

Interactions between gene activity and cell layers during floral development

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Summary

The *DEFICIENS* (*DEF*) gene is required for establishing petal and stamen identity in *Antirrhinum* and is expressed in all three layers of the floral meristem in whorls 2 and 3. Expression of *DEF* in a subset of meristem layers gives rise to organs with characteristic shapes and cell types, reflecting altered patterns and levels of *DEF* gene activity. To determine how the contributions of layers and gene activity interact, we exploited a *DEF* allele which carries a transposon insertion in the MADS box region to generate periclinal chimeras expressing alleles with different activities. By comparing the phenotype, development and expression patterns of these chimeras we show that expression of *DEF* in L1 makes a major contribution to morphology in whorl 2, irrespective of the allele. By contrast L1 expression is largely unable to rescue whorl 3, possibly because of a non-autonomous inhibitor of *DEF* activity in this whorl.

Keywords: ABC models, *Antirrhinum*, chimera, organ identity, transposon.

Introduction

The development of plant organs with defined shapes requires growth to be co-ordinated between several layers of cells. One approach to studying how this process is controlled has been to exploit periclinal chimeras in which a developmental gene has been inactivated in one or more meristem layers. Such chimeras have been used to look at how genes influence the shape of leaves and floral organs (Bouhidel and Irish, 1996; Jenik and Irish, 2001; McHale and Marcotrigiano, 1998; Perbal *et al.*, 1996; Sieburth *et al.*, 1998). However, a complication with this approach is that for genes that are normally expressed in more than one layer, chimeras affect the overall level of gene activity in the organ as well as the layer in which the gene is expressed. One way of distinguishing these effects would be to compare chimeras expressing alleles with different levels of gene activity. If expression in a given layer always has the same type of effect, irrespective of the allele involved, this would indicate that the effect is a consequence of the layer rather than the overall level of activity. We have explored this approach to study the role of *DEFICIENS* (*DEF*) in the control of floral organ development in *Antirrhinum*.

DEF encodes a MADS domain transcription factor involved in specifying organ identity in whorls 2 and 3 of *Antirrhinum* flowers (Schwarz-Sommer *et al.*, 1992). In *def*

mutants, sepals grow in place of petals and carpels in place of stamens, the B class phenotype in the ABC model of flower development (Coen and Meyerowitz, 1991). A similar phenotype is conferred by a second gene in *Antirrhinum*, *GLOBOSA* (*GLO*), which encodes a related MADS domain protein that can form a ternary complex with *DEF* and other MADS domain proteins (Egea-Cortines *et al.*, 1999; Gutierrez-Cortines and Davies, 2000; Tröbner *et al.*, 1992). Both *DEF* and *GLO* are expressed from a very early stage of floral development in whorls 2 and 3 of the floral meristem. Many of these properties are shared by the orthologues of *DEF* and *GLO* in *Arabidopsis*, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), respectively (Honma and Goto, 2001; Pelaz *et al.*, 2000, 2001; Riechmann and Meyerowitz, 1997; Theissen and Saedler, 2001).

Periclinal chimeras have been characterised which express the wild-type *DEF* allele in either the outer epidermal layer, L1, or both the internal layers, L2 and L3, of the meristem (Perbal *et al.*, 1996). The results show that *DEF* expression in L1 largely restores petal morphology and epidermal cell identity in whorl 2 but has little effect on whorl 3. By contrast, *DEF* expression in L2/L3 has less effect on petal morphology and partially restores stamen development in whorl 3. Some of the effects of L2/L3 expression

in whorl 2 can be accounted for by trafficking of DEF protein into the epidermal layer. Similar results have been reported for chimeras of *AP3* of *Arabidopsis*, although in this case no trafficking of protein between cell layers was detected (Jenik and Irish, 2001).

A complication with the analysis of chimeras is that the phenotypes obtained may reflect contributions from both the layer and total altered level of gene activity. These contributions are likely to be distinct, as the phenotypes of the chimeras are different from those seen in plants carrying alleles with reduced expression levels or activity (Schwarz-Sommer *et al.*, 1992). Moreover, transgenic *Antirrhinum* plants expressing *DEF* from an epidermal-specific promoter give similar qualitative phenotypes to L1 chimeras, even though the level of expression is unlikely to be exactly the same (Efremova *et al.*, 2001). However, expression of *AP3* from an epidermal promoter in transgenic *Arabidopsis* plants results in a greater degree of rescue than seen in L1 chimeras, highlighting the importance of the level as well as the layer of expression (Efremova *et al.*, 2001).

To investigate the contribution of each layer in the context of different gene activities, we compared chimeras expressing different *DEF* alleles in various layers. This was achieved by exploiting the *def-621* allele, which carries a transposon insertion in the MADS box region of *DEF*. Excision of the transposon during somatic development gives rise to chimeras that either carry a wild-type allele (precise excision) or defective *DEF* allele (imprecise excision). By comparing the phenotype, development and expression patterns of these chimeras, we show that expression of *DEF* in L1 makes a major contribution to morphology in whorl 2, irrespective of the allele. By contrast L1 expression is largely unable to rescue whorl 3, possibly because of a non-autonomous inhibitor of *DEF* activity generated in subepidermal mutant tissue.

Results

Pattern of *DEF* expression in the chimeras

Periclinal chimeras were derived by taking cuttings from branches of *def-621* plants in which petal development had been restored to various degrees. The whorl 2 organs exhibited a range of characteristics between petals and sepals, allowing the chimeras to be classified into five categories (Figure 1c–g).

