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

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Abstract

The flower-bearing branches of plants, the inflorescences, show enormous variation in the way the flowers are placed on the plant body. A set of genes that gives floral identity to meristems is widely conserved in the plant kingdom, but in petunia and Arabidopsis, two species that have divergent inflorescence architectures, their expression patterns are very different. Here we show that changes in the 5’ cis-regulatory regions of one of these genes attribute to the changes in inflorescence structure. This study shows an example of how a change in a cis-regulatory region can account for a change in body plan.
Introduction

The most eye-catching manifestation of evolution is the morphological diversity of plants and animals. Given that the proteins that regulate the development of major body parts, which are encoded by “toolkit” genes, are deeply conserved between animals with widely divergent morphologies, it was suggested that the evolution of distinct morphologies probably resulted from divergence of their expression patterns (Carroll, 2008). Because most toolkit genes control a multitude of developmental processes, alterations in the encoded proteins would have many pleiotropic effects and would usually cause, besides a “positive” phenotypic change, many negative side-effects. Therefore, it has been argued that a major factor driving the evolution of body shape are changes in regulatory cis-elements (enhancers) by which genes respond to specific transcription factors, as these often change gene expression in only a few specific tissues, and thus are less pleiotropic (Carroll, 2008). However, solid experimental data to support this theory have remained scarce (Carroll, 2008; Hoekstra and Coyne, 2007; Stern and Orgogozo, 2008).

Flowering plants (Angiosperms) display an enormous morphological diversity and they offer, because many species are amenable to genetic analysis and transgenesis, excellent possibilities to study the evolution of developmental mechanisms (Benlloch et al., 2007; Castel et al., 2010; Moyroud et al., 2010). For example, 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 (Castel et al., 2010; Weberling, 1989). Some species form solitary flowers, whereas others generate inflorescences that bear many flowers in a variety of different patterns (Rickett, 1954). Compound inflorescences are divided in three major classes, which differ in the position where flowers and shoots are formed. In (open) racemes, the apical meristem remains undifferentiated and flowers derive from lateral meristems, which consequently develop into flowers, but not in the apical meristem, ends in a flower.

Distinct inflorescence architectures are associated with differences in the expression patterns of floral meristem identity genes that specify floral meristem fate, such as LEAFY (LFY) and APETALA1 (AP1) from Arabidopsis thaliana (Benlloch et al., 2007; Moyroud et al., 2010). In racemose inflorescences, like those of Arabidopsis and Antirrhinum majus (snapdragon), LFY and AP1 (homologs) are expressed in lateral meristems, which consequently develop into flowers, but not in the apical meristem,
which therefore remains meristematic (Coen et al., 1990; Huijser et al., 1992; Mandel et al., 1992; Weigel et al., 1992). Mutations in LFY and/or AP1 (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). This indicates that in Arabidopsis, the time and place where flowers form is primarily regulated via the transcription of LFY and its direct target API (Benlloch et al., 2007; Moyroud et al., 2010; Wagner et al., 1999).

Also in species with cymose inflorescences, like the nightshades (Solanaceae) Nicotiana spp. (tobacco), Solanum lycopersicum (tomato) and Petunia hybrida (petunia) LFY homologs specify floral identity (Ahearn et al., 2001; Molinero-Rosales et al., 1999; Souer et al., 1998). The proteins encoded by these LFY homologs are highly similar to LFY with regard to their sequences and functional properties, but are expressed in very different patterns (Ahearn et al., 2001; Maizel et al., 2005; Molinero-Rosales et al., 1999; Souer et al., 2008; Souer et al., 1998). During development of the petunia inflorescence, for example, the LFY-homolog ABERRANT LEAF AND FLOWER (ALF) is first activated in the apical (floral) meristem and with a slight delay in the lateral sympodial meristem. Transcription of ALF is however, not the limiting factor that determines when and where flowers form in petunia, because (i) ectopic ALF expression from the cauliflower mosaic virus 35S promoter (35S:ALF) does not trigger precocious flowering and because (ii) ALF is also expressed strongly during the vegetative phase in leaf primordia.

A range of genetic data indicate that the limiting factor that controls the formation of flowers in petunia is the transcription of another gene, DOUBLE TOP (DOT), the ortholog of Arabidopsis UNUSUAL FLORAL ORGANS (UFO) (Souer et al., 2008). UFO and DOT are interchangeable F-Box protein components of an SCF ubiquitin ligase complex that binds to LFY and ALF in order to promote the transcription of downstream genes (Chae et al., 2008; Souer et al., 2008; Wang et al., 2003). Mutation of DOT or its tomato ortholog ANANTHA (AN) leads to complete loss of floral identity. In these species, the expression of DOT and AN marks the onset of flowering, both in time and in place (Lippman et al., 2008; Souer et al., 2008), whereas Arabidopsis UFO is expressed from the embryonic phase onwards in virtually every meristem (Lee et al., 1997; Long and Barton, 1998).

