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Genetic control of branching pattern and floral identity during *Petunia* inflorescence development

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SUMMARY

A main determinant of inflorescence architecture is the site where floral meristems are initiated. We show that in wild-type *Petunia* bifurcation of the inflorescence meristem yields two meristems of approximately equal size. One terminates into a floral meristem and the other maintains its inflorescence identity. By random transposon mutagenesis we have generated two mutants in which the architecture of the inflorescence is altered. In the *extra petals*$^-$ (*exp*) mutant the inflorescence terminates with the formation of a single terminal flower. Phenotypic analysis showed that *exp* is required for the bifurcation of inflorescence meristems. In contrast, the *aberrant leaf and flower*$^-$ (*alf*) mutant is affected in the specification of floral meristem identity while the branching pattern of the inflorescence remains unaltered. A weak *alf* allele was identified that, after bifurcation of the inflorescence meristem, yields a 'floral' meristem with partial inflorescence characteristics. By analysing independent transposon *dTph1* insertion alleles we show that the *alf* locus encodes the *Petunia* FLORICAULA/LEAFY homolog. In situ hybridisation shows that *alf* is expressed in the floral meristem and also in the vegetative meristem. Differences and similarities between these *Petunia* mutants and mutations affecting inflorescence architecture in other species will be discussed.

Key words: Floral meristem, *extra petals*, *aberrant leaf and flower*, *dTph1*, Cyme, Inflorescence meristem, *leafy*, *floricaula*

INTRODUCTION

In the plant kingdom, a wide variety of inflorescence structures exist that can be classified according to their overall architecture (Coen and Nugent, 1994). These different inflorescence architectures are a consequence of differences in the behaviour of meristematic cells. For example, *Arabidopsis* and *Antirrhinum* develop racemose inflorescences with a single main axis, where the apex remains meristematic and (multiple) flowers are generated from meristems appearing on the flanks of the apex. Other species, such as tulip, develop a determinate inflorescence where the complete apex transforms into a single terminal flower and meristematic activity is lost. In cymose inflorescences the apex also transforms into a terminal flower, but growth of the inflorescence continues from a new, secondary meristem that forms in the axil of the flower. Thus, multiple terminal flowers are generated on a single inflorescence. Species belonging to the Solanaceae, like *Petunia*, have been classified as cymose (Child, 1979; Weberling, 1989).

Floral meristem identity genes are likely to play a key role in determining inflorescence architecture, because their expression domain determines where flowers are formed. The two highly homologous genes *floricaula* (*flo*) and *leafy* (*lfy*) specify floral meristem identity in the racemose inflorescences of *Antirrhinum* and *Arabidopsis*, respectively (Coen et al., 1990; Weigel et al., 1992). Expression of *flo* and *lfy* is restricted to floral meristems that initiate on the flanks of the inflorescence meristem (Coen et al., 1990; Weigel et al., 1992). At later stages of flower development *flo* and *lfy* mRNAs still accumulate in floral organs. The observation that *lfy* encodes a nuclear protein that can bind DNA suggests that LFY is a transcription factor that activates a yet unknown set of target genes (Weigel, 1995). By analysing periclinal chimeras, that express *flo* in only one of the three meristematic layers, it was shown that *flo* acts in a cell non-autonomous manner (Carpenter and Coen, 1995; Hantke et al., 1995). The expression of the downstream floral organ identity genes, *deficiens* and *plena* is activated in all three cell layers in these *flo* chimeras. Whether this is the consequence of diffusion of the FLO protein itself, or via signalling by another diffusable factor remains to be resolved.

While *flo* and *lfy* are not expressed in vegetative meristems, the homologous genes from pea, tobacco and *Impatiens* are expressed in both floral meristems and leaf primordia (Hofer et al., 1997; Kelly et al., 1995; Poteau et al., 1997). The finding

that *NFL*, the tobacco homolog of *FLO/LFY* is expressed at the site where leaf primordia are initiated suggests a more general role for *flo*/*lfy* and homologs from other species in specifying determinacy of lateral organs and meristems (Kelly et al., 1995). However, the function of *nfl* during development of the simple tobacco leaf remains unknown, because no mutant is available. In pea the *unifoliata* (*uni*) gene encodes the FLO/LFY homolog. Similar to the *flo*$^-$ and *lfy*$^-$ mutants of *Antirrhinum* and *Arabidopsis*, the flowers of *uni*$^-$ mutants are converted into shoots. In addition, the compound pea leaf with tendrils is converted into a single lamina in *uni*$^-$ mutants. This phenotype was explained by assuming a role for *uni* in maintaining a transient phase of indeterminacy of lateral derivatives formed by the meristem (Hofer et al., 1997). Therefore, the function of *flo/lfy* and their homologous genes in other plant species seems distinct from direct determination of floral meristem identity.

Modification of the expression pattern of *lfy* can change an indeterminate inflorescence structure into a determinate floral meristem (Weigel and Nilsson, 1995). The constitutive expression of *lfy* in *Arabidopsis* leads to precocious formation of terminal flowers. Transformation of the same *lfy* construct in aspen leads to flowering within five months while wild-type aspen flowers after 8-20 years. Also in the *centroradialus*$^-$ (*cen*) mutant of *Antirrhinum* and the *terminal flower*$^-$ (*tfl*) mutant of *Arabidopsis* ectopic expression of *lfy* and *flo* in the apex leads to the formation of a terminal flower (Bradley et al., 1996, 1997). This shows that the activity of LFY or FLO is sufficient to convert the indeterminate inflorescence into a determinate floral meristem.

We have started the genetic analysis of cymose inflorescence development in *Petunia*. By random transposon mutagenesis we have isolated mutants in which inflorescence development is altered. Among those, mutants with terminal flowers, altered inflorescence or floral meristem organisation and aberrant meristem identity were found. Here we present the characterisation of two of those mutants. In the *extra petals*$^-$ (*exp*) mutant the inflorescence terminates by the formation of a single flower. We show that *exp* is required for bifurcation of the inflorescence meristem. In the *aberrant leaf and flower*$^-$ (*alf*) mutant floral meristem identity is altered. The *alf*$^-$ mutant has phenotypic characteristics highly similar to those of *flo*$^-$ and *lfy*$^-$ mutants. We describe the cloning and molecular characterisation of the *Petunia* homolog of *flo*/*lfy* and show that it is present at the *alf* locus. Like the pea gene *uni*, *alf* is expressed in both the floral meristem and the vegetative meristem, but no alterations are observed during vegetative development of *alf*$^-$ mutants.