To determine the meristem layers expressing *DEF* in these chimeras, *in situ* hybridisations were performed on each phenotypic category and compared to wild type and *def-621*. In wild-type floral meristems, *DEF* expression is first detected at late stage 3, when signal is observed in all three layers of the central dome (Schwarz-Sommer *et al.*,

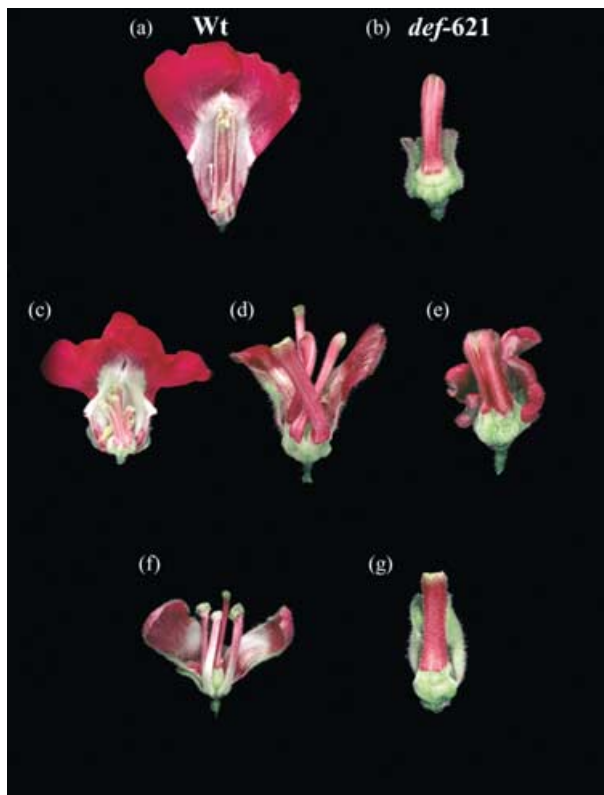


Figure 1. Phenotype of wild-type, *def-621* and chimeric flowers. Mature flowers were taken from wild-type (a), *def-621* (b) and the five classes of chimeric plants (c–g). In the chimeras, whorls 2 and 3 exhibited a range of characteristics from wild type and mutant. Sepals and the lateral and ventral organs of whorl 2 have been removed to reveal the internal organs.

1992). By stage 5, *DEF* expression is found in all layers of petal and stamen primordia, but is absent from the central region of the meristem, destined to form carpels (Figure 2a). In *def-621*, no expression is detected at any stage (not shown).

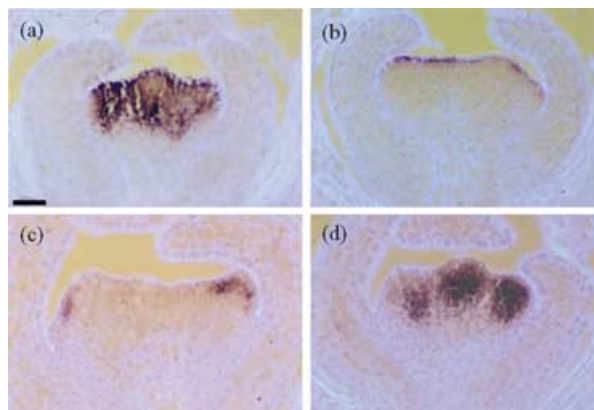


Figure 2. Expression of *DEF* in wild type and chimeras. In wild type (a), *DEF* was expressed in all three layers of the central dome. Note that the plane of section is not medial and is through cells destined to form petals and stamens. In the chimeras, strong *DEF* expression was restricted to L1 (b), L2 (c) or L2 and L3 (d). Scale bar, 100 μ m.

In the chimeras, three different patterns of *DEF* expression were found. In the first pattern, *DEF* was expressed only in the epidermal layer (L1) of the meristem dome at stages 3–4 (Figure 2b). At later stages, when the whorl 2 primordia were more advanced, expression could be seen in the epidermis of whorls 2 and 3 and also in subepidermal cells at the margins of whorl 2 organs. The subepidermal expression probably corresponded to regions of tissue derived from L1 by periclinal divisions (Perbal *et al.*, 1996; Satina, 1944). This L1 pattern of *DEF* expression was found in chimeras belonging to three of the five phenotypic categories: one near wild type (Figure 1c, comparable to the L1 chimera of Perbal *et al.*, 1996), one intermediate (Figure 1d) and one more extreme (Figure 1e).

The second pattern of expression, found in one phenotypic category (Figure 1g), consisted of strong *DEF* signal in L2, with little or no expression in L1 or L3 at stages 3–4 (Figure 2c). The third pattern, corresponding to a single phenotype (Figure 1f), consisted of strong *DEF* expression in both L2 and L3, with little or no expression in L1 at stages 3–4. This pattern is similar to that previously described for a L2/L3 chimera by Perbal *et al.* (1996) (Figure 2d).

Sequence of the excision alleles in the chimeras

The observation that three out of five phenotypic categories of plants were L1 chimeras suggests that the layer of *DEF* expression does not account for all aspects of phenotypic variation. One explanation is that the phenotypic differences reflect imprecise excision of Tam 3 from the *DEF* locus, leaving footprints that restore *DEF* function to various degrees. Revertant *DEF* alleles were therefore amplified by PCR from total DNA of the chimeras and the products directly sequenced.

The revertant allele amplified from the L1 chimera with the most wild-type phenotype had wild-type *DEF* sequence (Figure 3a), indicating that precise excision of Tam 3 had occurred, fully restoring *DEF* function in L1 (Figure 4c). This category was referred to as L1⁺. The intermediate L1 chimera had a 6-bp footprint at the Tam 3 excision site. This footprint encoded an additional Valine (V) and Threonine (T) (Figure 3c) and presumably only partially restored *DEF* gene function. This chimera was subsequently referred to as L1^a (Figure 4g). The PCR product from most extreme L1 chimera did not give a readable sequence, indicating that more than one type of Tam 3 excision event may have been present in the plant (Figure 4e). These results confirmed that the phenotypic variation observed within the L1 chimeras were due to different excision events of Tam 3 producing revertant alleles that restored *DEF* function to various degrees.