Souer et al. (2008) postulated that simultaneous expression of LFY and UFO (homologs) determines the moment (flowering time) and position (architecture) where flowers are formed and that the alterations in the expression patterns of both LFY and UFO homologs were a key factor for the divergence of flowering time
and inflorescence architecture (Souer et al., 2008). Consistent with this idea, the simultaneous constitutive expression of LFY and UFO or ALF and DOT results in a similar phenotype in both Arabidopsis and petunia: meristems terminate at early seedling stage in structures with floral characteristics (Parcy et al., 1998; Souer et al., 2008).

What caused the changes in the expression patterns of these toolkit flowering genes has, however, remained unknown. It could be by mutations in the regulatory elements of the genes (cis-regulatory changes), or, alternately, by changes in the upstream regulatory network of the LFY and UFO homologs (trans-regulatory changes).

Here we show, by promoter swap experiments in Arabidopsis and petunia, that the divergent expression of the LFY homologs is caused by alterations in the upstream trans-regulatory network. Conversely, the divergent expression of the UFO homologs is due to alterations in cis-regulatory elements, which make these genes responsive to distinct sets of transcription factors that appear conserved between the two species.
Results

The promoter regions sufficient for reporter gene studies

To study the genetic basis of the different expression patterns of the *LFY* and *UFO* homologs in *Arabidopsis* and petunia, we aimed to compare the activity of the promoters of these genes. First, we isolated the 5’ non-coding regions of the petunia *LFY* and *UFO* homologs *ALF* and *DOT* respectively: 2.8kb *ALF* promoter (*ALFp*) and 3.1kb *DOT* promoter (*DOTp*) by somatic transposon insertion mediated PCR. Subsequently, their activity was tested by fusing them to the *ALF* and *DOT* cDNA’s (*ALFp:ALF* and *DOTp:DOT*, Fig. S1) and introducing them into petunia *alf*<sup>W2167</sup> and *dot*<sup>A2232</sup> null mutants.

*alf* and *dot* mutants both have a strong flower-to-shoot phenotype (Fig. S2A and D) in which the apical shoot, which normally ends in a flower, behaves like a sympodial shoot: it does not form a flower, but forms another sympodial shoot over and over, which results in a green bushy phenotype (Souer *et al.*, 2008; Souer *et al.*, 1998).

In two out of nineteen independent transformant lines the *ALFp:ALF* transgene fully complemented the *alf* mutant phenotype (Fig. S2B). In other *ALFp:ALF* *alf* plants the mutant phenotype was complemented to various degrees. In four lines, the apical meristem terminated in an imperfect flower in which the second and third whorl organs were replaced by sepaloid organs (four lines, Fig. S2C). Another four lines, described elaborately in Castel (2010), had a semi-indeterminate phenotype, in which apical shoots did not terminate instantly after forming a sympodial shoot, but produced a second sympodial branch before ending in a sepaloid flower (four lines). The remaining ten *ALFp:ALF* *alf* lines were not complemented. Nevertheless, the two fully complementing lines showed that the transgene was able to complement the mutant, indicating that the 2.8 kb *ALFp* used contains sufficient regulatory information for wild type function of *ALF*.

In *DOTp:DOT* *dot* transformants, cymose branching was restored: apical meristems often terminated in a ‘green flower’ that lacked petals and stamens, but usually had a wild type carpel, and sometimes had mosaic organs containing sepaloid and petaloid tissue in the second whorl (Fig. S2D-F). The petaloid tissue was seen irregularly in all complementing lines.

This partial complementation indicates that the 3.1 kb *DOTp* fragment is able to rescue the meristem identity defect, but not all organ identity defects of *dot* mutants. The flower phenotype of the mutant plants that were complemented
best is similar to that of 35S:DOT dot (Souer et al., 2008). Although the dot phenotype was not fully rescued, the 3.1 kb promoter is sufficient to restore floral meristem identity and floral determinacy (Sablowski, 2007). For this experiment we are not so much interested in the role of DOT as floral organ identity gene, but more in its role as a meristem identity gene. Therefore we argued this part of the DOT 5’ non-coding region is sufficient for further use in this study.

The 2.3 kb region upstream of the translation start codon of LFY (LFYp) used in this study, when fused to the LFY cDNA, was shown to rescue the strong lfy-26 mutant (Blazquez et al., 1997), and the 3.8 kb UFO promoter (UFOp) 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.

