines formed imperfect 'green flowers', having sepaloid organs in place of petals and stamens (supplementary material Fig. S3C). Nine *pALF:ALF alf* lines displayed no rescue of the mutant phenotype at all. Nevertheless, the two fully complementing lines showed that the transgene could complement the mutant, indicating that the 2.8 kb *pALF* fragment contains sufficient regulatory information for wild-type function of *ALF*.

In *pDOT$^{3.1}$:DOT dot* transformants, cymose branching was restored in seven out of 15 independent lines: apical meristems often formed a 'green flower' that lacked petals and stamens, but usually had a wild-type carpel, and occasionally mosaic organs in the second whorl containing sepaloid and petaloid tissue (Fig. 1C). The remaining eight plants showed no complementation of *dot*. Extending *pDOT* with another 1.5 kb (*pDOT$^{4.6}$:DOT*) clearly improved the rescue of the *dot* organ identity defect (Fig. 1D). In eight of 15 independent transformants, cymose branching was restored similar to *dot pDOT$^{3.1}$:DOT*, but, in addition, more complete flowers with sepaloid and petaloid tissues and fertile stamens and carpels were produced. The remaining seven plants showed no complementation. Next, we replaced the *t35S* terminator, which is often used (Karimi et al., 2002), with 1 kb of the 3′ flanking region of *DOT* (*tDOT*) and repeated the complementation. In eight of 15 independent *dot pDOT$^{4.6}$:DOT:tDOT* transformants, both the cymose branching and floral organ development were fully restored (Fig. 1E). Four lines showed partial complementation, that is, complete restoration of cymose branching but formation of imperfect flowers similar to those in *pDOT$^{4.6}$:DOT:t35S* transformants. The remaining three plants were not complemented.

These results indicated that the 3.1 kb *pDOT$^{3.1}$* fragment was sufficient to restore floral meristem identity but not the organ identity defects in *dot*, similar to the Cauliflower Mosaic Virus 35S promoter (Souer et al., 2008). The addition of 1.5 kb of 5′ promoter region improved the *dot* complementation compared with *pDOT$^{3.1}$*, but the full restoration of all *dot* defects was reached only when we added 1 kb of 3′ region as well.

The 2.3 kb region upstream of *LFY* (*pLFY*) used in this study, when fused to the *LFY* cDNA, was able to rescue the strong *lfy-26* mutant (Blazquez et al., 1997), and the 3.8 kb *UFO* promoter (*pUFO*) drives *GUS* expression in a pattern identical to that of endogenous *UFO* (Lee et al., 1997). This indicates that these promoter regions contain all regulatory sequences necessary for promoter swap studies.

Comparison of *pALF* and *pLFY*
pLFY contains proximal and distal regions necessary for the correct expression of the gene (Blazquez and Weigel, 2000), and several transcription factors have been identified that interact with known regulatory regions in *LFY* (Lee et al., 2008; Yamaguchi et al., 2009). Alignments of *pLFY*, using the Phytozome portal (Goodstein et al., 2012), revealed sequence conservation in distal and proximal regions in *LFY* homologs from other *Brassicaceae*, but little or none in *LFY* homologs from other species, including *pFA* from tomato (supplementary material Fig. S4A). In a complementary approach, we compared *pALF* (2.8 kb) with *pFA* (3.8 kb) and *pLFY* (3.8 kb), using mVISTA (Frazer et al., 2004). These pairwise alignments showed that *pALF* and *pFA* share four conserved regions, whereas no clear similarity was seen with *pLFY* (supplementary material Fig. S4B). This suggests that *pLFY* and *pALF* share few
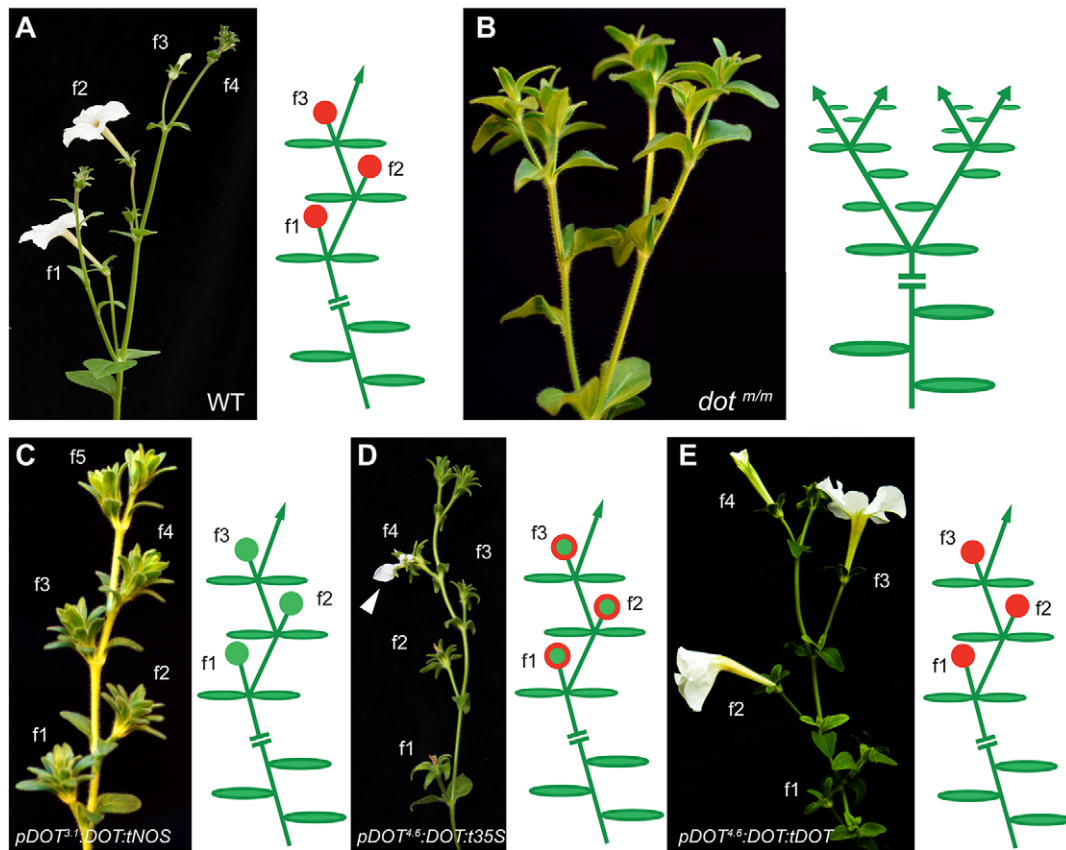
Fig. 1. Complementation of *dot* by *pDOT³·¹:DOT:tNOS*, *pDOT⁴·⁶:DOT:t35S* and *pDOT⁴·⁶:DOT:tDOT*. (A) Cymose inflorescence of wild-type petunia showing four consecutive flowers (f1, f2, f3, f4), with diagram showing the reiteration of modular sympodial units with flowers (red dots). (B) *dot* mutant in hybrid W138/W115 background, with diagram showing the conversion of flower-to-shoot (green arrows). (C-E) Complementation of *dot* by *pDOT³·¹:DOT:tNOS* (C) producing 'green flowers' (green dots in the diagram), *pDOT⁴·⁶:DOT:t35S* (D) with partial developed flowers (white arrow in the picture and green dots with red perimeter in the diagram) and full complementation of *pDOT⁴·⁶:DOT:tDOT* (E), respectively.