In L2 chimeras, two different revertant alleles were found, each containing 6-bp footprints. One was identical to the 6-bp footprint of the L1^a chimera and the chimera was there-



Figure 3. Sequence of *DEF* alleles. Sequence around Tam 3 excision site for wild-type *DEF* (a), the *def-621* allele carrying Tam 3 (b), imprecise excisions which partially restore *DEF* function recovered from L1 or L2 chimeras (c,d) and imprecise excision not restoring *DEF* function recovered from a L2⁺/L3 chimera (e). The target duplication caused by the insertion of Tam 3 (bold triangle) in *def-621* is indicated in capital letters. In this instance, Tam 3 inserted with a 6-bp duplication, but with an additional A to the right of the transposon. Alterations relative to *DEF* sequence are highlighted in bold. The predicted amino acid sequences are shown under each allele in the one-letter code.

fore referred to as L2^a (Figure 4h). The other, although it differed from the first in the 6th position, was predicted to give the same amino acid sequence (Figure 3d) and was also referred to as L2^a.

Polymerase chain reaction on the L2/L3 chimera did not give a readable sequence, presumably due to the presence of more than one excision event. However, an alternative approach to analysing the sequence in L2 was possible because of the appearance of more wild-type-looking fertile

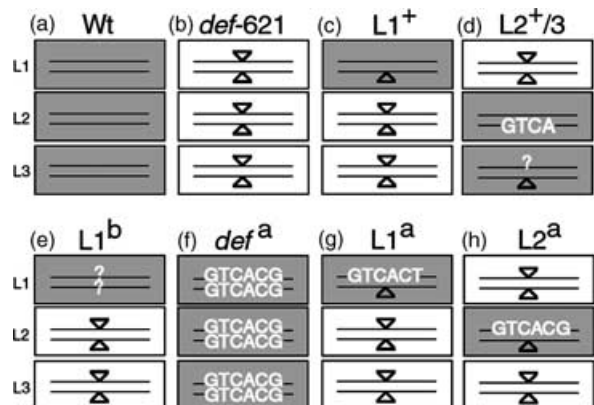


Figure 4. Summary of genotypes and *DEF* expression in the three meristem layers of wild type, *def-621*, *def^a* and the chimeras. Layers expressing *DEF* are shown in grey; known footprint sequences are given in white; white question marks indicate unknown excision events; *DEF* sequence is indicated by an uninterrupted black line and Tam 3 by a black triangle.

branches on the L2/L3 chimeras, which allowed self-seed to be collected. These derivatives expressed *DEF* not just in L2/L3 but also in L1 and were presumably caused by an additional excision event in L1. As the germline is derived from L2, the genotype of this layer could be determined by analysing the progeny from the fertile derivatives. These progeny segregated for wild type and mutant. Some of the wild types gave wild-type *DEF* gene sequence, while others did not give a readable sequence and were presumably heterozygotes. Sequencing the mutant progeny revealed a 4-bp footprint at the *DEF* locus, resulting in a frame shift and no *DEF* gene function (Figure 3e). Thus, the L2/L3 chimera was inferred to be heterozygous for two sequences in its L2: a wild-type allele (precise excision), presumably responsible for partially restoring petal development and a mutant allele caused by imprecise excision. The chimera was subsequently referred to as L2⁺/L3 (Figure 4d).

The phenotype of *DEF* chimeras thus depends on two factors: the layer expressing *DEF* and the nature of the excision allele. To analyse the individual contributions of these factors, the size, shape, development and morphology of whorls 2, 3 and 4 was determined. This was initially done for chimeras carrying the wild-type allele. The findings confirm the results obtained for an independent set of chimeras in a different genetic background by Perbal *et al.* (1996), but extend the analysis through descriptions of organ shapes and SEMs of development and cell-types.

Phenotype and development of plants carrying wild-type alleles

Whorl 2. To determine the morphology of all five whorl 2 organs, several flowers from each phenotypic category were dissected, flattened out between two glass plates and the images scanned into a computer (right-hand part of Figure 5). In the L1⁺ chimera, the length of whorl 2 organs was about 70% of wild type while width was not greatly affected (Figure 5c). In the L2⁺/L3 chimera, whorl 2 organs showed a greater reduction in both length and width compared to wild type (Figure 5d). Thus, the overall area of whorl 2 organs is affected most by *DEF* activity in L1, although expression in L2/L3 also has an effect.

The unification of the corolla and degree of lobing of whorl 2 organs was also studied. Wild-type *Antirrhinum* petals are united for part of their length to form a corolla tube with five lobes, while in *def-621*, the corolla is replaced by five separate sepals. This distinction between wild type and mutant was readily seen by stage 7 (compare Figure 6a,ii,b,ii). Similar to wild type, in L1⁺ chimeras the proximal regions of the organs were united to form a tube, while the distal regions were greatly expanded to form five separate lobes (Figure 1c). In contrast, the whorl 2 organs of