MATERIALS AND METHODS

Plant material

The *alf-S3018* (Doodeman et al., 1984) and *exp-W2115* (this work) mutants arose spontaneously in the progeny of the *Petunia* line W138. The *alf-G5509* and *alf-T2009* alleles were derived from a plant heterozygous for the *alf-S3018* and a wild-type allele. The *alf*$^+$*-X2011* revertant allele was isolated by cross-fertilisation of flowers on a revertant branch of an *alf-S3018*/*alf-G5509* plant. The *alf-W2167 dTph1* insertion allele was identified among 2000 *Petunia* plants by PCR with primers flo1 (5′-GGAATTCCCCATGGACCCAGAGGCTTTC-3′) and the out1 primer (5′-dGGGAATTCGCTCCGCCCCTG-3′) complementary to the terminal inverted repeat of *dTph1* as described previously (Koes et al., 1995). The *alf-X2586* mutant arose spontaneously among W138 progeny (Angenent et al., personal communication). Allelism was confirmed by crossing heterozygous plants harbouring different *alf*$^-$ alleles.

To obtain the *alf*$^-$/*exp*$^-$ double mutant *alf-G5509*/+ plants were crossed to *exp-W2115*/*exp-W2115* plants. Six of the F_1 plants were self-pollinated to obtain F_2 progenies and four of these segregated for *alf*$^-$ mutants. Because the *exp-W2115* is unstable, presumably transposon tagged, five out of six progenies segregated for *exp*$^-$ mutants. The *alf*$^-$/*exp*$^-$ double mutant phenotype was only seen in those F_2 families segregating for both *exp* and *alf*.

Scanning electron microscopy

SEM was performed as described previously (Souer et al., 1996).

Isolation and sequence analysis of the *alf* cDNA and genomic fragments

The *flop* cDNA was isolated by screening a *Petunia* W115 petal cDNA library with the *leafy* cDNA under low stringency conditions. Fragments of this clone were subcloned in pBluescript-SK plasmids (Stratagene) and sequenced.

For PCR amplification of insertion and footprint alleles the following primers were used:

flo1, 5′-GGAATTCCCCATGGACCCAGAGGCTTTC-3′
flo3, 5′-GGAATTCTGAGGAACCAGTGCAGCAG-3′
flo4, 5′-CGGGATCCTTAGTAGGGCATTTTTCACCAC-3′
flo5, 5′-CGGGATCCGGAGCTTTGGTGGGCACATAC-3′
flo6, 5′-GCTCTAGATGAACAATGCAGGGATTTC-3′

Amplification products were cloned in pBluescript-KS or pGEM plasmid vectors and sequenced. Sequencing was performed using asymmetric PCR with fluorescent M13 primers employing an Applied Biosystems DNA sequencer model 370A.

Expression analysis

Total RNA extractions and RT-PCR reactions were performed as described previously (Souer et al., 1996), except that the primers used were oligo $d(T)_{17}$ for first strand synthesis and flo3 and flo5 for PCR amplification. To obtain a quantitative response, *alf* transcripts were amplified in 25 PCR cycles, while for *gapdh* transcripts only 18 cycles were used.

In situ hybridisations were performed as described by Cañas et al. (1994). An *alf*-specific digoxigenin-labelled RNA probe was obtained by T7 polymerase-driven in vitro transcription from the full length cDNA in pBluescript-SK according to the instructions of Boehringer Mannheim. RNA transcripts were partly hydrolysed for 45 minutes in 60 mM Na_2CO_3, 40 mM $NaHCO_3$.

RESULTS

Development of the wild-type *Petunia* inflorescence

Members of the Solanaceae, like *Petunia* are classified as having cymose inflorescences that terminate in a flower. Growth continues from a sympodial meristem in the axis of this flower (Child, 1979; Napoli and Ruehle, 1996; Weberling, 1989). Development of the *Petunia* inflorescence is very similar in diverse inbred lines, although there are slight differences in the extent of axillary meristem dormancy and internode elongation. The *Petunia* wild-type inflorescence contains two leaf-like structures at each node, termed bracts (Figs 1 and 2B). At each node one flower develops in the axil of one bract. In the axil of the other bract, an inflorescence shoot develops that will reiterate this branching pattern (Figs 1 and 2A,B). When the flower and the inflorescence have

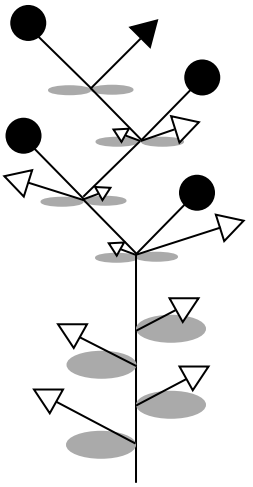

Fig. 1. Diagram showing a wild-type *Petunia* plant. The position of flowers is shown by closed circles, and the apical inflorescence meristem by a closed triangle. Leaves and bracts are indicated by large and small grey ovals respectively. Vegetative axillary meristems are shown by open triangles; smaller size indicates stronger dormancy.

grown out, axillary meristems develop in the axils of both bracts. These meristems are initially vegetative, forming a few leaves, before they transform into an inflorescence meristem that develops in a similar manner as the main inflorescence (Figs 1 and 2B). The extent of outgrowth of these axillary meristems depends on their position in the plant. At the acropetal nodes (near the apex) both meristems remain in a dormant state. At the most basipetal nodes (more distal from the apex) the axillary meristem in the axil of flower and bract will sprout, while the one in the axil of bract and inflorescence usually remains in a dormant state, unless the apex has been removed.

To better understand the formation of floral meristems we analysed the wild-type inflorescence by scanning electron microscopy (SEM). The inflorescence meristem simultaneously generates two bracts before a bifurcation of the central dome yields two halves (Fig. 3A,B). One half develops as a determinate floral meristem that soon after the bifurcation starts to generate sepals, the first floral organs. The other half remains meristematic and will continue with a new division, perpendicular to the last division, to form two new bracts and a new floral meristem. Therefore, the *Petunia* inflorescence does not completely meet the definition of cymose development as the inflorescence meristem does not terminate, but rather splits into two new meristems of which one is the determinate floral meristem. For this reason, we prefer to use the term inflorescence meristem instead of sympodial meristem since the meristem is formed by bifurcation and does not arise as an axillary structure. During the early stages of flower development shown in Fig. 3A,B, the axillary meristem that develops in the axils of older flowers is not visible yet, but will appear later.

The *alf* gene is required for floral meristem identity

Among progeny of the *Petunia hybrida* line W138, an unstable mutant was identified that exhibited a bushy phenotype and only rarely generated heavily malformed floral organs (Fig. 2A, right; Doodeman et al., 1984; Gerats et al., 1988; Gerats, 1991). Because of the proliferation of green leaf-like structures the mutant was called *aberrant leaf and flower* (*alf*). Contrary to what the name suggests, we could not detect differences between *alf*$^-$ and wild-type plants during their vegetative phase. The first moment that differences become evident is after transition of the vegetative shoot meristem to an inflorescence meristem. While the wild-type inflorescence generates bracts and flowers (Fig. 2B), the *alf*$^-$ inflorescence solely forms branches which develop bracts (Fig. 2C). After having formed several branches subtended by bracts, *alf*$^-$ inflorescences occasionally terminate by the formation of floral organs, most often carpels. The formation of axillary meristems in the axils of bracts subtending inflorescence branches appears normal in *alf*$^-$ plants.