cis-regulatory elements or that such elements are too small, or too different in sequence, to be detected by sequence comparison.

To distinguish between these possibilities, we generated stable *Arabidopsis* and petunia transformants containing β-glucuronidase (GUS) reporter genes driven by the *pALF* and *pLFY* fragments described above to the GUS coding sequence (*pALF:GUS* and *pLFY:GUS*; supplementary material Fig. S2) (Jefferson et al., 1987) and analyzed ten independent transformants for each gene/species combination, using histochemical GUS staining. Although the expression level varied between distinct transformants, the expression pattern was highly similar.

pALF:GUS and *pLFY:GUS* are expressed in seemingly identical patterns during vegetative growth both in a petunia and in an *Arabidopsis* background. That is, emerging petunia and *Arabidopsis* leaves expressed GUS, which quickly faded when the leaves grew older (Fig. 2A-D). We could observe *pLFY:GUS* expression from the third leaf on in *Arabidopsis*, but could not discern the gradual increase of *pLFY:GUS* expression during vegetative development (Blazquez et al., 1997). However, the rather small quantitative changes involved are difficult to distinguish by histochemical staining, in particular because the vegetative phase lasted rather short under the long-day conditions used.

Analysis of *pALF:GUS* and *pLFY:GUS* plants after the switch to flowering (Fig. 2E-H) showed that in a petunia inflorescence both transgenes were expressed in a similar pattern as the endogenous *ALF* gene, whereas in *Arabidopsis* inflorescences their expression patterns were similar to that of *LFY*. In petunia, both promoters are

highly active in the apical FMs and also in the SIMs (Fig. 2E,F). The slightly delayed expression of endogenous *ALF* in SIMs compared with the apical FM (Souer et al., 1998) was not observed with the limited resolution of *GUS* assays. This is not surprising because (i) the SIM emerges as a very small region between the bract and the apical FM, which both express *ALF*, and because (ii) *ALF* expression in the SIM is only briefly delayed (Souer et al., 1998; Castel et al., 2010). In *Arabidopsis* inflorescences, however, expression of *pLFY:GUS* and *pALF:GUS* was restricted to lateral FMs (Fig. 2G,H), the only difference being that *pALF:GUS* expression faded more quickly than *pLFY:GUS* activity at later stages. More importantly, *pALF:GUS* and *pLFY:GUS* are, like endogenous *LFY*, never active in the apical meristems of *Arabidopsis*.

These data indicate that *pALF* and *pLFY* are functionally very similar, indicating that the *ALF* and *LFY* expression patterns diverged through changes in upstream *trans*-regulatory factors.

Expression of *pLFY:LFY* in petunia and *pALF:ALF* in *Arabidopsis*

To obtain further evidence that *pALF* and *pLFY* have similar expression patterns, we performed functional assays in transgenic plants. Previous results revealed that the ALF and LFY proteins are functionally similar and interchangeable (Maizel et al., 2005; Souer et al., 2008). In *Arabidopsis*, ectopic expression of *LFY* or *ALF* triggers precocious flowering and transforms the apical IM into an FM, which converts the open raceme into a solitary flower (or a closed raceme) (Weigel and Nilsson, 1995; Souer et al., 2008). This
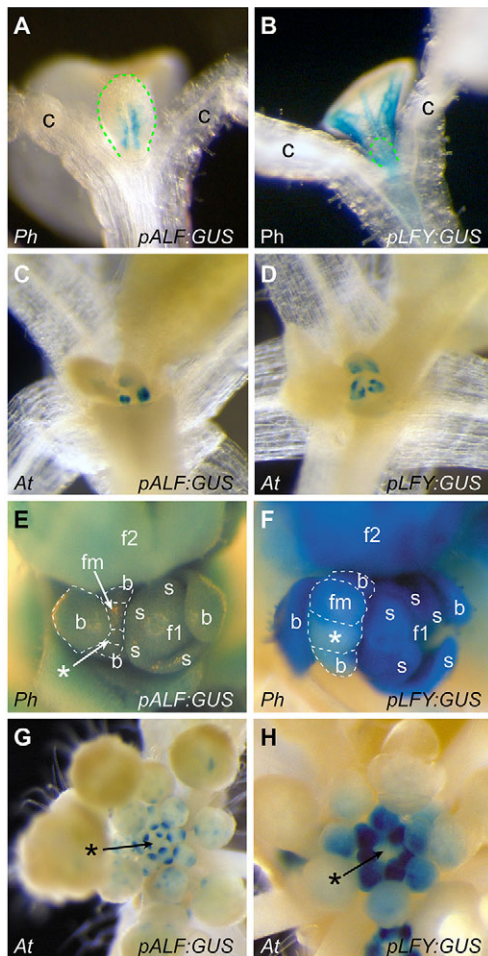
Fig. 2. pALF:GUS and pLFY:GUS expression patterns in petunia and Arabidopsis. (A,B) In young petunia (*Ph*) and in (C,D) *Arabidopsis* (*At*) seedlings, *pALF:GUS* and *pLFY:GUS* are active in emerging leaves, but not in the vegetative meristem. (E-H) In the reproductive stage, *pALF:GUS* (E) and *pLFY:GUS* (F) stained both sympodial inflorescence and young flowers of petunia, whereas in *Arabidopsis* (G,H), they were both expressed in FM, but excluded from the apical inflorescence meristem. Dashed lines indicate the outlines of organs that are poorly visible, subsequent flowers are indicated from young to old (f1, f2). C, cotyledon; asterisk, sympodial (E,F) or apical (G,H) meristem; fm, floral meristem; b, bract; s, sepal.