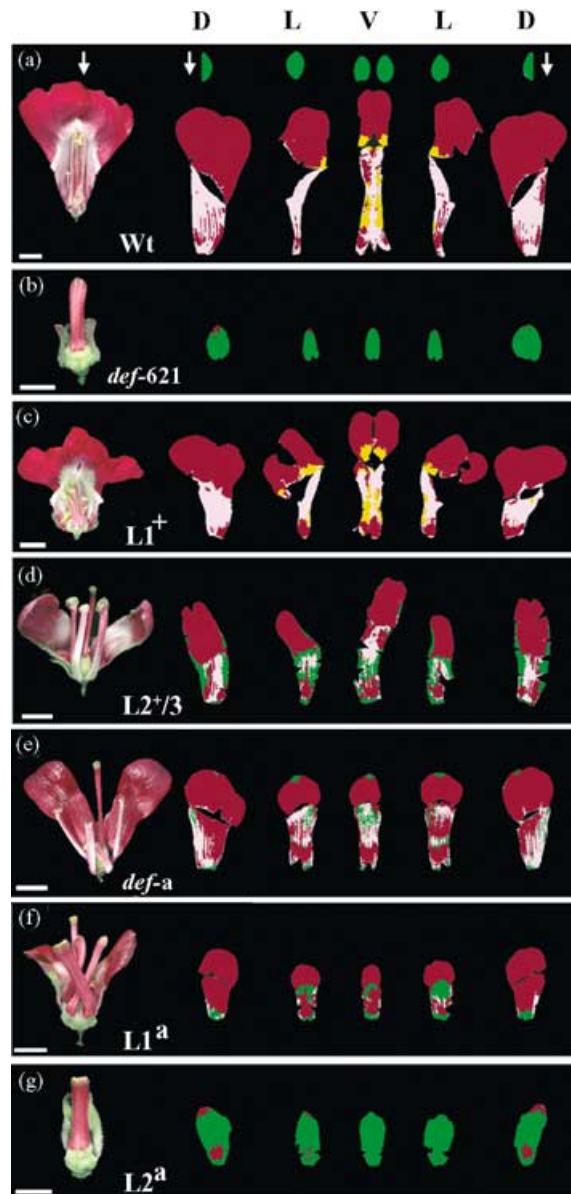


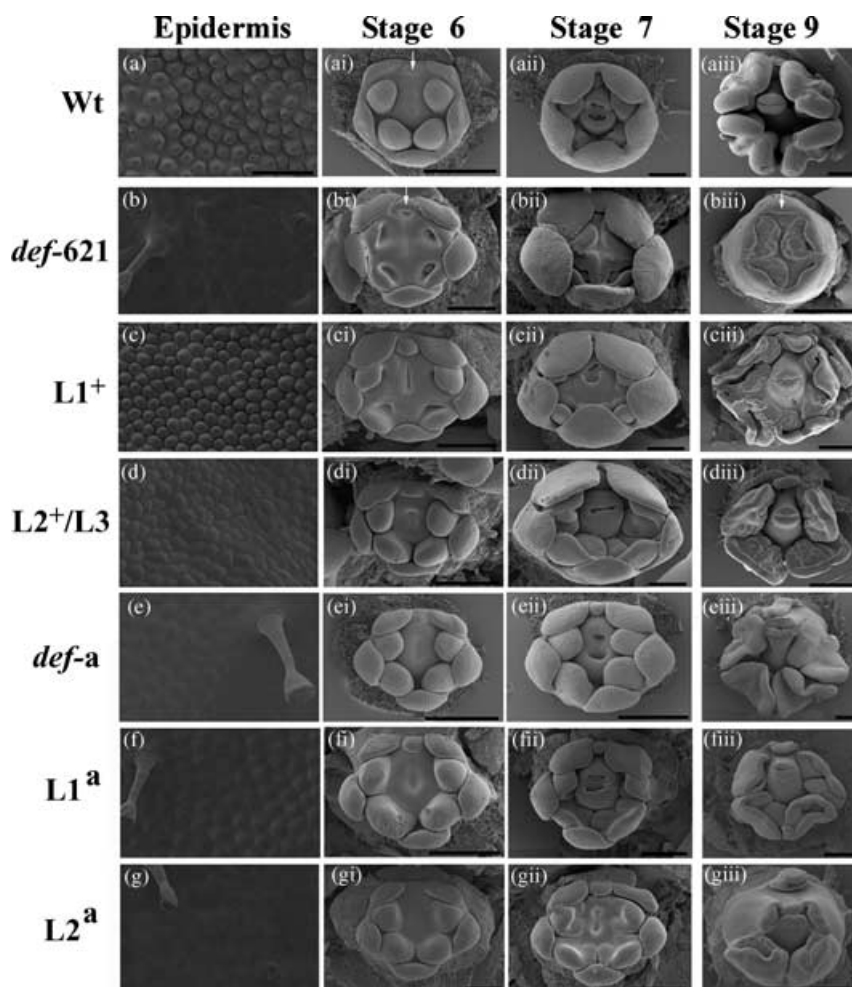
Figure 5. Phenotypes of chimeras and *def*^a compared to wild type and *def-621*.

Left shows mature flowers from which sepals and the lateral and ventral organs of whorl 2 have been removed. In the right-hand part of the figure, dorsal (D), lateral (L) and ventral (V) organs have been dissected and flattened out to reveal their adaxial surfaces. Pigmentation was enhanced in Adobe Photoshop so that regions of strong anthocyanin are shown in red; weak anthocyanin in pink; chlorophyll in green and aurone pigmentation in yellow. Panel (a) shows sepals of whorl 1 (above) and petals of whorl 2 (below) of wild type. The arrows indicate the dorsal midline. In panels b–g, only whorl 2 is shown. Scale bar, 5 mm.

the L2⁺/L3 chimera showed wider and deeper divisions between each organ (Figure 6d,ii) and the distal regions were not expanded to any great degree.

Cell morphology of mature whorl 2 organs was also investigated by light and scanning electron microscopy (SEM). The adaxial epidermis of wild-type petal lobes contains

Figure 6. SEM analysis of chimeras and *def*^a compared to wild type and *def-621*. Left column shows distal adaxial surface of whorl 2 organs. Remaining columns show floral meristems at stages 6, 7 or 9. Meristems are orientated such that the ventral organs are at the bottom of the picture. Whorl 1 was removed for all meristems and whorl 2 from stage 9 meristems. Arrows indicate dorsal organ in whorl 3. Scale bar: a–g, 100 μ m; ai–gi, aii–gii, 200 μ m; aiii–giii, 500 μ m.



conical cells (Figure 6a) that are highly pigmented with anthocyanin. The whorl 2 sepals of *def-621* show little or no pigmentation in the epidermis and the adaxial surface has stomata and glandular hairs, all characteristics typical of wild-type sepals (Figure 6b).

The distal regions of $L1^+$ organs had an epidermis that was typical of wild-type petals, consistent with *DEF* activity having been fully restored to $L1$ (Figure 6c). In $L2^+/L3$ chimeras, the cells of the adaxial epidermis were flattened rather than conical shaped, but no stomata or glandular hairs were found, indicating, along with the pale anthocyanin pigmentation, non-autonomous effects of *DEF* on the epidermis from the underlying wild-type $L2$ (Figure 6d).