To further examine these abnormalities we analysed *alf*$^-$ inflorescences by SEM. Fig. 3C shows that after the formation of bracts, a bifurcation of the *alf*$^-$ inflorescence meristem yields two meristems, which is similar to the wild-type inflorescence at this stage of development. However, in contrast to wild-type meristems, both meristems behave as inflorescence meristems as they will generate bracts on their flanks and bifurcate again to form new inflorescence meristems (Fig. 3C). Occasionally, *alf*$^-$ inflorescence meristems repeat the formation of bracts without secondary bifurcations (e.g. Fig. 3C, left meristem). These observations show that the *alf* mutant is affected in the transition from inflorescence meristem identity to floral meristem identity.

One progeny obtained by self-fertilisation of a *+/alf-S3018* plant segregated for mutants with a weaker *alf*$^-$ phenotype (*alf-T2009*, Fig. 2D). Upon crossing, *alf-T2009* and *alf-G5509* indeed appeared to be allelic (not shown). All *alf-T2009* inflorescences generate meristems which terminate in floral structures. On the periphery these flowers first develop a number of bracts or sepals after which a central carpel is generated that is usually malformed. In between this central carpel and bracts, chimeric organs develop which can contain sepal, petal, stamen and carpel tissue (Fig. 2E). SEM analysis revealed that after bifurcation of the *alf-T2009* inflorescence meristem, the floral meristem has partial characteristics of an inflorescence. This meristem repeats the formation of bracts as inferred from their position on the meristem (Fig. 3D). In contrast to the situation in *alf-G5509* inflorescences, in *alf-T2009* inflorescences no further bifurcation occurs. After having generated several bracts, the central portion of the meristem develops carpels as can be seen by the typical donut structure of the two carpel primordia (Fig. 3D). Therefore, in the *alf-T2009* mutant the initial specification of floral meristem identity is blocked as inferred by the repeated formation of bract primordia, but the meristem acquires in time more floral characteristics causing it to terminate with the formation of carpels. This phenotype, together with the observation that in the allelism test *alf-T2009*/*alf-G5509* heterozygous plants could be recognised as having an intermediate phenotype, shows that *alf-T2009* is a weak *alf* allele.

extra petals is required for bifurcation of the inflorescence meristem

In a random transposon-mutagenesis experiment we found another mutant with an altered inflorescence. This mutant was initially identified for its increased number of petals and, therefore, named *extra petals* (*exp*). While wild-type *Petunia*

flowers have five petals (Fig. 2H), *exp*⁻ flowers contain six full-grown petals (Fig. 2G). Often an additional one or two petals are recognisable that do contain a central vein, but remain very narrow, possibly due to mechanical constraints. About half of the flowers on *exp*⁻ plants contain six mature anthers, while the other half contains five anthers similar to the situation in wild-type flowers. The number of sepals and carpels in *exp*⁻ flowers was similar to the numbers found in wild-type plants. SEM analysis of young *exp*⁻ flowers shows that they contain seven to eight organ primordia in whorl 2 and six to eight primordia in whorl 3, while the number of sepal and carpel primordia in whorls 1 and 4 is similar to wild-type flowers (compare Fig. 3E,F with 3B).

To examine if the effect of *exp* on floral organ number is dependent on their identity, we generated the double mutant with the floral organ identity gene *green petals* (*gp*). The *gp* locus encodes a MADS-box protein and is the ortholog of *deficiens* from *Antirrhinum* and *apetela-3* of *Arabidopsis* (van der Krol et al., 1993). Loss of *gp* function results in the transformation of petals into sepal-like organs. *exp*⁻/*gp*⁻ flowers consisted of five sepals in whorl 1, seven to eight sepal-like organs in whorl 2, five to six anthers in whorl 3 and two carpels in whorl 4 (not shown), indicating that the *exp* mutation increases the number of floral organs in whorl 2 regardless of their identity.

Closer examination of *exp*⁻ plants showed that their inflorescence had an unusual architecture. The *exp*⁻ inflorescence (i.e. the structure between the first two bracts) consists of a single terminal flower that almost completely lacks the pedicel (Fig. 2H). Apparently, the formation of this terminal flower is due to the complete transformation of the apical inflorescence meristem into a floral meristem, as (1) no remains of the inflorescence meristem is detectable after this transition and (2) the flower is positioned apically (Fig. 3G). This is consistent with the observation that *exp*⁻ plants loose their apical dominance once a terminal flower is generated, similar to wild-type plants from which the inflorescence apex is manually removed. As a consequence, the dormancy of the vegetative meristems in the axils of leaves is broken and they will generate a series of leaves before they terminate with the formation of a single flower. Thus, *exp*⁻ plants generate multiple inflorescences that each consist of a single flower.

To further define the role of *exp* in inflorescence development we determined the phenotype of the *exp*⁻/*alf*⁻ double mutant. Fig. 2I shows that *exp*⁻/*alf*⁻ plants have, like *alf*⁻ mutants, an indeterminate inflorescence that only contains bracts and completely lacks flowers. In addition, the *exp*⁻/*alf*⁻ double mutant has lost the branching pattern that is typical of *alf*⁻ inflorescences, as it consists of a single branch bearing bracts (compare the two plants in Fig. 2I or Fig. 3H and 3C). In the axils of the older bracts vegetative meristems appear, similar to the situation in wild-type plants.

Taken together these data show that *exp* and *alf* function in two distinct processes. *exp* is required for bifurcation of the inflorescence meristem into two new meristems, but does not seem to have an effect on the identity of these meristems. On the other hand, *alf* is required to determine the floral identity for one of the two meristems, but does not seem to affect the initiation of new meristems.

Isolation of the *flo*/*lfy* homolog of *Petunia*

The phenotypic defects seen in *alf*⁻ mutant plants resemble those in *floricaula*⁻ and *leafy*⁻ mutants (Coen et al., 1990; Weigel et al., 1992) suggesting that the *alf* locus may contain the *Petunia* homolog of *lfy* and *flo*. To test this, we isolated the *flo*/*leafy* homolog of *Petunia* (*flop, floricaula/leafy ortholog Petunia*). The *flop* cDNA was isolated by screening a petal cDNA library of the *Petunia* hybrida line W115 with the full length *lfy* cDNA under low stringency conditions.