Promoter swap studies

If the divergent expression of two homologs is due to changes in cis-regulatory regions, then the promoters will maintain their specific expression pattern regardless of their host. If, on the other hand, the divergence is due to changes in trans-regulatory factors, then the expression pattern of a given promoter will be dependent on the host.

To determine which is the case for LFY and ALF and for UFO and DOT, we performed promoter swap experiments. We fused the four promoters to the coding sequence of reporter gene β-glucuronidase (GUS, Fig. S1) (Jefferson et al., 1987) and stably transformed the four constructs to both Arabidopsis and petunia. Several lines for each of the constructs in each species were examined using GUS staining. Figure S3 shows schematic representations of endogenous ALF, LFY, DOT and UFO expression for reference.

ALFp and LFYp activity
Endogenous ALF mRNA is already detected at the flanks of the young vegetative shoot apical meristem (SAM) in (incipient) leaf primordia and in young leaves (Souer et al., 1998). LFY mRNA is also detected in leaf primordia, initially at low levels and at steadily increasing levels as vegetative development progresses (Blazquez et al., 1997).

We observed that ALFp:GUS and LFYp:GUS are expressed in identical patterns during vegetative growth both in a petunia and in an Arabidopsis background. That is, emerging petunia and Arabidopsis leaves expressed GUS, and this expression
quickly faded when the leaves grew older (Fig. 1). We could already observe \( LFYp:GUS \) from the third leaf on in Arabidopsis, but could not discern the gradual increase of \( LFYp:GUS \) expression during vegetative development. However, the rather small quantitative changes involved (Blazquez et al., 1997) are difficult to distinguish by histochemical staining, in particular because the vegetative phase lasted rather short under the long day conditions that we used.

After the switch to reproductive growth, \( ALF \) mRNA is expressed in apical meristems of petunia, which develop into flowers (Castel, 2009; Souer et al., 1998), whereas expression in the sympodial meristem, which first generates the next sympodial shoot before turning into a flower, is slightly delayed (Castel et al., 2010; Souer et al., 2008). \( LFY \) in \( Arabidopsis \), is never expressed in the apical inflorescence meristem, but is strongly up regulated in lateral floral meristems even before they are anatomically visible (Blazquez et al., 1997).

Analysis of \( ALFp:GUS \) and \( LFYp:GUS \) plants after the switch to flowering (Fig. 2) showed that the activity of both promoters in a petunia background reflects the \( ALF \) expression pattern, while in an \( Arabidopsis \) background they generate a \( LFY \)-like \( GUS \) expression pattern. In petunia, both promoters are highly active in the apical flowers, and also in the sympodial meristems (Fig 2A-B). The delayed expression of endogenous \( ALF \) in sympodial meristems (Souer et al., 1998) was not observed with the limited resolution of \( GUS \) assays. This is not surprising given that (i) the sympodial meristem emerges as a very small region between the bract and the apical floral meristem, which both express \( ALF \), and (ii) \( ALF \) expression in the sympodial meristem is only briefly delayed (Castel et al., 2010; Souer et al., 1998).

In \( Arabidopsis \), \( LFYp:GUS \) and \( ALFp:GUS \) are active in lateral floral meristems only (Fig. 2C-D). \( ALFp \) activity fades from the center of the \( Arabidopsis \) flower more quickly than \( LFYp \) activity does. More importantly, \( ALFp:GUS \) and \( LFYp:GUS \) are never active in apical meristems of \( Arabidopsis \), similar to endogenous \( LFY \).

In summary: the \( ALFp \) behaves like \( LFYp \) in \( Arabidopsis \), and the \( LFYp \) behaves like the \( ALFp \) in petunia, indicating that the promoters themselves are functionally similar. This implies that the divergent expression patterns of these promoters in their cognate hosts result from changes in upstream, \( trans \)-regulatory factors.