Whorl 3. Whorl 3 of wild type comprises four separate stamens (two ventral and two lateral) and an arrested dorsal stamen (stamenode, arrowed in Figure 6ai). In *def-621*, from stage 6 onwards, five carpel primordia were seen in place of wild-type stamen primordia (Figure 6bi–iii). The dorsal primordium was smaller than the other carpel primordia (arrowed in Figure 6bi), although unlike the stamenode of wild type, its development was not

normally arrested (arrowed in Figure 6biii). By stage 9, the carpel primordia of whorl 3 were visibly united (Figure 6biii).

In $L1^+$ chimeras, early development of whorl 3 was more similar to *def-621* than wild type but unlike the situation in *def-621*, development of the dorsal primordium was eventually arrested and the styles were not united (Figure 6ciii). In $L2^+/L3$ chimeras, development of whorl 3 was as wild type until stage 7, when normal anther thecae failed to develop in the chimera (compare Figure 6aii,dii). The dorsal primordium was retarded, as wild type, but eventually developed more fully into a thin strap of carpeloid tissue. The other primordia formed four separate stamen-like organs (Figure 1f) with ovules and stigmatic tissue on their adaxial surface (Figure 6diii).

Thus, in contrast to whorl 2, *DEF* activity in $L1$ is unable to restore near wild-type identity to whorl 3, although it can arrest the most dorsal primordium and prevent united growth of the styles. Expression of *DEF* in $L2/L3$ can partially restore stamen identity to the organs of whorl 3 and prevent united growth, but not completely arrest development of the dorsal organ.

Whorl 4. Development of a wild-type flower terminates with the production of two united carpels in whorl 4. By contrast, in *def-621* the flower is usually terminated after whorl 3. This difference was detectable at stage 6, when carpel primordia were visible in the centre of a wild-type floral meristem but not in the mutant (compare Figure 6ai,bi). In both the L1⁺ and L2⁺/L3 chimeras, the fourth whorl was restored (Figure 6ciii,diii).

*Phenotype and development of plants carrying the imprecise *def^a* allele*

To evaluate the effects of the imprecise *def^a* allele expressed in various layers, we first had to determine the phenotype of plants carrying the allele in all three layers. To achieve this, the L2^a chimera was crossed to wild type and the F₂ analysed. The progeny segregated for wild type and mutants with a weak *def* phenotype (*def^a* homozygotes).

In plants homozygous for the *def^a* allele, whorl 2 organs were reduced in both width and length (compare right-hand part of Figure 5a,e). As in strong *def* mutants, whorl 2 organs of *def^a* homozygotes were not usually united (Figure 6eii). The distal regions of the whorl 2 organs formed lobes, although they were not as highly expanded or distinct as wild-type petal lobes (Figure 5e). SEMs revealed that the epidermis had flat cells and glandular hairs (Figure 6e). Thus, impairing *DEF* activity in all three layers, through the *def^a* allele, reduces the overall length and width of whorl 2 organs and prevents united growth and the establishment of wild-type cell morphology. However, it does not prevent petal lobing or pigmentation.

Whorl 3 of *def^a* homozygous plants contained four separate stamen-like structures with thick filaments (Figure 5e, left). Their development was indistinguishable from wild type until stage 7 when thecae failed to develop in the mutants (compare Figure 6aii,eii). The mature whorl 3 organs produced no pollen and had ovules on their adaxial surface. The most dorsal primordium of whorl 3 was retarded like wild type. Thus, even though *DEF* activity was impaired by the *def^a* allele, the shape and size of all the whorl 3 organs resembled wild type, although they were infertile. Whorl 4 appeared to develop as wild type in *def^a* homozygotes (Figure 6ei–iii).

*Phenotype and development of plants chimeric for the *def^a* allele*

The area of whorl 2 organs in the L1^a chimera was reduced relative to *def^a* homozygotes, much as that of the L1⁺ chimera was reduced relative to wild type (compare Figure 5a,c,e,f). The L2^a chimera had five sepal-like organs in whorl 2 which were slightly larger than those of *def-621* (Figure 5b,g). The distal regions of the whorl 2 organs of

the L1^a chimera formed lobes comparable to those of *def^a* homozygotes, whereas in the L2^a chimera there appeared to be no distinct lobes. Thus, as for the wild-type allele, the overall area and lobing of whorl 2 organs is affected most by *DEF* activity in L1.

The distal regions of whorl 2 organs in the L1^a chimera were comparable with those of *def^a* homozygotes with respect to anthocyanin pigmentation and cell morphology (compare Figures 5e,f and 6e,f). In the L2^a chimera, anthocyanin pigmentation was restricted to epidermis above the veins in proximal regions and along the organ margins in distal regions of whorl 2 organs. Stomata and glandular hairs were present on the adaxial epidermis, typical of wild-type sepals and *def-621* (compare Figure 6b,g). Thus, restoring *def^a* function to L1 could rescue the *def^a* epidermal cell morphology in this layer (comparable to the restoration of the wild-type epidermal cell morphology by the wild-type allele in the L1⁺ chimera). However, restoring *def^a* function to L2 did not restore epidermal cell morphology or pigmentation to that of *def^a* homozygotes. Thus, in the context of *def^a*, there was no indication at the phenotypic level of non-autonomous effects of the L2 on L1, unlike the situation with wild-type *DEF* alleles. This may reflect the much weaker effects of the *def^a* allele.

As with *def^a* in all three layers, whorl 3 primordia initially looked like stamens in the L1^a chimera and there was retardation of the dorsal primordium (Figure 6fi). However, at later stages, in contrast to *def^a* mutants, whorl 3 organs of the L1^a chimera resembled carpels more than stamens (Figure 6fii–iii). In the L2^a chimera, five carpel primordia could be seen in whorl 3, from stage 7 onwards (Figure 6gii–iii). Thus, as with the wild-type *DEF* allele, activity of *def^a* is required in all three layers to give stamenoid morphology to whorl 3. However, expression of *def^a* in L1 did restore production of four separate organs in whorl 3 and a more stamen-like appearance at earlier stages. This is surprising as even the wild-type *DEF* allele was unable to do this when expressed in L1 (compare Figure 6ci,fi) and indicates that interactions between layers and levels of gene activity may not be additive in all cases.