Fig. 2. Phenotype of wild-type, *alf*⁻, *exp*⁻ and *exp*⁻/*alf*⁻ plants. (A) Wild-type (left) and *alf-G5509* (right) plants. (B) Wild-type inflorescence. All flowers are subtended by bracts, but some have been removed to show the branching pattern. (C) *alf-G5509* inflorescence. Some bracts have been removed to show the branching pattern. The arrow indicates the first bifurcation of the inflorescence. (D) Inflorescence of a plant carrying the weak *alf-T2009* allele. Some bracts have been removed. (E) Detail of floral structures found at inflorescence branches of *alf-T2009* homozygous plants. (F) Wild-type *Petunia* flower. (G) *exp*⁻ flower. The flower has six instead of five petals. (H) Wild-type (left) and *exp*⁻ (right) plants. The *exp*⁻ inflorescence has terminated in a single flower. Note that the flower lacks a pedicel. (I) *exp*⁻/*alf*⁻ (left) and *alf*⁻ (right) plants. The *exp*⁻/*alf*⁻ inflorescence is unbranched but indeterminate. Some leaves have been removed. ax, axillary branches; br, bract; cal, carpeloid organs; if, inflorescence shoot; le, leaf; pet, petaloid organ.

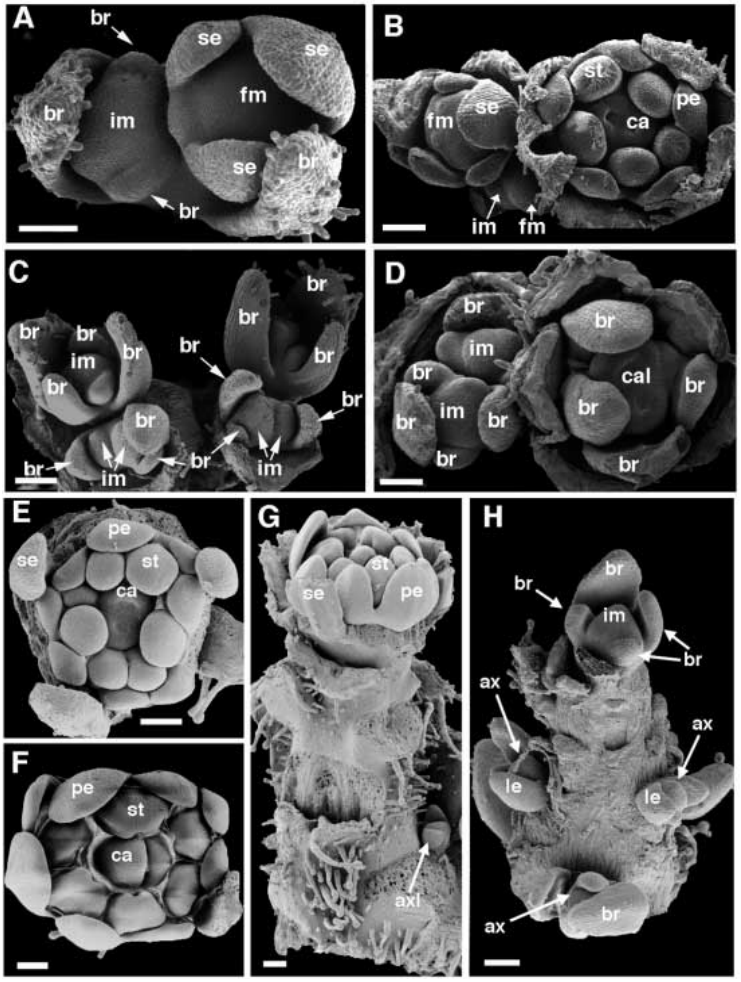

Fig. 3. SEM analysis of wild-type, *alf*⁻, *exp*⁻ and *exp*⁻/*alf*⁻ inflorescences.(A) Wild-type inflorescence. The inflorescence has generated a floral meristem (right) on which the first three sepal primordia have arisen. On the inflorescence meristem (left) the bract primordia have just initiated perpendicular to the bracts formed on an earlier node. (B) Wild-type inflorescence slightly later than in A. Bifurcation of the inflorescence has occurred. The floral meristem has initiated all floral organ primordia. Sepals that enclose the oldest flower at this stage were removed. (C) *alf-G5509* inflorescence. The bifurcation of the *alf*⁻ inflorescence meristem is similar to that in wild-type inflorescences. However, both meristems behave as inflorescence meristems as shown by the repetitive formation of bracts and new bifurcations of each meristem. (D) *alf-T2009* inflorescence. After bifurcation of the inflorescence meristem the 'floral' meristem continues to form bracts but secondary bifurcation does not occur. This meristem soon terminates with the development of carpels (right). (E) Young *exp*⁻ flower. Extra petal and stamen organs develop on the floral meristem (compare with wild-type flower in panel B) Some sepals were removed. (F) *exp*⁻ flower slightly later in development. All sepals have been removed. (G) Side-view of an *exp*⁻ branch. A single terminal flower has developed at the apex. The young meristem on the right is an axillary meristem that has developed in a leaf axil. (H) Side-view of an *exp*⁻/*alf*⁻ inflorescence. The inflorescence meristem keeps developing bract primordia but does not bifurcate. In the axils of older bracts, axillary meristems have developed. ax, meristem in axil of bract; axl, meristem in axil of leaf; br, bract; ca, carpel; cal, carpel-like; fm, floral meristem; im, inflorescence meristem; le, leaf; pe, petal; se, sepal; st, stamen. The scale bar equals 100 μm in all panels.

By Southern blot analysis under low stringency conditions only a single band could be detected after hybridisation with the *flop* cDNA (data not shown). Furthermore, upon screening a *Petunia* inflorescence cDNA library with the *flop* cDNA, only cDNAs derived from identical transcripts were isolated. Therefore, we presume that the *flop* gene is single copy in *Petunia*.

The *flop* cDNA fragment is 1412 bp long, potentially encoding a protein of 412 amino acids (Fig. 4). The deduced amino acid sequence of the *flop* gene has 82% identity with FLO, 72% with UNI, 66% with LFY, and 65% with BOFH, the FLO/LFY homolog of *Brassica oleracea*. FLOP is most homologous to the tobacco NFL1 protein sharing 93% identity (Fig. 5). By PCR and sequence analysis we determined that the positions of the two introns in the *flop* gene are identical with those in *flo*, *lfy* and *nfl* (Coen et al., 1990; Kelly et al., 1995; Weigel et al., 1992), consistent with the view that they are orthologs.

The *flop* cDNA is transcribed from the *alf* locus

To determine if the *alf*⁻ phenotype was caused by mutation of *flop*, we performed a PCR reaction with *flop* specific primers on genomic DNA isolated from a family of plants segregating for the mutable *alf-S3018* allele. Compared to wild-type, the amplification products of *alf-S3018* contained an insertion of approximately 300 bp. Sequence analysis of this fragment showed that a *dTph1* element was inserted in the protein coding sequence, 1 bp upstream the exon2/intron2 boundary at bp 873 (Fig. 4).