predicts that if an ALF (trans)gene retains (part of) its wider petunia expression pattern, when introduced in *Arabidopsis*, this should alter the flowering time and/or inflorescence architecture. Hence, we introduced *pLFY:LFY* and *pALF:ALF* into wild-type *Arabidopsis* Columbia. We found that none of the 20 primary transformants analyzed for each construct displayed aberrations in inflorescence architecture or flowering time (supplementary material Fig. S5A-C). This underlines that in *Arabidopsis pALF* is not expressed ectopically when compared with *pLFY*.

The same reasoning predicts that if *pLFY* were expressed in a more restricted pattern than *pALF*, when introduced in petunia, a *pLFY:LFY* transgene would not be able to fully rescue *alf* mutants. Therefore, we transformed *alf* with a *pLFY:LFY* transgene. We found that in six out of 12 independent *pLFY:LFY alf* lines floral identity of the apical meristem was restored, resulting in a normal cymose architecture (supplementary material Fig. S6A-D). Two of these *pLFY:LFY* lines had aberrant 'green flowers' with supernumerary whorls containing only sepals, two other lines had flowers with near-perfect flowers, except that the third whorl

consisted of petaloid stamens, whereas two transformants had perfect wild-type flowers with only a few small sections of petal tissue on the stamens (supplementary material Fig. S6C-H). These findings provide further support that in petunia *pLFY* is indeed active in the appropriate regions to compensate for the loss of *ALF* activity.

Functional comparison of *pDOT* and *pUFO*

A GUS gene driven by *pDOT*[3.1] showed no expression during the seedling stage, neither in petunia nor in *Arabidopsis* (Fig. 3A,B), showing that *pDOT*[3.1] reproduces the expression of the parental gene, independently of the host plant species. The same was observed for *pDOT*[4.6]:*GUS* (Fig. 3C,D).

In the petunia inflorescence, *pDOT*[3.1]:*GUS* was expressed within the apical FMs only, on the adaxial side of the sepal primordia, but never in the center of FMs, nor in the emerging SIM (Fig. 4A). In *pDOT*[4.6]:*GUS* transformants, *GUS* expression was much stronger and stained the entire FM, whereas no expression at all was seen in the emerging SIM (Fig. 4B). Even when stained briefly (30 min), a strong GUS signal was seen in the entire flower dome. However, when we analyzed *pDOT*[4.6]:*GUS* expression by *in situ* hybridization, we observed *GUS* mRNA only at the sepal/petal boundary (Fig. 4C). This suggests that the strong GUS activity seen in the center of the FM does not reflect the *GUS* mRNA expression pattern, but might result from intercellular movement of the GUS enzyme or an X-gluc reaction product or from transmission of (cytosolic) GUS protein through cell division into daughter cells, which becomes more evident at high *GUS* expression levels. Evidently, the extra promoter sequences included in the *pDOT*[4.6] construct contain some enhancers that drastically increase its activity compared with *pDOT*[3.1], without altering its expression pattern.
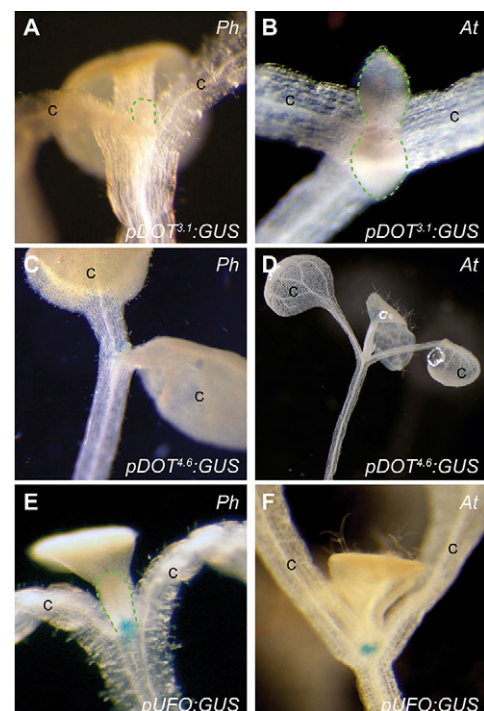
Fig. 3. Expression of *pDOT*[3.1]:*GUS*, *DOT*[4.6]:*GUS* and *pUFO:GUS* in vegetative meristems. (A-D) Transgenic petunia (*Ph*) and *Arabidopsis* (*At*) seedlings do not express *pDOT*[3.1]:*GUS* or *pDOT*[4.6]:*GUS*. (E,F) *pUFO:GUS* is expressed in the SAM of young petunia and *Arabidopsis* seedlings, respectively. Dashed lines outline the youngest visible leaves. C, cotyledon.
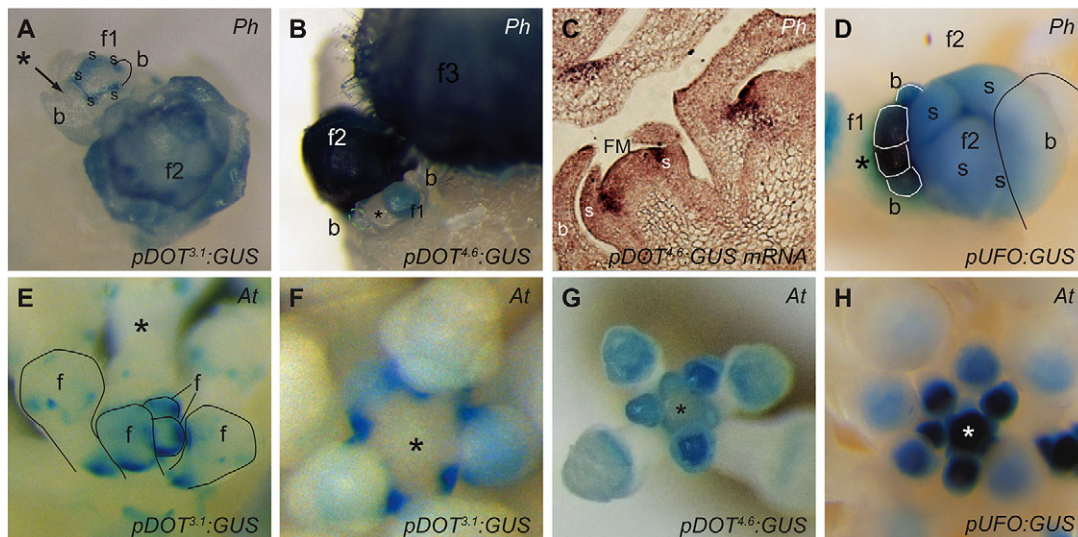
Fig. 4. Expression of *pDOT^{3.1}:GUS*, *pDOT^{4.6}:GUS* and *pUFO:GUS* in the inflorescences. (A) *pDOT^{3.1}:GUS* in petunia (*Ph*) inflorescence. GUS expression is first seen as a stripe at the base of the first emerging sepal primordium and at a later stage, when all sepal primordia are visible, as a pentagon. (B) *pDOT^{4.6}:GUS* petunia inflorescence. GUS activity is seen in the entire FM, but not in the SIM (asterisk). (C) *In situ* hybridization of a *pDOT^{4.6}:GUS* petunia inflorescence section, showing that *GUS* mRNA is restricted to the sepal/petal boundary. (D) *pUFO:GUS* in petunia inflorescence, showing expression in both SIM (asterisk) and young flower primordia (f1, f2). (E) Side view and (F) top view of an *Arabidopsis* (*At*) *pDOT^{3.1}:GUS* inflorescence: GUS activity was observed only in FM. (G) In *Arabidopsis*, the expression of *pDOT^{4.6}:GUS* is seen between sepals end petals of FMs but is excluded from the apical IM. (H) In flowering *pUFO:GUS Arabidopsis*, both the apical IM (asterisk) and young flowers primordia strongly expressed *GUS*. In older flowers blue staining was confined to the base of the flower. Dashed lines indicate the outlines of organs that are poorly visible, subsequent flowers are indicated from young to old (f1, f2). asterisk, sympodial (A,B,D) or apical (E-H) meristem; fm, floral meristem; b, bract; s, sepal.