Whorl 4 developed as wild type in the L1^a and the L2^a chimeras (Figure 6fi–iii,gi–iii). Thus, even partial restoration of *DEF* function by the *def^a* allele in L1 or L2 was enough to promote carpel formation in whorl 4 and the wild-type determinacy of the flower.

Pattern of GLO expression in the wild-type allele chimeras

Differences between the development of wild type and chimeras first became apparent after stage 6, the point at which the *DEF/GLO* heterodimer is thought to be required to maintain *DEF* and *GLO* transcript levels (Zachgo *et al.*, 1995). To investigate whether these differences were accompanied by altered patterns in *GLO* expression, *in situ*

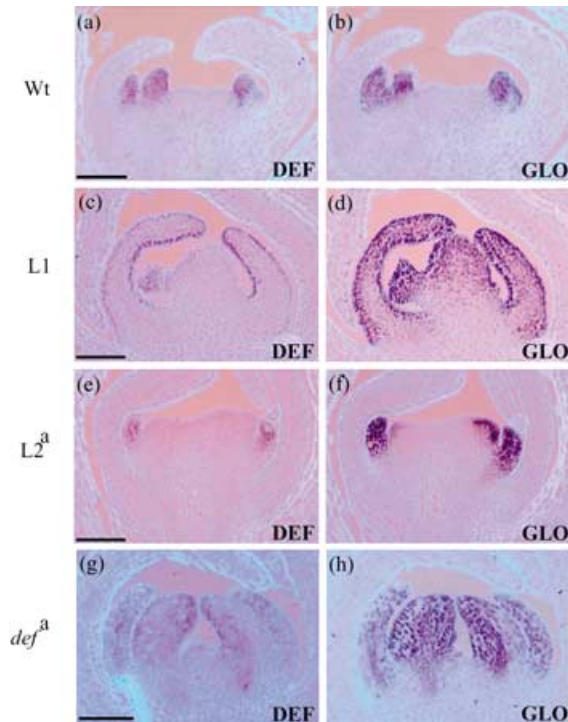


Figure 7. Expression patterns of *DEF* and *GLO* in wild-type, chimeras and *def^a* homozygotes.

Consecutive sections were probed with digoxigenin-labeled *DEF* or *GLO* antisense RNA. In wild-type floral meristems, *DEF* and *GLO* expression coincides in all layers of whorls 2 and 3. In the L1 chimeras (L1⁺ shown in c–d), strong *GLO* expression became restricted to L1-derived cells, the epidermis and organ margins of whorls 2 and 3. In the L2^a chimera, strong *GLO* expression was found in all layers of whorl 2, but was restricted to subepidermal regions of whorl 3 (e, f). In *def^a* homozygotes, *DEF* expression was weaker than that in wild type, but *GLO* expression appeared to be at normal levels. Scale bar, 100 μ m.

hybridisation was performed on consecutive sections of wild-type and chimeric material, using *DEF* and *GLO* RNA probes.

In wild type, the expression patterns of *DEF* and *GLO* coincide in all three layers of developing petals and stamens (Figure 7a,b). In the chimeras, irrespective of which layer expressed *DEF*, *GLO* expression was as wild type up to stage 6 (not shown). Differences in the pattern of *GLO* expression only emerged after the presence of *DEF* was required to maintain normal levels of *GLO* expression. In the L1⁺ chimera after stage 6, strong *GLO* expression became restricted to L1-derived tissue: high levels of expression could be seen in the epidermis and in internal layers at the margins of whorl 2 organs (Figure 7d). Low levels of *GLO* expression observed in non-L1 derived tissue could represent basal levels of *GLO* that cannot be up-regulated in the absence of *DEF*.

In the L2⁺/L3 chimera after stage 6, *GLO* expression was detected in all three layers of developing whorl 2 organs, consistent with the non-autonomous effects of *DEF* on L1

from L2 (Figure 8a,b,f,g). However, in whorl 3 primordia, *GLO* expression was strongest in L2/L3 (Figure 8b,g).

To explore this difference between whorls 2 and 3, expression of the floral meristem identity gene *FLORICAULA* (*FLO*) was also examined. In wild-type floral meristems, *FLO* is not detected at any stage in developing stamens (Figure 8d,i; Coen *et al.*, 1990). However, in the L2⁺/L3 chimera *FLO* was found to be expressed in L1 of the third whorl organs, corresponding to the region where *GLO* expression was reduced (arrowed in Figure 8c,h). This ectopic *FLO* expression correlated with feminisation of the whorl 3 organs, where ovules and stigmatic tissue could be found on the adaxial surface of the anther sacs (Figure 1f). This indicates that in the L2⁺/L3 chimera *DEF*/*GLO* is not established at sufficient levels in L1 to antagonise expression of *FLO* in whorl 3.

In plants homozygous for *def^a*, expression of *DEF* was reduced compared to wild type, while *GLO* expression appeared to be as wild type (Figure 7g,h). Despite this, *FLO* was ectopically expressed in stage 6–7 stamen primordia (Figure 8e,j). This suggested that the *def^a* allele was capable of upregulating and maintaining *GLO* expression in whorls 2 and 3, but not of completely repressing *FLO* expression in whorl 3. Expression patterns of *GLO* found in the L1^a and the L2^a chimeras were comparable to those found in the L1⁺ and the L2⁺/L3 chimeras, respectively (Figure 7d,f).

Discussion

By comparing the effects of expressing two different *DEF* alleles in various layers, we have shown that each layer makes a distinctive contribution to organ development. These contributions are different for whorls 2 and 3.