To prove that the insertion of this *dTph1* element caused the *alf*⁻ phenotype, we screened a *Petunia* population by PCR for independent insertions in the *flop* gene (Koes et al., 1995). Among 2000 plants we found one plant carrying a *dTph1* insertion in the first exon of *flop* (*alf-W2167*, Fig. 4A,B). Selfing of this +/*alf-W2167* plant yielded progeny that segregated 3:1 for wild-type and *alf*⁻ phenotypes. A third independent mutant, *alf-X2586*, was found among W138 progeny (Angenent et al., personal communication). By PCR and sequence analysis we identified a *dTph1* element in the second exon of *flop* (Fig. 4A,B). Crosses of heterozygotes harbouring different *alf* alleles, segregated 3:1 for wild-type and *alf*⁻ mutants, confirming that the mutants represent alleles of one locus.

The finding that three independent transposon insertions in *flop* all cause *alf*⁻ phenotypes showed that *flop* originated from the *alf* locus. For this reason we will from now on refer to the *flop* gene as *alf*. Because the three *alf* insertion alleles show identical phenotypic alterations we presume that they are all null alleles.

Sequence analysis of *alf* alleles

To determine the consequence of sequence alterations in the *alf* gene we sequenced a number of excision alleles originating from

alf-S3018. A revertant allele, *alf*$^{+}$*-X2011*, was obtained by cross-fertilisation of (nearly) wild-type flowers that formed on a revertant branch of an *alf-S3018*/*alf-G5509* heterozygote. The resulting progeny segregated 3:1 for wild-type and *alf*$^{-}$ phenotypes indicating that the reversion had occurred in the L2 layer of the parental plant. PCR and sequence analysis of genomic DNA of an *alf*$^{+}$*-X2011* homozygote showed that the *dTph1* element had perfectly excised from the *alf* gene (Fig. 4C). Sequence analysis of the weak *alf-T2009* allele revealed that *dTph1* excision had created a 3 bp footprint that leaves the reading frame intact. An extra threonine residue is now present at position 287, apparently resulting in a protein with partial activity (Fig. 4C). One of the selfings derived from a plant heterozygous for *alf-S3018* and a wild-type allele yielded a family with *alf*$^{-}$ plants that had lost the *dTph1* element in the *alf* gene as determined by PCR. Sequencing of this new *alf-G5509* allele showed that the footprint left by the transposable element shifted the reading frame thereby creating a stop codon at position 290, two amino acids after the original *dTph1* insertion site (Fig. 4C). This shows that small changes around amino acid 288 at least partially influence the activity of *alf* and that the C-terminal part after amino acid 288 is required for *alf* activity.

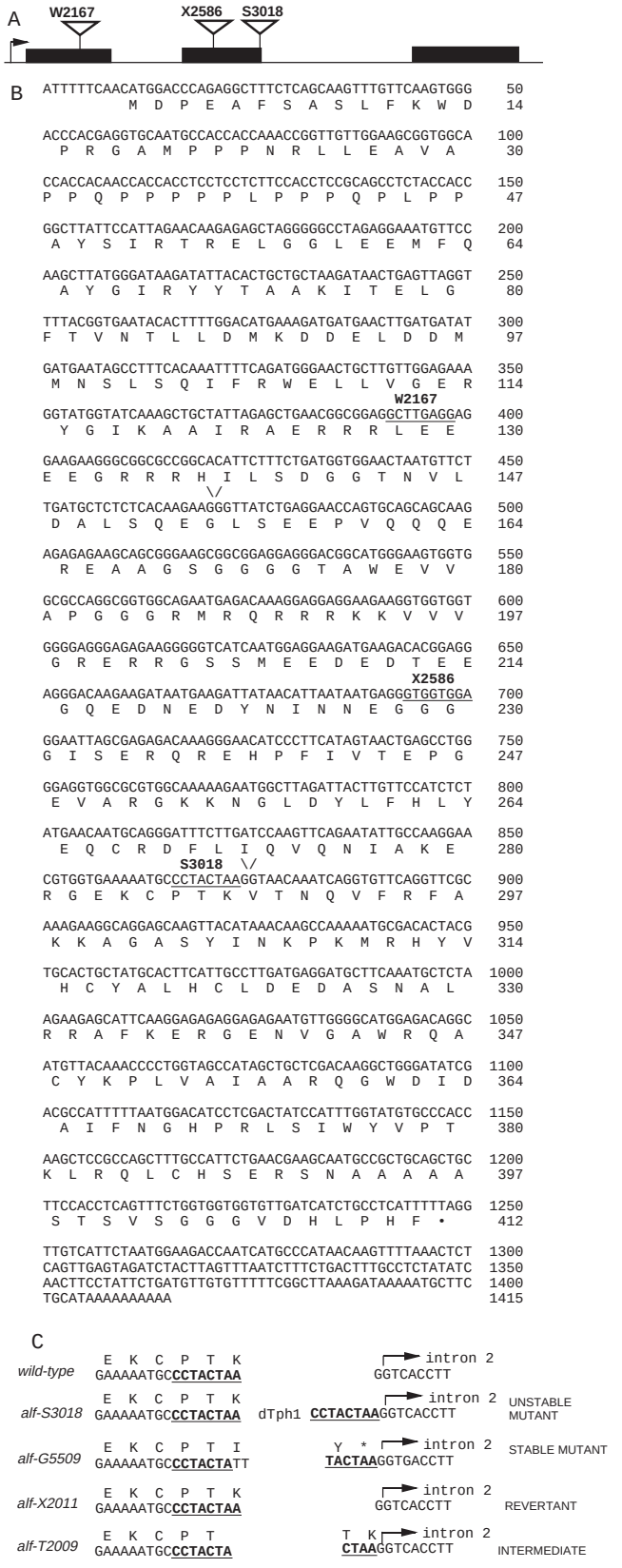

Fig. 4. mRNA sequence and structure of the *alf* gene, the *flo*/*lfy* homolog of *Petunia*. (A) Genomic map of the *alf* gene. Thick bars represent exon sequences. The position of *dTph1* insertions is indicated by open triangles. The arrow indicates the direction of transcription. (B) The deduced amino acid sequence is shown below the nucleotide sequence. The target site duplication sequence of *dTph1* insertion alleles is underlined. The position of the introns is indicated by two slashes (\/) after nucleotides 470 and 874, respectively. The GenBank accesion number for the *alf* cDNA is AF030171. (C) Sequences of footprint alleles created after excision of *dTph1* from the insertion allele *alf-S3018*. The target site duplication is underlined.

Expression pattern of *alf*

To establish the expression domain of *alf*, we performed RT-PCR on total RNA extracted from various plant parts. We detected *alf* transcripts in young flowers, vegetative apices and inflorescence apices (Fig. 6, lane 1, 3 and 5). No expression was found in roots, while only low expression could be detected in leaves. Therefore, *alf* is expressed in both the inflorescence and the vegetative meristem.