In *Arabidopsis*, *pDOT^{3.1}:GUS* was expressed in lateral FMs and excluded from the apical meristem (Fig. 4E,F). In weak *pDOT^{4.6}:GUS* expressors, GUS activity was restricted to the FM exactly at the sepal/petal boundary and was never seen in the IM (Fig. 4G). In strong expressors, GUS signal was present in both apical inflorescence and lateral flower meristem, in the whole dome rather than in a ring shape (supplementary material Fig. S7A). The different results between weak versus strong expressors probably reflect a quantitative difference in GUS staining rather than qualitative. Indeed, detection of *GUS* mRNA by *in situ* hybridization of strong expressors showed that mRNA expression was most strongly expressed at the sepal/petal primordia boundary, whereas a weaker signal was observed throughout the FM and the apical IM (supplementary material Fig. S7B). The latter might either result from aspecific binding of the probe, or might reflect low activity of *pDOT^{4.6}* in the IM and center of the FM in *Arabidopsis*.

We also introduced *pDOT^{4.6}:GUS:tDOT* in petunia and *Arabidopsis*. However, among 90 stable petunia transformants, which were generated in four independent transformation experiments, none showed any *GUS* expression, whereas 32 transformants had the typical *dot* loss-of-function phenotype, indicating that the transgene(s) silenced the endogenous *DOT* gene and itself. When we transformed the same construct into *Arabidopsis*, none of the 30 independent transformants showed any *GUS* expression, but mutant (*ufo*) phenotypes were not seen. This suggests that, for unknown reasons, the *pDOT^{4.6}:GUS:tDOT* constructs triggers RNA interference at high frequency.

In *Arabidopsis*, *UFO* mRNA is already expressed in heart-stage embryos (Long and Barton, 1998) and persists in seedlings in a cup-shaped domain surrounding the central part of the SAM (Lee et al., 1997). When fused to *GUS*, *pUFO* was already active during embryogenesis of petunia in a ring around the root meristem and in the apical meristem (supplementary material Fig. S8), and in the seedling

stage, *pUFO* remained active in the vegetative SAMs both in an *Arabidopsis* and petunia background (Fig. 3E,F). This means that *pUFO* reproduces during the vegetative phase the expression of the parental gene from which it is derived, irrespective of the host plant species. Within the petunia inflorescence *pUFO:GUS* was strongly expressed in both the IM and FM (Fig. 4D), and in *Arabidopsis* in both the apical IM and lateral FMs, similar to *UFO* (Fig. 4H). Moreover, we observed that in all *Arabidopsis* and petunia *pUFO: GUS* transformants (i.e. in both weak and strong expressors) GUS activity localized in young FMs throughout the entire meristem dome, whereas in expanding flowers the signal faded from the center (Fig. 4D,H). These data imply that the divergent expression of *DOT* and *UFO* within FMs also results from differences in their promoters.

In summary, *pDOT^{3.1}:GUS*, *pDOT^{4.6}:GUS* and *pUFO:GUS* largely recapitulate the divergent expression patterns of the corresponding endogenous *DOT* and *UFO* genes, regardless of the host species. This implies that their different expression patterns in vegetative meristems, IMs and FMs are caused by alterations in *cis*-regulatory elements (CREs; that is, individual transcription factor bindings sites, or clusters of such sites, known as enhancers).