The two alleles used for comparison were the wild-type *DEF* allele and *def^a*, an allele which arose by imprecise excision of Tam 3. The *def^a* allele carries a two amino acid insertion in the N-terminal half of the MADS domain, adjacent to conserved residues that are thought to make direct contact with DNA (Riechmann and Meyerowitz, 1997). It therefore most likely produces a protein with reduced DNA binding activity. Plants homozygous for *def^a* have organs intermediate between petals and sepals in whorl 2. Organs in whorl 3 have a stamen-like morphology but are infertile and bear ovules on their adaxial surface. The phenotype is comparable to that conferred by *def^{nic}*, which also has an amino acid change in the DNA-binding region of the MADS domain, resulting in a loss of DNA-binding activity *in vitro* (Schwarz-Sommer *et al.*, 1992).

For both *DEF* and *def^a* alleles, restricting expression to L1 has a similar effect in slightly reducing whorl 2 organ area, while the pattern of lobe development and epidermal cell types is similar to that seen in plants expressing the allele in all layers. By comparison, restricting expression to

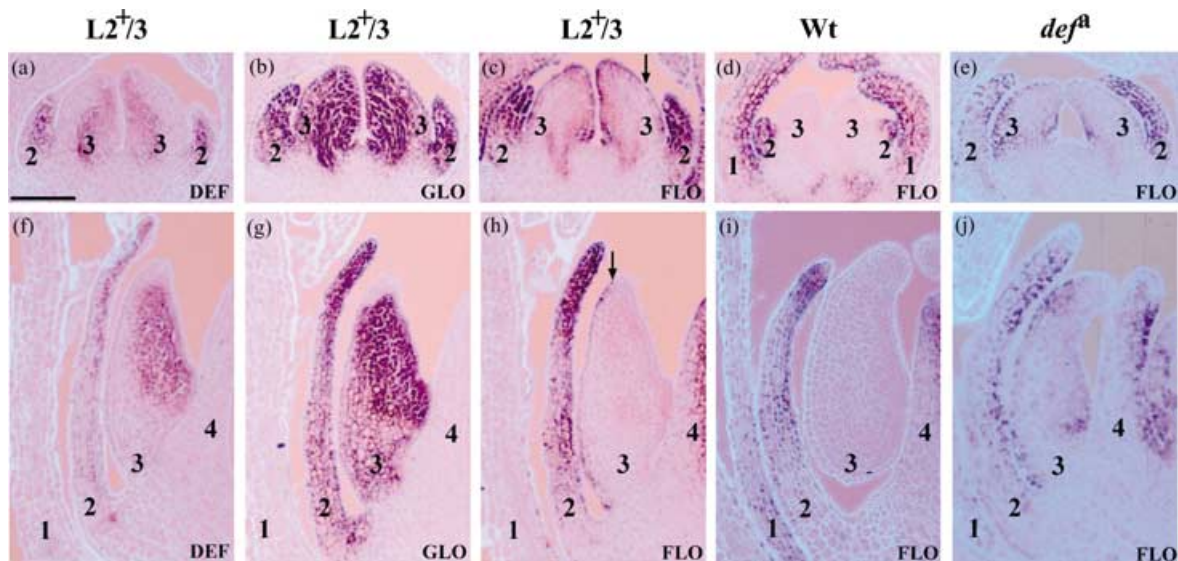


Figure 8. Expression patterns of *DEF*, *GLO* and *FLO* in the $L2^+/L3$ chimera, wild type and *def^a* homozygotes.

Consecutive sections were probed with *DEF*, *GLO* or *FLO* antisense RNA. In the $L2^+/L3$ chimera (a,f), strong *DEF* expression was restricted to L2 and L3 in whorls 2 and 3. Expression of *GLO* was found in all three layers of whorl 2, but was absent or only very weakly expressed in L1 of whorl 3 (b,g). In wild-type floral meristems, *FLO* was expressed in whorls 1, 2 and 4 and was absent from whorl 3 (d,i). In the $L2^+/L3$ chimera, ectopic expression of *FLO* was found in whorl 3 (arrowed in c,h). Ectopic expression of *FLO* was also found in *def^a* (e,j). Scale bar, 100 μ m.

subepidermal layers results in a greater reduction in organ area and degree of lobing.

The results show that *DEF* expression in L1 makes a greater contribution to overall morphology of whorl 2 organs than does expression in underlying layers, irrespective of the allele. This implies that *DEF* target genes in L1 are largely responsible for organ growth properties. The size difference between wild-type petals and sepals mainly reflects differences in the duration rather than the rate of growth (Vincent and Coen, in preparation), suggesting that some of these target genes are involved in the timing of growth arrest.

Although *DEF* in L1 chimeras influences target genes primarily in the epidermis, it also affects underlying layers as these grow along with the epidermis in L1 chimeras. This is unlikely to be caused by movement of the *DEF* protein, as no trafficking of *DEF* from L1 into subepidermal layers has been detected (Perbal *et al.*, 1996). Nor is it accounted for by the contributions of L1 to subepidermal layers by periclinal divisions at the organ margins, as plants expressing *DEF* under the control of an epidermis-specific promoter show similar growth properties to L1 chimeras (Efremova *et al.*, 2001). Rather, it appears to reflect downstream co-ordination between target gene activity in the epidermis and underlying cells. Similar conclusions have been arrived at from the analysis of plants expressing *AP3* in outer cell layers of *Arabidopsis* (Efremova *et al.*, 2001; Jenik and Irish, 2001).

DEF expression in subepidermal layers also restores some aspects of petal growth and pigmentation to whorl 2. This may reflect trafficking of *DEF* protein into L1 (Perbal *et al.*, 1996) and/or influence on target genes in L2/L3.

For whorl 3, expression of wild-type *DEF* in L1 alone is unable to rescue stamen development (Efremova *et al.*, 2001; Perbal *et al.*, 1996). This is not simply a consequence of reduced overall levels of *DEF* because *def^a* homozygotes have a weaker whorl 3 phenotype than $L1^+$ chimeras even though the whorl 2 phenotype is stronger than that of $L1^+$ chimeras. A possible explanation for the inability of *DEF* expression in L1 to restore stamen development is that the mutant subepidermal layers produce an inhibitor of *DEF* function that can move into the L1. The inhibitor would normally have to be counteracted by *DEF* in subepidermal cells at an early stage to restore stamen development, as *DEF* function is needed very early on to rescue whorl 3 (Zachgo *et al.*, 1995). Alternatively, *DEF* activity in L1 may play only a minor role in whorl 3 development.