\( DOTp \) and \( UFOp \) activity
In \( Arabidopsis \), \( UFO \) mRNA is already expressed in heart stage embryos (Long and Barton, 1998), and in seedlings \( UFO \) expression persists in a cup-shaped domain surrounding the central part of the SAM (Lee et al., 1997). \( DOT \) expression, in contrast, is never observed during the vegetative phase (Souer et al., 2008).
When fused to GUS, the DOTp showed no expression during embryogenesis of petunia, while the UFOp was active in the apical meristem (Fig. S4). Strikingly, UFOp:GUS is also expressed in a ring around the root meristem of petunia embryos (Fig. S4C). During the seedling stage DOTp:GUS was not active, neither in petunia nor in Arabidopsis (Fig. 3A-B), whereas UFOp:GUS was active in the vegetative SAMs of both species (Fig. 3C-D). This means that the DOTp and UFOp reproduce during the vegetative phase the expression of the parental gene from which they are derived, irrespective of the host plant species.
During inflorescence development, UFO mRNA is observed in the apical meristems of Arabidopsis in a cup-shaped pattern. In the lateral floral meristems UFO is expressed in the center of the floral meristems from floral stage 2 onwards (Lee et al., 1997), and when the flower develops, expression in the center ceases and then becomes confined to a narrow ring at the adaxial base of the sepals (Lee et al., 1997). In petunia, DOT mRNA is first seen in the apical floral meristem in a stripe at the adaxial side of the first sepal primordium, just before the sepal is anatomically visible. This stripe expands to a pentagon as the other sepals emerge, and becomes eventually restricted to a narrow ring between the sepal and the petal base (Castel, 2009; Souer et al., 2008). Although the expression patterns of DOT and UFO in later stages of flower development are quite similar, DOT is, in contrast to UFO, never expressed in the center of the flower.

In a petunia background, the DOTp was active within floral meristems on the adaxial side of the sepal primordia, but never in the center of floral meristem, nor in the emerging sympodial meristem (Fig. 4A). UFOp:GUS, in contrast, was active throughout all meristems of petunia (Fig. 4B). In an Arabidopsis background, DOTp:GUS was expressed in lateral floral meristems and excluded from the apical meristem. Within flowers the expression pattern was highly similar to that in petunia flowers: DOTp:GUS activity was seen in a ring adaxially of the sepal base, but not in the center of the floral meristem (Fig. 4C and Fig. S5). In strong DOTp:GUS expressors, GUS staining was also observed at the base of the pedicel, in a horseshoe-shaped pattern with the open side pointing towards the meristem (Fig. 4C, inset). UFOp:GUS in Arabidopsis was active throughout the apical SAM and the lateral floral meristems in a pattern similar to that of the endogenous UFO gene (Fig. 4D).
Summarizing, DOTp:GUS and UFOp:GUS largely recapitulate the divergent expression patterns of the DOT and UFO gene, irrespective of the host species. However, the expression of the DOTp in the Arabidopsis apices seems to be an exception to this, as DOTp is not active in the apical meristem, but at the abaxial base of the pedicels (see discussion). It is clear the UFO and DOT promoter have diverged since the last common ancestor of petunia and Arabidopsis, resulting in clearly different expression patterns in vegetative, inflorescence and floral meristems.

Expression of promoter:cDNA constructs in petunia

To assess whether the changes in the cis-regulatory elements of UFO and DOT are important for the divergent inflorescence architectures of racemes and cymes, we made promoter:cDNA constructs of LFY and UFO and introduced them into petunia. Based on the above results and because LFY and ALF, and UFO and DOT encode functionally interchangeable proteins (Souer et al., 2008) we expected (i) that alf mutants can be equally well complemented by LFYp:LFY as by ALFp:ALF, and (ii) that UFOp:UFO in wild type petunia would cause precocious flowering and inflorescence architecture defects.

We found that LFYp:LFY was able to restore the specification of flowers in alf mutants even more efficiently than ALFp:ALF (Fig. S6), which showed that the LFYp is
indeed active in the appropriate regions to compensate for the lacking ALF activity. In six out of twelve independent LFYp:LFY alf lines the determinacy of the apical meristem was restored: in two LFYp:LFY lines the repetitive branching of the apical meristem as seen in alf (Fig. S6A) was rescued, and green flowers were formed that had supernumerary whorls consisting of only sepals (Fig. S6C and F); in two other lines all whorls of the flower were present, but the third whorl consisted of petaloid stamens (Fig. S6D and G); and in two cases the otherwise wild type flowers only showed small sections of petal tissue on the stamens (Fig. S6H).

The UFOp:UFO transgene in wild type petunia readily caused these transgenic plants to flower early (Fig. 5A-B). The normally cymose inflorescence (Fig. 5C) was transformed into a single flower with supernumerary petals and stamens (Fig. S7A-B) that was subtended by extra leaf-like organs directly under the sepal whorl (Fig. 5B and D). When UFOp:UFO was transformed into a dot background, single flowers were formed as early as in a wild type background (Fig. S7C-D). However, in a dot background these flowers lacked petals and stamens and consisted of whorls of sepals around a central carpel (Fig. S7D inset), indicating that UFOp, like DOTp could not drive transgene expression at sufficiently high levels during late stages of floral meristem development, when floral organs are formed. When UFOp:UFO transformants were crossed to plants constitutively expressing LFY, the precocious flowering and flower defects were enhanced (Fig. 5E).