To determine the spatiotemporal expression pattern of *alf*, we performed in situ hybridisations at various stages of development (Fig. 7). By analysing serial sections of vegetative meristems we detected *alf* expression in a ring of cells, a few cells wide, around the central zone (Fig. 7A). This expression domain coincides with the places where leaf primordia are initiated. *alf* transcripts remain present in young leaf primordia, but disappear when the leaves grow older.

In the inflorescence meristem, *alf* expression is restricted to the site where bracts and the floral meristem will arise (Fig. 7B). The region of the inflorescence meristem that remains meristematic is seen as a zone that lacks *alf* expression. Slightly later in development *alf* is expressed inside the bracts and at the peripheral zone of the floral meristem, where sepals will form (not shown). When sepals are formed the expression domain moves towards the centre of the floral meristem to the sites where petal and stamen primordia will arise, but is absent from the centre of the floral meristem (Fig. 7C). Later, signals were detected mainly in petal, stamen and carpel primordia (Fig. 7D). The expression in stamens seems unequally distributed, as some sections lacked signal while in subsequent sections expression

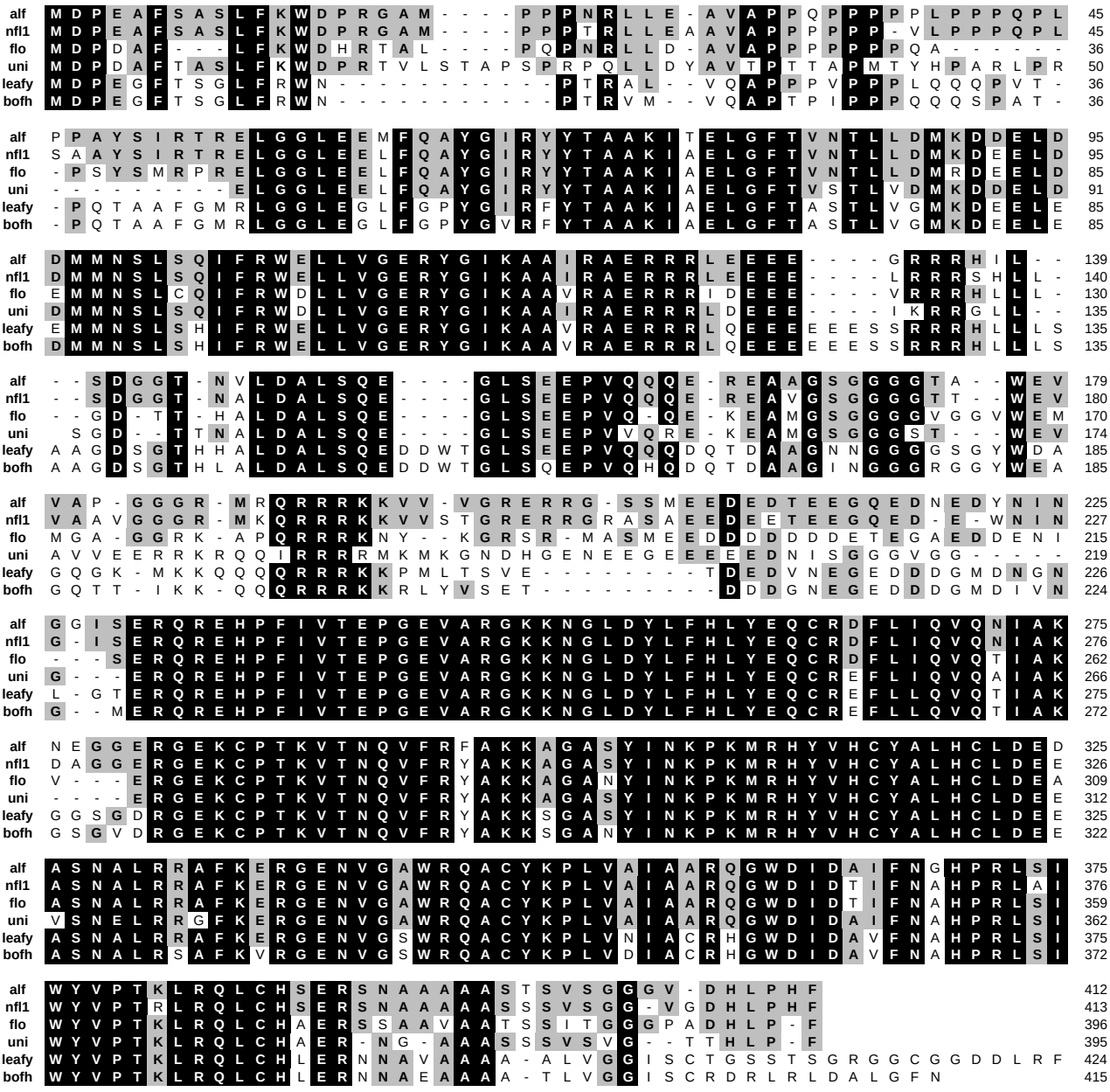

Fig. 5. Alignment of the deduced amino acid sequence of *alf* with NFL (Kelly et al., 1995), FLO (Coen et al., 1990), UNI (Hofer et al., 1997), LFY (Weigel et al., 1992) and BOFH (Anthony et al., 1996). Amino acids that are identical in all six sequences are shown in black boxes. In addition, amino acids identical to ALF are shown in grey boxes to reveal the higher similarity of ALF with NFL.

could be detected. Around stage five of flower development (Angenent et al., 1995), when the gynoecium is fused at the top and the style and stigma start to develop, *alf* signal is apparent in petals and carpels (Fig. 7E). In addition, also on the bottom of the placenta strong *alf* signals can be detected.

In conclusion, *alf* is transcribed in the inflorescence at the sites where floral meristems and organ primordia are formed and in vegetative meristems at the sites where leaf primordia are initiated.

Transcription of *alf* is not auto-regulated

In *Antirrhinum*, FLO was reported to (auto)regulate its own expression (Carpenter et al., 1995). To determine if a similar control exists in *Petunia*, we analysed the expression of *alf* by RT-PCR in plants harbouring different *alf* alleles. The levels of *alf* transcripts in inflorescences homozygous for the weak *alf-T2009* allele, the unstable *alf-W2167* allele and the stable recessive *alf-G5509* allele are similar to those in homozygous (Alf^{+}/Alf^{+}) or heterozygous (Alf^{+}/alf-*W2167*) wild-type inflorescences (Fig. 6). This indicates that ALF does not control the accumulation of its own mRNA.