Functional heterologous complementation

To obtain direct evidence that the changes in the CREs of *pUFO* and *pDOT* are important for the divergent racemose and cymose inflorescence architectures, we introduced promoter:cDNA constructs (*pUFO:UFO* and *pDOT^{3.1}:DOT*) into *Arabidopsis* and petunia. Based on the above results and because *UFO* and *DOT* encode functionally interchangeable proteins (Souer et al., 2008), we expected that *pDOT^{3.1}:DOT*, which is sufficient to restore inflorescence architecture in petunia, would not alter flowering time in wild-type *Arabidopsis*, whereas *pUFO:UFO* would cause precocious flowering and inflorescence architecture defects in wild-type petunia.

About 20 primary *Arabidopsis* transformants of each construct were investigated, and none of them showed any aberrant phenotypical features or altered flowering time compared with empty vector controls (supplementary material Fig. S5). By contrast, the introduction *pUFO:UFO* in wild-type petunia always resulted in early flowering (Fig. 5A,B) and conversion of the cymose inflorescence (Fig. 5C) into a solitary flower with supernumerary petals and stamens subtended by extra leaf-like organs (bracts) directly under the sepal whorl (Fig. 5D; supplementary material Fig. S9A,B). When *pUFO:UFO* was transformed into a *dot* background, a solitary 'green flower' was formed – as early as in a wild-type background (supplementary material Fig. S9C,D) – that consisted of whorls of sepals around a central carpel lacking petals and stamens (supplementary material Fig. S9C,D). This indicates that *pUFO* could not drive transgene expression at sufficiently high levels during later stages of FM development, when floral organs are formed. When *pUFO:UFO* transformants were crossed to plants expressing *35S:LFY*, the precocious flowering was enhanced (Fig. 5E).

ANANTHA and *FIMBRIATA* activity in petunia and *Arabidopsis*

Arabidopsis and petunia are distantly related eudicot species that belong to the Rosids and Asterids, respectively. To study at which time point during evolution *pDOT* and *pUFO* diverged, we analyzed homologous promoters from *Antirrhinum* (*pFIM*; 3.6 kb) and tomato (*pAN*; 5 kb), which are both Asterids. *Antirrhinum* is a member of the *Plantaginaceae* (order Lamiales) and has a racemose inflorescence, whereas tomato belongs, like petunia, to the *Solanaceae* (order Solanales).

In tomato, *AN* is expressed in a very similar pattern as *DOT* in petunia (Lippman et al., 2008). During the vegetative stage *pAN:GUS* did not show any activity in a petunia or *Arabidopsis* background (Fig. 6A,B), identical to the native expression pattern of *AN* in tomato. In petunia inflorescences, the expression of *pAN:GUS* was similar to that of *pDOT*$^{4.6}$. That is, in low *pAN:GUS* expressors GUS activity was seen in a pentagonal domain on the sepals/petals boundary (supplementary material Fig. S10A), in strong expressors it was seen in the entire FM, and in both cases it was never seen in the SIM (Fig. 6E). In weak *Arabidopsis*

expressors, *pAN:GUS* expression was visible in the FM in the sepals/petals boundary and not in the apical IM (Fig. 6F). In strong expressors GUS stained both FM and IM (supplementary material Fig. S10A,B).

In *Antirrhinum*, *FIM* is already expressed during the vegetative phase (supplementary material Fig. S11), and during reproductive growth its expression is restricted to the (lateral) FM and excluded from the apical IM (Simon et al., 1994). In petunia seedlings we never observed expression of *pFIM:GUS* (Fig. 6C), whereas in *Arabidopsis* seedlings *pFIM:GUS* was expressed at the base of newly formed leaves (Fig. 6D). The expression pattern of *pFIM:GUS* in inflorescences was highly similar to that of *pDOT*$^{4.6}$*:GUS*. In weak petunia *pFIM:GUS* expressors, we observed GUS activity in emerging flower primordia first as a stripe at the base of incipient sepals (supplementary material Fig. S10C) and slightly later, when all sepal primordia were visible, as a ring in the flower dome (Fig. 6G). In strong expressors GUS activity stained the whole flower dome, including the FM center (supplementary material Fig. S10D). However, we never observed GUS activity in the emerging inflorescence meristem. Also in the *Arabidopsis* inflorescence, *pFIM:GUS* expression was confined to the typical ring pattern at the sepal/petal boundary, and was never observed in the FM center or in the apical meristem (Fig. 6H), similar to *DOT*$^{4.6}$*:GUS*.

In summary, these data indicate that *pAN* and *pDOT* contain very similar, if not identical, regulatory sequences, because they are active in indistinguishable patterns, whereas those in *pFIM* are very similar, but not fully identical, because *pFIM* responds to transcription activators in the base of young *Arabidopsis* leaves, whereas *pDOT* does not.