Promoter:cDNA constructs in Arabidopsis

Finally, to verify whether the activity of the promoters used in this study interfered with development of Arabidopsis, all four promoter:cDNA fusions were introduced into wild type Arabidopsis thaliana Columbia. About twenty primary transformants of each construct were investigated, and none of them showed any aberrant phenotypical features (Fig S8A-F). The genomic constructs did not trigger adventitious flowering in Arabidopsis either, as determined by counting of the leaves of the primary rosette and the cauline leaves of the primary shoot, and comparing these numbers with those of Arabidopsis containing an empty vector transgene (Fig. S8G). These findings are therefore consistent with the observation that the ALFp and LFYp are expressed in a similar way in Arabidopsis: if the ALFp were expressed during vegetative growth in Arabidopsis, like endogenous ALF is in petunia, ALFp:ALF in Arabidopsis would have triggered adventitious flowering (Weigel and Nilsson, 1995).
Figure 5. 

$UFOp:UFO$ converts the cymose petunia inflorescence to a single flower.

(A) Wild type petunia plant before flowers can be observed with the naked eye. (B) Transgenic $UFOp:UFO$ petunia, which flowers early and produces single flower inflorescences. This plant is at approximately the same age as the plant in (A).

(C) Wild type inflorescence in which consecutive flowers ($f_1$, $f_2$, $f_3$) of the cymose inflorescence can be observed. (D) In $UFOp:UFO$ petunia, the cymose inflorescence was reduced to a solitary flower with extra organs. Arrows indicate the extra organs in the sepal whorl. (E) Double transgenic 35S:LFY $UFOp:UFO$ petunia flower extremely early, after forming two true leaves. The first whorl contains petaloid sepals (arrowhead).
Discussion

In plants with fundamentally different inflorescence architectures, orthologous floral meristem identity genes often have very different expression patterns consistent with the different positions where flowers appear (Benlloch et al., 2007; Lippman et al., 2008; Souer et al., 2008). These expression patterns may have diverged by changes in the cis-regulatory regions or by changes in the upstream trans-regulating factors (Carroll, 2008). Here we show that the cis-regulatory regions of LEAFY (LFY) orthologs from species evolutionarily as distant as Arabidopsis (an Asterid) and petunia (a Rosid) are still functionally similar and functionally exchangeable. This indicates that the cis-regulatory regions of these genes remained conserved, and suggests that instead alterations in the upstream trans-regulatory network were responsible for the divergence of the LFY and ALF expression patterns. This is in marked contrast to the expression patterns of UNUSUAL FLORAL ORGANS (UFO) and its petunia ortholog DOUBLE TOP (DOT); their expression patterns diverged by changes in 5’ cis-regulatory elements and we provide direct evidence that these alterations were an important step in the development of the cymose inflorescence of petunia.

Reporter genes driven by the promoters of LFY (LFYP) and of its petunia ortholog ABERRANT LEAF AND FLOWER (ALF, ALFP) are in Arabidopsis expressed in the same meristems as the LFY (endo)gene, and in petunia display the same expression pattern as the endogenous ALF gene. The observation that LFYP:LFY can fully rescue alf mutants further strengthens the idea that ALFP and LFYP are exchangeable. These data strongly suggest that ALF and LFY are regulated by similar cis-elements and respond to the same transcription factors, although formally we can not rule out the remote possibility that they respond via different cis-elements to distinct transcription factors that happen to have similar expression patterns.

In this respect it is interesting that several species of the Brassicaceae, to which also Arabidopsis belongs, express their LFY homologs within the apical inflorescence meristem, which nevertheless does not acquire floral identity for reasons that are unknown (Shu et al., 2000; Sliwinski et al., 2007; Yoon and Baum, 2004). Reporter gene studies in which the cis-regulatory sequences of the LFY homologs of these Brassicaceae were fused to GUS and introduced into Arabidopsis showed that in the case of Ionopsidium acaule, the apical expression is due trans-regulatory differences: the IaLFY promoter is not active in the inflorescence meristem of Arabidopsis. In two other Brassicaceae species, however, the apical expression of the LFY homolog is caused by an alteration in the cis-regulatory elements: the promoters are also active in the SAMs of Arabidopsis. The authors speculated that the alteration was due to loss of an element responsive to TERMINAL FLOWER1, a conserved floral repressor that is
expressed in the apical meristem of *Arabidopsis* and represses *LFY* there (Bradley *et al.*, 1997; Sliwinski *et al.*, 2007; Yoon and Baum, 2004).

