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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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ABSTRACT
Higher plant species diverged extensively with regard to the moment (flowering time) and position (inflorescence architecture) at which flowers are formed. This seems largely caused by variation in the expression patterns of conserved genes that specify floral meristem identity (FMI), rather than changes in the encoded proteins. Here, we report a functional comparison of the promoters of homologous FMI genes from Arabidopsis, petunia, tomato and Antirrhinum. Analysis of promoter-reporter constructs in petunia and Arabidopsis, as well as complementation experiments, showed that the divergent expression of LEAFY (LFY) and the petunia homolog ABERRANT LEAF AND FLOWER (ALF) results from alterations in the upstream regulatory network rather than cis-regulatory changes. The divergent expression of UNUSUAL FLORAL ORGANS (UFO) from Arabidopsis, and the petunia homolog DOUBLE TOP (DOT), however, is caused by the loss or gain of cis-regulatory promoter elements, which respond to trans-acting factors that are expressed in similar patterns in both species. Introduction of pUFO/UFO causes no obvious defects in Arabidopsis, but in petunia it causes the precocious and ectopic formation of flowers. This provides an example of how a change in a cis-regulatory region can account for a change in the plant body plan.

KEY WORDS: Inflorescence, Cyme, Raceme, Evo-Devo

INTRODUCTION
Flowering plants (Angiosperms) display an enormous morphological diversity and, because many species are aseismatic to genetic analysis and transgenesis, they offer excellent possibilities to study the evolution of developmental mechanisms and morphological change (Benlloch et al., 2007; Castel et al., 2010; Moyroud et al., 2010; Della Pina et al., 2014). Angiosperms differ widely with regard to the moment (i.e. the season and age of the plant) they switch from vegetative growth to flowering, as well as the number of flowers that are formed and their position on the plant body (Weberling, 1989; Castel et al., 2010). Distinct species may form solitary flowers or inflorescences that bear many flowers in a variety of different patterns (Rickett, 1954). Compound inflorescences are divided into three major classes depending on the position where flowers and shoots are formed. In (open) racemes, the apical meristem remains undifferentiated and flowers derive from lateral meristems that form at its periphery. In cymes, flowers are formed from apical meristems, and inflorescence growth continues from lateral meristems, called sympodial inflorescence meristems (SIMs), which ultimately will also acquire floral identity after having formed a subsequent lateral SIM. Panicles go through several rounds of producing lateral meristems before each meristem ends in a flower (Rickett, 1954; Prusinkiewicz et al., 2007; Castel et al., 2010).

Distinct inflorescence architectures are associated with differences in the expression patterns of floral meristem identity (FMI) genes that specify floral meristem (FM) fate (Benlloch et al., 2007; Moyroud et al., 2010). In racemes, FMI genes, such as LEAFY (LFY) and APETALA 1 (API) of Arabidopsis, and FLORICAULA (FLO) of Antirrhinum majus, are expressed in lateral meristems, which develop into flowers, but not in the apical meristem, which remains meristematic (Coen et al., 1990; Huijser et al., 1992; Mandel et al., 1992; Weigel et al., 1992). Mutations in LFY and/or API (partially) convert lateral flowers into shoots (Mandel et al., 1992; Weigel et al., 1992), whereas constitutive expression results in precocious flowering and conversion of apical meristems into flowers (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995). Hence, in Arabidopsis, the time and place where flowers form are primarily regulated via the transcription of LFY and its direct target API (Wagner et al., 1999; Benlloch et al., 2007; Moyroud et al., 2010).

Also, in species with cymose inflorescences, such as the nightshades (Solanaceae) Nicotiana spp. (tobacco), Solanum lycopersicum (tomato) and Petunia hybridra (petunia), LFY homologs specify floral identity (Souer et al., 1998; Molinero-Rosales et al., 1999; Ahearn et al., 2001). The encoded proteins are structurally and functionally highly similar to LFY, but are expressed in different patterns (Souer et al., 1998, 2008; Molinero-Rosales et al., 1999; Ahearn et al., 2001). In tomato, for instance, FALSIFLORA (FA) is already expressed during the vegetative phase in (incipient) leaf primordia, and during reproductive development in both (apical) FMs and (lateral) SIMs (Molinero-Rosales et al., 1999). In petunia inflorescences, the LFY-homolog ABERRANT LEAF AND FLOWER (ALF) is first activated in the apical FM and with a slight delay in the lateral SIM (Souer et al., 1998). However, the transcription of ALF is not the limiting factor that determines when and where flowers form in petunia, because (i) ectopic ALF expression does not trigger the formation of precocious or ectopic flowers, and because (ii) ALF is, like FA in tomato, expressed prior to flowering in leaf primordia (Souer et al., 1998).