Pairwise sequence comparisons revealed four regions in *pDOT* (blocks 1-4) that have high similarity to *pAN*. Blocks 1 and 3, which contain predicted binding sites for MADS-box and SQUAMOSA PROMOTER BINDING PROTEIN (SBP)-like transcription factors, are also found in *pFIM* and homologs from diverse Rosid species, but not in *pUFO* and homologs from other *Brassicaceae* (Fig. 6I and supplementary material Figs S12 and S13). A similar analysis for *pUFO* revealed conservation of several regions among *Brassicaceae*, but no similarity with any of the other Asterids or Rosids in Phytozome (supplementary material Fig. S13B).
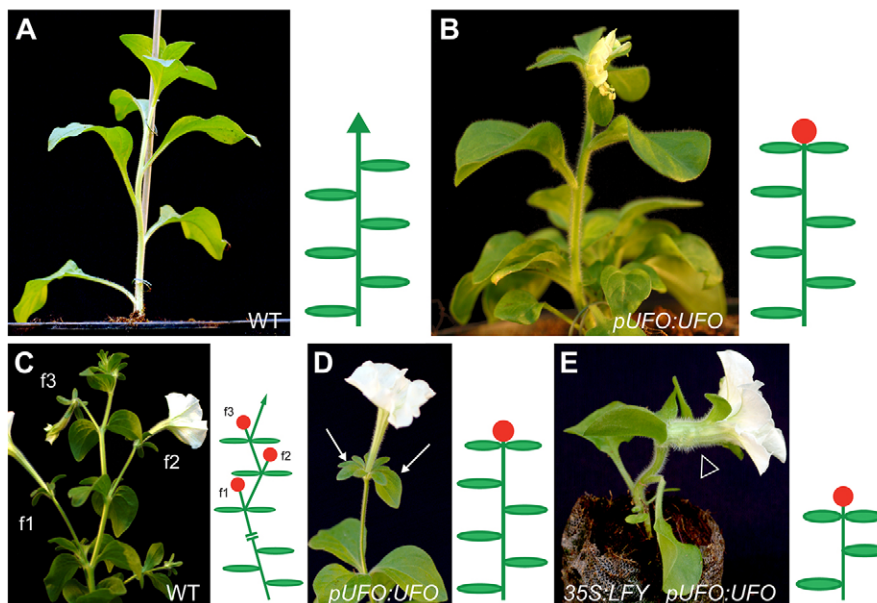
Fig. 5. *pUFO:UFO* converts the cymose petunia inflorescence to a single flower. (A) Wild-type petunia plant during the vegetative phase, with diagram showing the production of leaves. (B) *pUFO:UFO* transformant of the same age, which flowers early and produces a terminal flower (red dot in the diagram). (C) Cymose inflorescence of wild-type petunia, showing three consecutive flowers (f1, f2, f3), with diagram showing the reiteration of modular sympodial units with flowers (red dots). (D) In *pUFO:UFO* petunia, the cymose inflorescence was reduced to a solitary flower with extra organs (red dot in the diagram). (E) Double-transgenic *35S:LFY pUFO:UFO* petunia flowers extremely early, after forming two true leaves. The first whorl contains petaloid sepals (arrowhead).
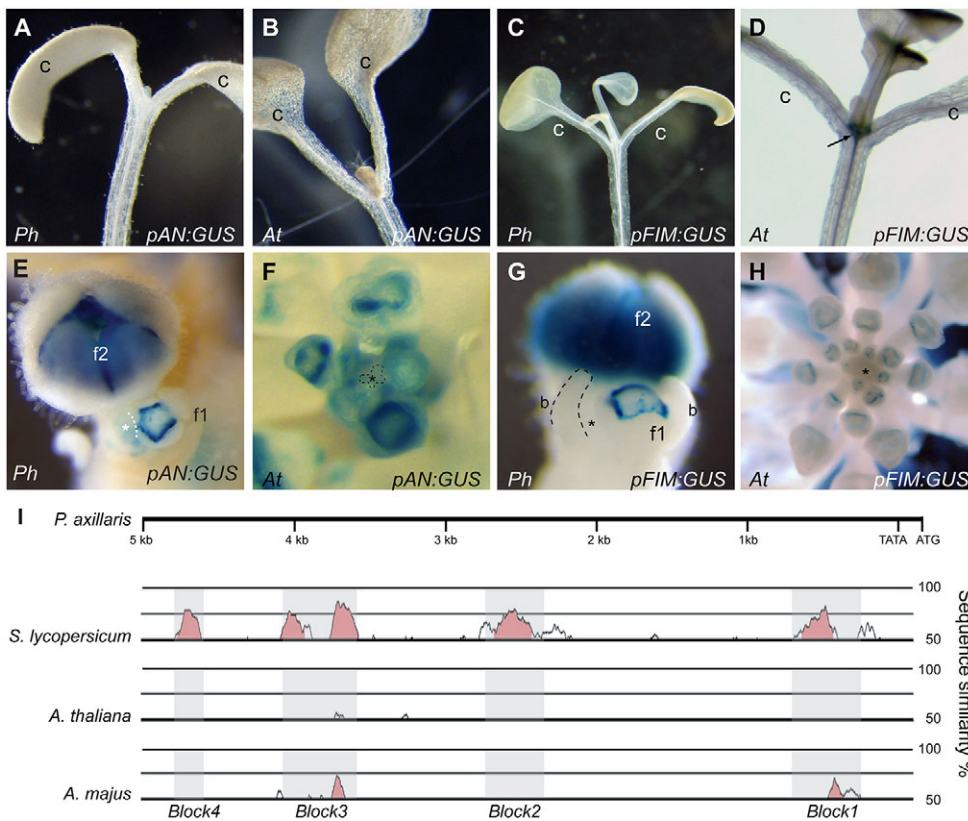
Fig. 6. Expression of *pAN:GUS* and *pFIM:GUS* in petunia and *Arabidopsis*. (A-D) Expression in the vegetative phase. (A) *pAN:GUS* is not expressed in petunia (*Ph*) or (B) *Arabidopsis* (*At*) seedlings. (C) *pFIM:GUS* is inactive in petunia seedlings, and is in *Arabidopsis* (D) expressed at the base of young leaves (arrow). (E-H) Expression in reproductive phase. (E,F) *pAN:GUS* is expressed in a pentagonal pattern at the sepal/petal boundary in FMs of (E) petunia and (F) *Arabidopsis*. (G) Petunia expresses *pFIM:GUS* in floral meristems at the sepal/petal boundary, but not in the SIM. (H) *Arabidopsis* expresses *pFIM:GUS* in floral meristems, at the sepal boundary, but not in the apical IM. C, cotyledons; asterisk, sympodial (E,G) or apical (F,H) meristem. (I) Sequence comparison from *pDOT* from *Petunia axillaris* with *pAN* (*S. lycopersicum*), *pUFO* (*A. thaliana*) and *pFIM* (*A. majus*), using mVISTA (Frazer et al., 2004). Sequence similarity is indicated with white peaks if 50-70% and beige peaks if above 70%. The four blocks that are conserved between petunia, tomato and *Antirrhinum* are highlighted by grey shading.

DISCUSSION

Differences in the spatio-temporal regulation of meristem identity genes caused the divergence of inflorescences with regard to the positions where flowers and shoots are formed (Benlloch et al., 2007; Lippman et al., 2008; Souer et al., 2008). Here, we show that the modification of FMI gene expression patterns results from variation in their transcriptional regulation, due to alterations in CREs of FMI genes as well as alterations in the upstream regulatory genetic network.