When we presume that the *ALFp* still responds to the same upstream regulating factors, it is striking that it still contains the element(s) that is responsible for *TFL1*-mediated repression of this promoter in the SAM, when species closely related to *Arabidopsis* seem to have lost this element. The repression of *LFY* by *TFL1* in *Arabidopsis* persists from the vegetative to the reproductive phase (Bradley *et al.*, 1997) and is conserved in *Antirrhinum* (Bradley *et al.*, 1996; Bradley *et al.*, 1997). However, in nightshade species the situation appears to be different. The *TFL1* homologs from petunia were never investigated, but the *TFL1* homologs from tobacco and tomato, *CET4* and *SELF PRUNING (SP)* respectively, are expressed only in vegetative axillary meristems and not in the apical (floral) or lateral (sympodial inflorescence) meristems (Amaya *et al.*, 1999; Thouet *et al.*, 2008). Furthermore, the self pruning mutant of tomato is not affected in branching of the flower truss, but the vegetative sympodial meristems, which form in the axils of the leaf below the inflorescence, switch to flowering ever faster, until they produce a flower truss without producing leaves (Pnueli *et al.*, 1998). These data indicate that within the cymose flower clusters of nightshades, the delay in expression of the *LFY* homologs in the sympodial inflorescence meristems is not caused by repression by *TFL1* homologs, but by another factor. This suggests that even if the *ALFp* contains the elements responsive to *TFL1*, this element is not needed for the brief delay in *ALF* expression in the sympodial inflorescence meristem, but was conserved for other reasons, such as the development of axillary shoots.

In contrast to the results with *ALFp:GUS* and *LFYp:GUS*, we found that reporter genes driven by *DOTp* and *UFOp* maintain their distinct patterns even in a heterologous host plant. This indicates that the expression patterns of *UFO* and *DOT* diverged via alterations in their *cis*-regulatory elements. Most likely this involved either the gain or loss of *cis*-regulatory elements (binding sites) that respond to distinct transcription factors with different expression patterns. It is striking that the *UFOp:GUS* expression pattern in petunia is very similar to that of *UFOp:GUS* and *UFO* in *Arabidopsis* as, for example, *UFOp:GUS* is also in a petunia background expressed during the vegetative phase, or the center of young floral meristems, even when *DOT* is not active there. This suggests that the unknown *trans*-regulatory factors that drive *UFO* expression are conserved and have similar expression patterns in both species, but do not activate *DOT* at the these sites because *DOT* lacks the responsive *cis*-elements.

Although its is difficult to compare the expression patterns of a gene in two hosts with a different morphology in detail, the expression patterns of *DOTp* and
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*UFO* are largely independent of the host species. The main exception to this similarity is the expression of *DOTp:GUS* in flowering *Arabidopsis*. While the *UFO* is active in a large domains in all meristems, *DOTp:GUS* is only expressed in the apical (floral) meristem in petunia. *DOT* expression starts around the time that the inflorescence meristem transforms into a floral meristem as a single stripe in the axil of the first appearing organ primordium, which in wild type will develop into the first appearing sepal (Fig. 4A) (Souer et al., 2008). In an *Arabidopsis* background, however, no *DOTp:GUS* expression was seen in the apical (inflorescence) meristem. Instead, the *DOTp* appeared active at the base of the pedicels of *Arabidopsis* flowers in a horseshoe-shaped domain (Fig. 4C, inset) in an area that could be considered the axil of the cryptic bract (Long and Barton, 2000). Interestingly, in some *Arabidopsis* mutant backgrounds such as *lfy, ap1* or *ufo*, the cryptic bracts, do develop into real flower-subtending bracts (Levin and Meyerowitz, 1995; Mandel et al., 1992; Weigel et al., 1992). Markedly, the first flower in *ufo* is often subtended by a bract and later flowers have squamules (stipules for a cryptic bract) (Levin and Meyerowitz, 1995).

In an *alf* mutant background, *DOT* is also expressed in the axils of organ primordia that are formed on the flank of the apex, although at lower levels than in wild type, but in *alf* such primordia develop into bracts (Castel, 2009; Souer et al., 2008). Although the *DOTp:GUS* expression in the axil of the cryptic bract in *Arabidopsis* seems at first sight to be quite different from the area in which the *DOTp* is active in petunia, this expression may be comparable to the *DOT* expression in the axil of the bract in *alf*.

Although *DOTp:DOT* was able to specify floral identity in *dot* and restore determinacy of the apical meristem, neither *DOTp:DOT* nor *UFOp:UFO* was able to restore the formation of petals and stamens in a *dot* mutant background. A few small, irregularly appearing patches of petal tissue were seen, but these we considered to be the result of somatic transposon excisions. A similar flower phenotype with green flowers with a central carpel was observed in *35S:DOT dot* (Souer et al., 2008). Endogenous expression of *DOT* in the axils of sepals is extremely high, and we assume that these extreme levels cannot be reached by *DOTp* or *UFOp*. The genomic *DOT* fragment used in this study, with 3.1 kb of promoter, possibly lacks one or more regions necessary to reach these high wild type *DOT* mRNA levels, although its pattern does reflect that of endogenous *DOT*.