Surprisingly, expression of *def^a* in L1 alone appears to show greater rescue of stamen development at early stages than expression of *DEF* in L1. According to the non-autonomous inhibitor model, this could be accounted for if the product of the *def^a* allele forms a complex whose action is more resistant to a subepidermal inhibitor. Thus, in $L1^a$ chimeras, although *def^a* allele is defective relative to *DEF*, its greater resistance to the inhibitor could allow it to function more effectively early on to promote stamen development.

As described previously, *DEF* expression in L2/L3 is able to restore stamen development to a greater degree than expression in L1 (Perbal *et al.*, 1996). This indicates that some features of stamen development depend on the activity of *DEF* target genes in L2/L3. Nevertheless, restoration is not complete as the whorl 3 organs are feminised,

possibly because the epidermal layers retain some carpel identity. This is consistent with the expression patterns of downstream genes: *FLO*, which is normally inhibited by the activity of B class genes in whorl 3 (Coen *et al.*, 1990), is ectopically expressed in the whorl 3 epidermis of L2⁺/L3 chimeras.

Expression of either *DEF* or *def*⁹ in L1 is sufficient to arrest dorsal organ development in whorl 3. In wild-type flowers, arrest of this organ depends on the combined effect of expressing BC class genes with *CYCLOIDEA* in dorsal regions of the meristem (Carpenter and Coen, 1990; Luo *et al.*, 1996). The results therefore indicate that with regard to *DEF* activity, this combination in L1 is able to act non-autonomously to bring about arrest in all layers, irrespective of the *DEF* allele. Retardation of the whorl 3 dorsal organ was also observed at early stages in L2⁺/L3 chimeras, although a thin strap-like carpel grew later on, indicating that *DEF* expression in these layers can bring about partial arrest.

Very little *DEF* activity appears to be needed for restoring development of whorl 4, as this occurs in all the chimeras, irrespective of the allele. The absence of whorl 4 in *DEF* mutants is thought to reflect a role of B class genes in preventing premature arrest of the floral meristem (Bowman *et al.*, 1992; Krizek and Meyerowitz, 1996; Schultz *et al.*, 1991; Zachgo *et al.*, 1995). The results presented here indicate that the targets of this process are very sensitive to expression of *DEF*.

In summary, expression of *DEF* in different cell layers of the meristem makes characteristic contributions to floral development, irrespective of the allele. In whorl 2, expression in L1 plays the more important role in controlling the overall growth and morphology of the organs. It is possible that growth properties of all organs with an extended lamina, such as petals or leaves, is determined primarily by gene activity in epidermal layers, which sets the size of the outer skin, and this is co-ordinated with growth of internal cells through signalling. According to this view, genes such as *LAM1* in tobacco, which can modify leaf growth when expressed in subepidermal layers (McHale and Marcotrigiano, 1998), may act through the co-ordination step. In whorl 3, the subepidermal layers appear to play a greater role in controlling morphology. This may mean that the L1 is less important for growth of these organs, which lack an extended lamina. Alternatively, it could reflect production of a non-autonomous inhibitor that prevents expression of *DEF* in L1 from exerting its effects.

Experimental procedures

Antirrhinum stocks and scanning electron microscopy

The mutant *def-621* was derived from stock 98 (*niv*^{rec-98}), through a large-scale transposon mutagenesis program at the John Innes

Institute (Carpenter and Coen, 1990). Cuttings were taken from flowering spikes showing altered phenotypes as described in Carpenter *et al.* (1995). SEM was carried out on plastic replicas of flowers and developing meristems as described in Green and Linstead (1990).

In situ hybridisation

Methods of digoxigenin labeling of RNA probes, tissue preparation and *in situ* hybridisation were as described in Bradley *et al.* (1993). Probes to detect *FLO* and *GLO* were prepared as described in Coen *et al.* (1990) and Tröbner *et al.* (1992), respectively. The probe used to detect the *DEF* transcript was prepared from pJAM 2140, an 84-bp *DEF* cDNA fragment upstream of the transposon insertion site.

DNA and PCR analysis

DNA extractions were carried out as described in Ingram *et al.* (1997). PCR was carried out in a programmable thermal controller (PTC-100 MJ Research Inc.). Reactions were made in 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM MgCl₂; 0.01% (w/v) gelatin; 0.2 mM each of dATP, dCTP, dGTP, and dTTP; 0.002% (w/v) Tween 20. Sixty nanograms of DNA and 2U Taq polymerase were added per 50 µl reaction. Samples were heated to 95°C before addition of polymerase to minimise primer dimerisation. Reactions were overlaid with 20 µl mineral oil and 35 cycles of 30 sec at 95°C, 2 min at 55°C and 40 sec at 72°C, were performed. Samples were then heated to 72°C for 10 min before being stored at 4°C. Two primers specific for the *DEF* gene upstream of the Tam 3 insertion site were used: (i) 5'-CCT ATC ACA GTT TTA GGA AAG-3'; (ii) 5'-GGA GAG AAA GGA AAC TGG-3'. Two primers specific for the *DEF* gene downstream of the Tam 3 insertion site were used: (iii) 5'-GTG AAG CTT CTG AGT ACT TGG-3'; (iv) 5'-GTA CGT ACG CAG TTG TTG GG-3'. Primers specific for the left- and right-hand ends of Tam 3 were: (v) 5'-CAC GGC CCA ATT CAC ATC TTT-3'; (vi) 5'-CTC GGC ACG TTT CAC ATC TTT A-3'. PCR products were electrophoresed in 0.8% (w/v) agarose gels, purified by Wizard columns (Promega) and then sequenced with the ABI automatic sequencer (Perkin Elmer) using oligos B + D.

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