Our data suggest that CREs involved in the transcriptional activation of *DOT* reside in both the 3′ and 5′ flanking regions of the gene. The 3.1 kb promoter (*pDOT$^{3.1}$*) fragment contains major CREs that are sufficient to reproduce the *DOT* expression pattern and to rescue FM identity when fused to the *DOT* coding sequence, but not the identity of petals and stamens within the developing flower. The phenotype of weak *dot* mutants indicates that petal development is, of all DOT-regulated processes, the most dependent on full DOT activity, and is associated with an extremely high abundance of *DOT* mRNA in the cells at the sepal/petal boundary (Souer et al., 2008). Given that *pDOT$^{3.1}$* is active in the correct pattern, its inability to drive petal development is most likely due to an insufficiency to drive the very strong expression needed for petal development, rather than a shortcoming in the pattern of expression. Indeed, expression of *DOT* in a wider pattern, either from *pUFO* (supplementary material Fig. S8D) or *p35S* (Souer et al., 2008), also results in 'green flowers' lacking petal and stamen identity. By contrast, expression from *pDOT$^{4.6}$*, which is expressed in the same pattern as *pDOT$^{3.1}$* but at much higher levels, leads to partial rescue of petal and stamen identity and, if combined with the 3′ flanking sequence of *DOT*, to full rescue. These findings suggest that the CREs in the distal promoter region (−3000 to −4600) are largely redundant with those in the proximal region (−1 to −3000), because

they both promote *DOT* transcription in the same tissues. This is in line with recent data obtained with a larger set of *pDOT* constructs (S. Della Pina, E. Souer and R. Koes, unpublished data). The same may hold true for the 3′ flanking region of *DOT*, although we cannot exclude that (part of) the effect of *tDOT* results from enhanced mRNA processing and/or stability.

The most obvious difference between *DOT* and *UFO* is that the latter is expressed in all meristems during embryogenesis, vegetative and reproductive growth, whereas *DOT* expression is restricted to a defined region in FMs. That *pDOT:GUS* and *pUFO:GUS* reproduce these different expression patterns, regardless of the host plant used, indicates that the divergent expression of *DOT* in petunia and *UFO* in *Arabidopsis* is caused by differences in their 5′ flanking sequences. Furthermore, it indicates that the CREs that activate *pUFO* in the apical meristem (SAM) of embryos, seedlings and inflorescences (IM) respond to conserved transcription factors that are expressed in the same tissues in petunia.

The finding that in petunia, but not in *Arabidopsis*, the *pUFO: UFO* transgene causes precocious flowering, and the formation of solitary flowers provides direct evidence that alterations in CREs of a single gene may impinge major architectural differences. It is, however, difficult to link directly morphological changes during evolution to the regulatory divergence of *pUFO* and *pDOT*. Many plant families contain species with inflorescences described as racemes and cymes (Watson and Dalwitz, 2007), suggesting that these structures evolved multiple times independently. However, the details are hard to reconstruct with certainty because inflorescence architecture is (often) misclassified for a variety of reasons (Castel et al., 2010). In addition, assessing when the regulatory differences in *pDOT* and *pUFO* arose during evolution requires data on the regulation of *DOT/UFO* homologs in many more (related) species than currently available. Moreover, as floral

identity is specified by the combined action of several genes, not all changes in the expression of a single gene will necessarily alter development immediately, as outlined below.

The divergent expression patterns of *DOT*, *UFO* and *FIM* within the FM are intriguing because they seem to have similar functions within the flower. *UFO* and *FIM* are initially expressed throughout the FM, overlapping with the expression of subordinate organ-identity genes that specify petal and stamen fate (Simon et al., 1994; Lee et al., 1997; Schultz et al., 2001), whereas in petunia and tomato FMs, the *DOT* and *AN* mRNA expression patterns have little or no overlap with those of the downstream B and C-type genes (Schultz et al., 2001; Souer et al., 2008). Hence, we hypothesized that DOT protein moves between cells in the FM (Souer et al., 2008), which might also explain why (small) changes in their expression pattern in the flower have limited consequences for development. It is conceivable that the CREs and transcription factors driving *UFO* expression in the FM center are the same as those driving *UFO* expression in the other meristems, whereas *UFO* expression at the sepal/petal boundary might reply on distinct CREs and transcription factors similar to those driving the expression of *DOT*, *FIM* and *AN* in the same domain in their hosts. In young FMs of *Antirrhinum*, *FIM* is expressed in a thick, 8-cell-wide ring with only a small, 2-cell-wide hole in the center (Simon et al., 1994; Schultz et al., 2001), which is more similar to *UFO* than *DOT* expression. However, in petunia and *Arabidopsis* flowers, *pFIM:GUS* is expressed in a thin ring at the sepal boundary, similar to *DOT* and *pDOT:GUS*, suggesting that this difference between *FIM* and *DOT* expression is mostly due to alterations in the upstream *trans*-regulatory network. Nevertheless, there are clear functional differences between *pFIM* and *pDOT*, as *pFIM* is active in leaves of *Arabidopsis* seedlings, in contrast to *pDOT*. Whether expression of the *Impatiens UFO*-homolog – which is expressed in leaf primordia, like *pFIM* in *Arabidopsis* and within the petal primordia rather than at their boundary (Pouteau et al., 1998) – diverged from *FIM* and *DOT* by *cis*- or *trans*-regulatory changes remains to be established.