DISCUSSION

Development of the wild-type *Petunia* inflorescence

A remarkable transition observed during plant development is

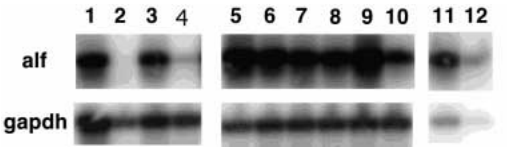

Fig. 6. RT-PCR analysis of *alf* RNA in different tissues and different genotypes. (Top) PCR amplification with *alf* specific primers. (Bottom) PCR amplification with *gapdh* specific primers. Lane 1, floral buds ≤1 cm.; lane 2, roots; lane 3, vegetative apices; lane 4, leaves; lane 5, wild-type inflorescences; lane 6, *alf-T2009* inflorescences; lane 7, *alf-W2167*/*alf-W2167* inflorescences; lane 8, *alf-W2167*/*Alf*$^{+}$ inflorescences; lane 9, *Alf*$^{+}$/*Alf*$^{+}$ inflorescences; lane 10, *alf-G5509*/*alf-G5509* inflorescences; lane 11, ten-fold dilution of the RNA used for lane 9; lane 12, 100-fold dilution of the RNA used for lane 9.

the shift from the vegetative to the reproductive phase. This shift is characterised by the development of an inflorescence meristem that generates floral meristems. In racemose inflorescences these floral meristems axillary while in cymose inflorescences the floral meristem develops in a terminal position. In cymes, new (sympodial) meristems are thought to form in the axils of these flowers leading to the typical zig-zag branching pattern of cymose inflorescences. Our results show that this is, at least in *Petunia*, an incorrect view. In *Petunia*, floral meristem development involves a bifurcation, not a termination of the inflorescence meristem, yielding two meristems of about the same size. One of these meristems will grow out to form a flower. This pattern by which floral meristems are initiated is highly similar to flower development in tomato, where a bifurcation of inflorescence meristems also occurs (Allen and Sussex, 1996). In pea, flower formation also involves bifurcation of the inflorescence meristem (Tucker, 1989). In Fig. 8, the pattern by which the wild-type *Petunia* inflorescence develops is drawn schematically as a reiterative cycle.

In this paper, we described two genes each of which is required for one of the two major processes that determine the final architecture of the *Petunia* inflorescence: *exp*, which is required for bifurcation of the inflorescence meristem, and *alf*, which is required for floral meristem identity.

alf determines floral meristem identity

By in situ hybridisation we show that the expression of *alf* in the inflorescence marks the formation of a floral meristem, before the bifurcation of the apex makes it anatomically visible. At this early stage, the zone of the inflorescence that expresses *alf* comprises a bit more than half of the meristem (Fig. 7B). The bifurcation itself is independent of *alf*, since it also occurs in *alf*$^{-}$ mutants, thereby maintaining the branching pattern. Another marker for bifurcation of the *Petunia* inflorescence is the *no apical meristem* gene (*nam*) that is expressed in a stripe of cells that separate the inflorescence and floral meristem (Souer et al., 1996). Also in this case the floral meristem appears as a slightly larger zone than the inflorescence meristem. However, when the bifurcation of the inflorescence can be seen microscopically, the two new meristems appear approximately equal in size (Fig. 3A,B). This suggests that the border cells of the expression domain of *alf* and *nam* in the inflorescence are not incorporated in the

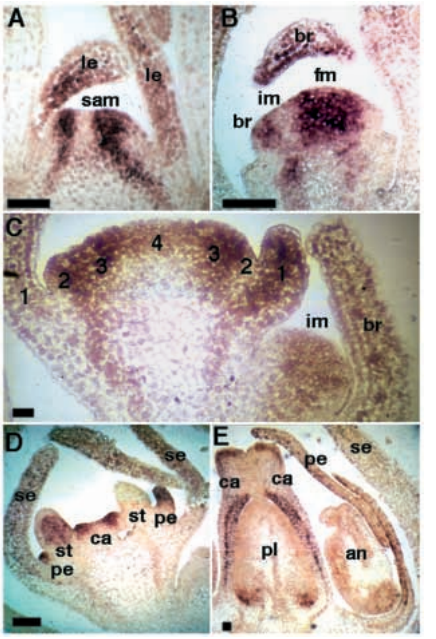

Fig. 7. In situ localisation of *alf* mRNA in vegetative meristems and inflorescences of a wild-type *Petunia*. (A) Young *Petunia* plantlet. Expression in the vegetative meristem is detected at the site where leaf primordia will appear. (B) Inflorescence meristem. A sector of *alf*-expressing cells marks the site where the floral meristem will be developed. Strong expression is also detected in the bract primordium (left). The structure overlying the apex is a bract from an earlier node. (C) Young floral meristem. Sepal primordia are formed in which *alf* is expressed. The zone on the meristem were later petal and stamen primordia will arise also expresses *alf*. An inflorescence meristem without *alf* expression is seen on the right in the axil of a bract. (D) Young flower. *alf* expression is detected in all floral organ primordia. Note that the stamen on the right lacks detectable *alf* expression. (E) Part of an older flower. *alf* expression is detected in the developing stigma, the carpel wall and the bottom of the placenta. In petals *alf* expression can still be detected. an, anther; br, bract; ca, carpel; fm, floral meristem; im, inflorescence meristem; le, leaf; pe, petal; pl, placenta; sam, shoot apical meristem; se, sepal; st, stamen. The scale bar equals 100 μm in all panels.

floral meristem or that the inflorescence cells divide faster at this stage of development.

Although the *Petunia* inflorescence does not meet the definition for cymose development, it also does not develop a straight main axis as in racemose inflorescences (Figs. 1 and 2B). The structure of repetitive bifurcations that makes up the *Petunia* inflorescence is best seen in *alf*$^{-}$ mutants. Because in wild-type plants the vigorous growth of the inflorescence pushes the flower aside, only a weak zig-zag pattern remains.

Expression of *lfy* and *flo* in the racemose inflorescences of *Arabidopsis* and *Antirrhinum* occurs on the flanks of the inflorescence dome, while *alf* is expressed in the terminal part of the inflorescence dome (Fig. 7B). Thus, the expression pattern of these three homologs reflects the different behaviour of inflorescence apices that results in different inflorescence architectures. *Antirrhinum* mutants harbouring the *flo-640* frame shift allele contain little or no *flo* transcripts. Carpenter et al. (1995) suggested that *flo* may be autoregulated. However, *alf*, like *lfy* (Weigel et al., 1992), does not appear to be

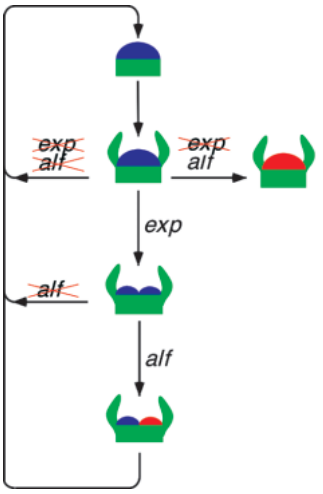

Fig. 8. Model explaining the action of *alf* and *exp* during inflorescence development in *Petunia*. The wild-type situation runs through the largest cycle, with the inflorescence meristem (blue) repeating the top to bottom pattern. The inflorescence meristem (blue) develops two bracts (green). *exp* causes the meristem to split in two halves. The action of *alf* is restricted to one of these meristems causing it to acquire a determinate floral meristem identity (red). In the absence of *exp*, bifurcation is omitted, but *alf* converts the meristem into a single terminal flower. In the absence of *alf*, bifurcation of the inflorescence meristem still occurs but floral meristem identity is not acquired. When both *alf* and *exp* are inactive and both the bifurcation and floral meristem identities are lost, the inflorescence keeps forming bracts without any branching.

autoregulated since the *alf* transcript levels are normal in *alf*$^-$ plants. (Fig. 6). Therefore, regulation of *alf* and *flo* seems to differ. Alternatively, the transcript derived from the *flo-640* allele may be highly unstable due to the sequence alterations caused by the transposon footprint (de Vetten et al., 1997; van Hoof and Green, 1996).