The limiting factor that controls the formation of flowers in petunia is DOUBLE TOP (DOT), which is orthologous to Antirrhinum FIMBRIATA, Arabidopsis UNUSUAL FLORAL ORGANS (UFO) and tomat0 ANANTHA (AN) (Souer et al., 2008).
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 (pDOT3.1) to the ALF and DOT cDNAs, and introduced the pALF:ALF and pDOT3.1:DOT genes (supplementary material Fig. S2) in petunia alfW2167 and dotA2232 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 pDOT3.1: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 (pDOT4.6: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 pDOT3.1: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 pDOT4.6: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 pDOT3.1:DOT:t35S transformants. The remaining three plants were not complemented.

These results indicated that the 3.1 kb pDOT3.1 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 pDOT3.1, 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 Phytozone 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
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
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 sepal, 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\textsuperscript{3.1} showed no expression during the seedling stage, neither in petunia nor in Arabidopsis (Fig. 3A,B), showing that pDOT\textsuperscript{3.1} reproduces the expression of the parental gene, independently of the host plant species. The same was observed for pDOT\textsuperscript{4.6}:GUS (Fig. 3C,D).

In the petunia inflorescence, pDOT\textsuperscript{4.6}: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\textsuperscript{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\textsuperscript{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\textsuperscript{4.6} construct contain some enhancers that drastically increase its activity compared with pDOT\textsuperscript{3.1}, without altering its expression pattern.
In Arabidopsis, pDOT3.1:GUS was expressed in lateral FMs and excluded from the apical meristem (Fig. 4E,F). In weak pDOT4.6:GUS expressors, GUS activity was restricted to the FM exactly at the sepal/petal 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 pDOT4.6 in the IM and center of the FM in Arabidopsis.

We also introduced pDOT4.6: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 pDOT4.6: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, pDOT3.1:GUS, pDOT4.6: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 pDOT3.1:DOT) into Arabidopsis and petunia. Based on the above results and because UFO and DOT encode functionally interchangeable proteins (Sour et al., 2008), we expected that pDOT3.1: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 phenotypic 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;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;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 pDOT;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).

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**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).
**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 (pDOT1) 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 development, rather than a shortcoming in the pattern of expression. Indeed, expression of DOT in a wider pattern, either from pUFO (supplementary material Fig. SSD) or p35S (Souer et al., 2008), also results in ‘green flowers’ lacking petal and stamen identity. By contrast, expression from pDOTas, which is expressed in the same pattern as pDOTas 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 iDOT 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 UFO 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 rely 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; Moliner-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 lcf-LFY in Ionopsisidium 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 pLcrLFY 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 pLscLFY 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 alf2167 and dot42232 dTPH1 transposon insertion alleles were in the non-transformable petunia line W138, and have been described in detail previously (Souer et al., 1998, 2008). ALF2167/1 and DOT42232/1 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 (Finzymes) 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 resequenced before introduction into the transformable petunia line W115 or homoygous 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 C58;1 (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
Biotechnology). 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 background, the 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 majority of the experiments. E.K., S.D.P., R.C. E.S. and R.K. carried out the experiments, analyzed and interpreted data. E.K., S.D.P. and R.K. wrote the paper. All authors read and commented on the manuscript prior to submission.

Competing interests
The authors declare no competing or financial interests.

Author contributions
R.K., E.K., S.D.P. and E.S. designed experiments. E.K. and S.D.P. carried out the experiments, analyzed and interpreted data. E.K., S.D.P. and R.K. wrote the paper. All authors read and commented on the manuscript prior to submission.

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Supplementary material
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