Variations in the expression patterns of *ALF*/*LFY* homologs are as important for morphological divergence as those of *DOT*/*UFO* homologs, but, again, not all the variation relates necessarily to developmental changes. Many species, with few exceptions (Coen et al., 1990), express their *LFY* homologs in vegetative tissues with different spatio-temporal patterns, where they have no apparent (architectural) role that is obvious from mutant phenotypes (Weigel et al., 1992; Kelly et al., 1995; Souer et al., 1998; Molinero-Rosales et al., 1999), except for a clade of legumes where *LFY* is involved in the development of compound leaves (Champagne et al., 2007). Several *Brassicaceae* with (rosette-flowering) indeterminate racemose inflorescences express their *LFY* homologs in the lateral (floral) meristems, and in the apical IM, which nevertheless remains indeterminate (Shu et al., 2000; Sliwinski et al., 2007). Transgenic experiments showed that the different expression of *IacLFY* in *Ionopsidum acuale* compared with *LFY* in *Arabidopsis* is due to a difference in the upstream regulatory network, whereas in *Idahao scapigera* and *Leavenworthia crassa* it traced to divergence of their *LFY* promoters, which prevents repression of *pIscLFY* and *pLcrLFY* in the IM by *TERMINAL FLOWER1* (*TFL1*) (Yoon and Baum, 2004; Sliwinski et al., 2007).

In this light, it is remarkable that the CREs in *pALF* and *pLFY* are so conserved, given that *Arabidopsis* and petunia are distantly related dicots with different inflorescence architectures and *ALF*/*LFY* expression patterns. This indicates that the divergent expression of *ALF* and *LFY* originates from differences in the upstream regulatory

network that remain to be identified, and that *pALF* and *pLFY* are, despite the lack of obvious sequence similarity, functionally similar. The latter was unexpected, as it suggests that *pALF* still contains the CRE(s) for *TFL1*-mediated repression. In *Arabidopsis*, this repression persists from the vegetative to the reproductive phase (Bradley et al., 1997) and is conserved in *Antirrhinum* (Bradley et al., 1996, 1997), but apparently not in nightshades. The *TFL1* homolog from petunia was never investigated, but homologs from tobacco and tomato, *CENTRORADIALIS4* (*CET4*) and *SELF PRUNING* (*SP*), respectively, are expressed only in vegetative axillary meristems and not in the FM or SIMs (Amaya et al., 1999; Thouet et al., 2008). Moreover, inactivation of *SP* only affects the development of the vegetative sympodial meristems (Pnueli et al., 1998), which are lacking in petunia (Castel et al., 2010), but not the cymose flower truss.

As *Arabidopsis* and petunia are distantly related species, *pALF* and *pLFY* most likely represent the ancestral state in dicots, whereas variants like *pLcrLFY* and *pIscLFY* are probably derived. Because the latter variants do not affect the spatial FMI regulation, they are most likely accompanied by compensatory alterations in the expression of *LcrUFO* and *IscUFO* or other FMI genes that remains to be established.

MATERIALS AND METHODS
Isolation of *pALF* and *pDOT*
The 5′ flanking regions of *ALF* and *DOT* were isolated using somatic transposon insertion-mediated PCR (SOTI-PCR) (Rebocho et al., 2008). To analyze sequence conservation across eudicot species we used the Phytozome portal (Goodstein et al., 2012) and a web-based version of mVISTA (Frazer et al., 2004).

Plant material
The *alf*[W2167] and *dot*[A2232] *dTPH1* transposon insertion alleles were in the non-transformable petunia line W138, and have been described in detail previously (Souer et al., 1998, 2008). *ALF*[W2167/+] and *DOT*[A2232/+] were crossed to the transformable line W115. *alf* and *dot* mutants were selected by phenotype from F2 progenies, their genotype confirmed by PCR and used for transformation. The phenotypes of *alf* and *dot* mutants in the hybrid W115/W138 background are comparable to those in line W138.

Construction of transgenes and plant transformation
The coding sequences of *ALF*, *DOT*, *LFY* and *UFO* were amplified from the vectors described previously (Souer et al., 2008), the *GUS* sequence was amplified from pGreenK vector (Karimi et al., 2002), and 5′ upstream/downstream non-coding regions were amplified from petunia W138 line and *Arabidopsis thaliana* Columbia genomic DNA. Phusion High-Fidelity DNA Polymerase (Finnzymes) was used for all amplification steps. Further details on transgene construction and the primers that were used can be found in supplementary Materials and Methods and Table S1.

All transgenes were (re)sequenced before introduction into the transformable petunia line W115 or homozygous *alf* and *dot* mutants using *Agrobacterium tumefaciens* (strain AGL0)-mediated leaf disk transformation (Horsch et al., 1985). *Arabidopsis thaliana* Columbia was transformed with *Agrobacterium tumefaciens* strain C58C1 (MP90) using the floral dip method (Clough and Bent, 1998), and transformants were selected on Murashige and Skoog medium (Duchefa) containing 50 mg/l kanamycin monosulfate.

All plants were grown in a greenhouse. For comparisons of phenotypes plants were grown side by side to exclude the possibility that any phenotypic differences resulted from variations in greenhouse conditions.

RNA extraction and quantitative real-time PCR
Total RNA was isolated from the SAM of 2-week-old seedlings or inflorescence apices of *Arabidopsis* and *Antirrhinum* using an RNAeasy extraction kit (Qiagen) and treated with DNA-free DNase (Roche) to remove residual genomic DNA. Transcript levels were quantified with *Eco* Real-time PCR system (Illumina) using Power SYBR Green (Applied

Biosystems). The primers used are shown in supplementary material Table S2. Normalization was performed based on the expression of *ACTIN*.

Whole-mount *GUS* staining

We accurately followed the whole-mount *GUS* staining protocol as described in Weigel and Glazebrook (2002). Untransformed W115 was always included as negative control. The stained tissue was examined under binoculars. The brightness of the digital images as a whole was adjusted for optimal visibility of the organs and blue staining using Adobe Photoshop software, when necessary.

Plant photography

Plant images were taken with a FujiFilm FinePix S2 Pro digital camera. In the figures, the background was blacked out using Adobe Photoshop.

Statistical analysis of flowering time

We measured the flowering times of primary *Arabidopsis* (Columbia) transformants by the number of rosette and cauline leaves at bolting. The plants were grown under a long-day regime (16 h light/8 h darkness). The counted leaf numbers were statistically analyzed using One-Way ANOVA in SPSS.

GenBank accession numbers

Sequences of the genes used in this study can be found in the EMBL/GenBank database under the following accession numbers: *ALF* promoter (JF274656), *ALF* (AF030171), *DOT* promoter (JF274657), *DOT* (EU352681), *LFY* (NP200993) and *UFO* (NM102834).

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