In this study we show that in two species with fundamentally different inflorescence architectures, two orthologous genes, *ALF* and *LFY*, can specify floral fate in different positions on the plant body by divergent expression patterns that are caused by changes in their upstream trans-regulatory factors. Contrastingly, analysis of the upstream non-coding regions of *DOT* and *UFO* has shown that the changes

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in cis-regulatory elements of this key floral initiator have been indispensable for maintenance of the cymose inflorescence structure of petunia.

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Supporting data

Figure S1. Schematic representation of the transgenes used in this study. All constructs are in the pRD400 vector that was described by Datla et al. (Datla et al., 1992), except DOTp:DOT which is in the V148 Gateway™ vector (see Materials and Methods). The primers used for construction are indicated with arrows, sequences are listed in Table S1, numbers indicate the order in which digested PCR products were cloned into the vector. Black bars, 5’ non-coding regions; blue bars, GUS coding sequence; red bars, NOS or 35S terminator; green bars, ALF, DOT, LFY or UFO coding sequence; bent arrow, ATG start codon; closed circle, stop codon.
**Figure S2.** Complementation of alf by ALFp:ALF and of dot by DOTp:DOT.  
(A) alf mutant in hybrid W138/W115 background. (B) alf fully complemented by the ALFp:ALF transgene; both inflorescence structure and flowers are indistinguishable from wild type. Note that the outgrowth of axillary shoots (ax) is not repressed like in (A). (C) alf partially complemented by ALFp:ALF. The cymose inflorescence structure is restored, but the second and third whorl of the flower consist of sepals instead of petals and stamens. (D) dot mutant in hybrid W138/W115 background. (E-F) Complementation of dot by DOTp:DOT. The cymose inflorescence structure is restored (E) and flowers with sepals in the first three whorls and carpels (arrow) in the fourth whorl are generated. The organs in the second whorl regularly contained petal tissue (arrowhead). Consecutive flowers are numbered from old to young (f1, f2, ...); b, bract.

**Figure S3.** Schematic representation of LFY, UFO, ALF and DOT expression.  
(A-B) Expression in the vegetative SAM (left), in the flowering meristems and young floral meristems (middle) and in a stage 3 flower (right). LFY (yellow) and UFO (blue) expression in Arabidopsis is represented in (A), ALF (yellow) and DOT (blue) expression in petunia in (B). In green areas expression of the two genes overlaps. Numbers indicate floral meristems of stages as defined by Smyth et al. (Smyth et al., 1990); br, bract; fm, floral meristem; lp, incipient leaf primordium; lp, leaf primordium; sam, shoot apical meristem; se, sepal; sm, sympodial meristem.
Figure S4. *UFOp:GUS* is expressed in mature petunia embryos. (A B) Wild type W115 (A) and *DOTp:GUS* (B) embryos stained for GUS. No blue signal was observed. (C) *UFOp:GUS* is expressed in the SAM (arrow) and the root of mature petunia embryos. c, cotyledon.

Figure S5. *DOTp:GUS* is expressed in petunia and *Arabidopsis* flowers in a similar pattern. (A-B) In well-developed petunia (A) and *Arabidopsis* (B) flowers, *DOTp:GUS* is expressed in a ring-shaped pattern at the base of the flower. *Ph*, *Petunia hybrida*; *At*, *Arabidopsis thaliana*.

Figure S6. Complementation of *alf* by *LFYp:LFY*. (A) *alf* inflorescence in W115/W138 hybrid background. Outgrowth of axillaries is suppressed. (B) Inflorescence of wild type W115/W138 hybrid. (C) *alf* partially complemented by *LFYp:LFY*; note that the cymose inflorescence structure was restored, but that flowers consist of whorls of sepals. See also (F). (D) Almost full complementation of *alf*, only small flower defects remained. See (G-H). (E) Wild type W115/ W138 hybrid flower. (F) Partially complemented flower as seen on inflorescences such as in (C). (G-H) Nearly completely complemented flower of *LFYp:LFY alf*. In (H) the flower was opened to show presence of all floral organs. Some stamens contained ‘flags’ of petal tissue (arrow), or appeared filamentous (arrowhead). Consecutive flowers are numbered from old to young (f1, f2, ...); b, bract; ax, axillary; c, carpel.
**Figure S7.** The effects of $UFOp:UFO$ in petunia.