During subsequent stages of flower development *alf* becomes sequentially active in the organ primordia in all 4 floral whorls, although the expression in stamen primordia is not uniform. The *alf* expression pattern is very similar to that of *lfy*. In contrast, *flo*, is only expressed in whorls 1, 2 and 4, while expression of *nfl* remains limited to whorls 1 and 2 (Kelly et al., 1995). Since the mutants usually lack floral meristems the role of these genes in floral organ development remains obscure.

alf expression in vegetative meristems

Like homologs isolated from tobacco, pea and *Impatiens*, *alf* is also expressed in vegetative meristems, while *flo* and *lfy* are inactive there. Recently, the analysis of the pea mutant *unifoliata* and the expression pattern of the *uni* gene revealed a role for *uni* in maintaining cells in a transient phase of indeterminacy (Hofer et al., 1997). Apart from the floral phenotype, which resembles that of *flo*$^-$ and *lfy*$^-$ mutants, the compound leaf with tendrils is converted into simple single lamina in *uni*$^-$ mutants. By in situ hybridisation it was shown that *uni* is still expressed in relatively old leaf primordia, where it presumably maintains cells in this transient indeterminate state to facilitate the formation of a compound leaf.

Wild-type *Petunia*s develop simple leaves consisting of a single lamina. The expression of *alf* in leaf primordia is lost rapidly after the outgrowth of the leaf primordia. Therefore, it seems likely that the expression of *alf* in leaves points towards an old function of *alf* in leaf development that has been lost during evolution in *Petunia*. A similar situation might exist in tobacco, where NFL expression was detected in leaf primordia. In tomato, the compound leaf is unaltered in the *falsiflora*$^-$ (*fa*) mutant, although it has been suggested that it encodes a FLO homolog (Allen and Sussex, 1996; Coen and Nugent, 1994). Molecular analysis of the *fa*$^-$ mutant and the *flo* homolog of tomato might clarify this contradiction.

exp and inflorescence development

We have shown that the wild-type inflorescence forms floral meristems by bifurcation of the inflorescence meristem. In the *exp*$^-$ mutant each inflorescence branch terminates with the formation of a single flower, thereby converting the normally indeterminate inflorescence into a determinate one. The *exp*$^-$ mutant thereby resembles to some extent the phenotype of the *cen*$^-$ and *tfl*$^-$ mutants of *Antirrhinum* and *Arabidopsis* (Bradley et al., 1996, 1997). In the *cen*$^-$ mutant about ten flowers are produced before the inflorescence meristem generates a terminal flower. The *cen* gene is expressed in the sub-apical region of the inflorescence meristem where it represses the activation of *flo* in the inflorescence meristem. In turn, the expression of *cen* seems to be dependent on FLO as *cen* is not expressed in a *flo*$^-$ mutant (Bradley et al., 1996). Therefore, *cen* seems to maintain the inflorescence identity of the apical dome after floral induction by regulating the expression domain of *flo*. Although the phenotype of the *cen*$^-$/*flo*$^-$ mutant has not been reported, one would expect that, given the cascade of regulation of *flo* and *cen* and their role in inflorescence development, the *flo*$^-$/*cen*$^-$ double mutant would be indistinguishable from the *flo*$^-$ single mutant.

Our analysis of the *exp*$^-$/*alf*$^-$ double mutant suggests a completely different role for *exp* than for *cen*. Similar to the situation in the *exp*$^-$ single mutant (Fig. 8, arrow to the right), bifurcation of the inflorescence is lost in the *alf*$^-$/*exp*$^-$ double mutant (Fig. 8, smallest cycle). In addition to the loss of bifurcations, the loss of ALF function in an *exp*$^-$ background converts the terminal flower into an indeterminate inflorescence meristem. Therefore, it seems that *exp* is required for bifurcation of the inflorescence meristem alone and has no influence on meristem identity.

The *nam* gene of *Petunia*, which is involved in establishing the border of the apical meristem and of floral organ primordia, is expressed in a stripe of cells in between the inflorescence and floral meristem, before bifurcation of the inflorescence meristem has occurred (Souer et al., 1996). In *nam*$^-$ mutants, however, no alterations in inflorescence architecture can be detected presumably because of redundancy between *nam* and *nam* homologous genes (Souer, 1997). Given the function of *nam* and *exp* in separating meristems and organ primordia, *nam* and *exp* might function in the same pathway, where *exp* is a specific separator of inflorescence and floral meristems and *nam* has a function throughout the plant body.

When bifurcation is lost, as in *exp*$^-$ plants, the complete apical dome is converted into a flower. This suggests that *alf* is expressed throughout the apex in the *exp* mutant. Since our analysis of *exp* and the *exp*/*alf* double mutant points towards a

role for *exp* in bifurcation of the inflorescence meristem and not in floral meristem identity, this might be an indirect effect of the lack of bifurcation. It could be that *alf* is still transcribed in part of the apical dome only, but induces floral meristem identity in the whole apex because it acts non-autonomously at the moment bifurcation has been omitted. Preliminary results indicate that the induction of floral organ development in *exp*$^-$ floral meristems is unequally distributed on the apex, with organ initiation starting in one part of the meristem first. This part presumably reflects the site in the apex where the *alf* gene is transcribed. In *Antirrhinum*, it was shown that FLO is able to induce expression of floral organ identity genes non-autonomously (Carpenter and Coen, 1995; Hantke et al., 1995). Establishing the expression domain of *alf* in *exp*$^-$ plants might clarify this point.

The conversion of the whole apical meristem into a floral meristem in *exp* mutants causes the formation of extra floral organs in whorl 2 and 3. The formation of extra floral organs in whorl 2 is independent of organ identity as it also occurs in the *gp*/*exp* double mutant, where petals are replaced by sepals. Possibly, the formation of extra floral organs is a secondary effect that is caused by extra space due to the conversion of the whole apex into a floral meristem.

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