(A-B) $UFOp:UFO$ flowers have supernumerary organs. Wild type petunia flowers (left) have five petals (A) and five stamens (B), whereas $UFOp:UFO$ flowers have six or more. Carpels (c) appeared wild type. (C) Repetitively branching inflorescence of $dot$ in W115/W138 background. (D) $UFOp:UFO$ converted the dot inflorescence to a single, aberrant flower, which terminated in a wild type carpel (inset).
Figure S8. The *promoter:cDNA* transgenes have no effect on *Arabidopsis* development. (A) The inflorescence of *Arabidopsis* containing the *promoter:cDNA* transgene appeared wild type, like those of the empty vector control (left). (B-F) Wild type flowers were observed in *Arabidopsis* inflorescences transformed with the *promoter:cDNA* constructs. (G) Bar plot showing the mean number of rosette plus cauline leaves of primary transformants: empty vector control ($n=8$, $\mu=16.75$, S.E.M.$=1.46$), *ALFp:ALF* ($n=22$, $\mu=17.18$, S.E.M.$=0.791$), *LFYp:LFY* ($n=32$, $\mu=17.69$, S.E.M.$=0.505$), *DOTp:DOT* ($n=21$, $\mu=16.71$, S.E.M.$=0.557$), and *UFOp:UFO* ($n=22$, $\mu=18.18$, S.E.M.$=0.616$). Error bars represent the standard error of the mean (S.E.M.). The difference between the means is not significant in a two-tailed test with $\alpha=0.05$, $P \geq 0.783$ for each of the comparisons in One-Way ANOVA.
### Table S1. Primers used for construction of transgenes

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Materials and Methods

Isolation of $ALF_p$ and $DOT_p$

The 5' flanking regions of $ALF$ and $DOT$ were isolated using somatic transposon insertion-mediated PCR (SOTI-PCR) (Rebocho et al., 2008). The sequences were submitted to GenBank (see below).

Plant material

The $alf^{W2167}$ and $dot^{A2232}$ $dTPH1$ transposon alleles arose spontaneously in non-transformable petunia line W138, and were described in detail previously (Souer et al., 2008; Souer et al., 1998). $ALF^{W2167/+}$ and $DOT^{A2232/+}$ heterozygotes were crossed to the transformable line W115 and heterozygous progeny were identified by PCR with primers flo22 #1287 and flo53 #3107 ($ALF$), and puf14 #1511 and puf3 #0827 ($DOT$). All primer sequences are listed in Table S1. Heterozygotes were subsequently selfed to obtain transformable $alf$ and $dot$ mutants. 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

All constructs were made by digestion and ligation into vector $pRD400$ (Datla et al., 1992) according to the scheme in figure S1, except for the $DOT_p$:DOT construct, which was made by introduction of $DOT_p$:DOT into the Gateway™ TOPO Entry vector (Karimi et al., 2002) according to the instructions of the manufacturer, and subsequent recombination into V148, which is a binary vector that contains a 35S terminator only. All primers used for cloning are listed in Table S1.

We amplified the cDNA coding sequences of $ALF$, $DOT$, $LFY$ and $UFO$ from the vectors described by Souer et al. (2008), and of $GUS$ from pGreen5K (Hellens et al., 2000), and the upstream non-coding regions from petunia line W138 or Arabidopsis thaliana Columbia genomic DNA. Phusion High-Fidelity DNA Polymerase (Finnzymes) was used for all amplification steps.

All transgenes were (re)sequenced before introduction into the transformable petunia line W115 ($GUS$ constructs), or to F1 (heterozygous wild types) and F2 (homozygous $alf^{W2167}$ or $dot^{A2232}$ mutants) hybrids of line W115 and line W138 (promoter:cDNA constructs). We used Agrobacterium tumefaciens (strain AGL0) mediated leaf disc 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 mono-sulfate.

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

Whole mount $GUS$ staining

We accurately followed the whole-mount $GUS$ staining protocol as described in (Weigel and Glazebrook, 2002). Untransformed W115 was always taken along as negative control. The stained tissue was examined under binoculars. If necessary, the brightness of the digital images as a whole was adjusted for optimal visibility of the organs and blue staining using Adobe® Photoshop® software.
Plant photography
Pictures of plants were taken with a FujiFilm FinePix S2 Pro digital camera. In figures the background was blacked out using Adobe® Photoshop® software.

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 hours light/8 hours darkness). The counted leaf numbers were statistically analyzed using One-Way ANOVA in SPSS (SPSS for Windows, Rel. 16.0.1. 2007. Chicago: SPSS Inc.).

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).
chapter 3

References


