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Non-cell-autonomous function of the *Antirrhinum* floral homeotic proteins *DEFICIENS* and *GLOBOSA* is exerted by their polar cell-to-cell trafficking

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SUMMARY

In *Antirrhinum majus*, petal and stamen organ identity is controlled by two MADS-box transcription factors, DEFICIENS and GLOBOSA. Mutations in either of these genes result in the replacement of petals by sepaloid organs and stamens by carpelloid organs. Somatically stable *def* and *glo* periclinal chimeras, generated by transposon excision events, were used to study the non-cell-autonomous functions of these two MADS-box proteins. Two morphologically distinct types of chimeras were analysed using genetic, morphological and molecular techniques. Restoration of *DEF* expression in the L1 cell layer results in the re-establishment of DEF and GLO functions in L1-derived cells only; inner layer cells retain their mutant sepaloid features. Nevertheless, this activity is sufficient to allow the expansion of petal lobes, highlighting the role of DEF in the stimulation of cell proliferation and/or cell shape and elongation when expressed in the L1 layer. Establishment of DEF or GLO expression in L2 and L3 cell layers is accompanied by the recovery of petaloid identity of the epidermal cells but it is insufficient to allow petal lobe expansion. We show by in situ immunolocalisation that the non-cell-autonomy is due to direct trafficking of DEF and GLO proteins from the inner layer to the epidermal cells. At least for DEF, this movement appears to be polar since DEF acts cell-autonomously when expressed in the L1 cell layer. Furthermore, the petaloid revertant sectors observed on second whorl mutant organs and the mutant margins of petals of L2L3 chimeras suggest that DEF and GLO intradermal movement is limited. This restriction may reflect the difference in the regulation of primary plasmodesmata connecting cells from the same layer and secondary plasmodesmata connecting cells from different layers. We propose that control of intradermal trafficking of DEF and GLO could play a role in maintaining of the boundaries of their expression domains.

Key words: flower development, MADS-box proteins, periclinal chimeras, immunolocalisation, *Antirrhinum, DEFICIENS, GLOBOSA*

INTRODUCTION

Organogenesis during plant development is initiated by an increased rate of division of cells located in certain regions of the apical meristems. In most angiosperms, meristems are composed of three cell layers (Satina et al., 1940; Sussex, 1989). In the outermost layer (L1), cells primarily divide anticlinally and give rise to the epidermis. Occasionally, L1 cells divide periclinally to form the subepidermal tissue, such as at the margins of petals (Satina, 1944). In the L2 layer, cells divide anticlinally within the meristem and in all planes during organogenesis. Cells of the L3 layer divide in all planes. The proportional contribution of the L2 and L3 layers to the plant body varies in the different organs (Dermen, 1953; Huala and Sussex, 1993). Inflorescence meristems retain the same layering as vegetative meristems; however, normally, only L2 cells give rise to the germinal cells (Stewart, 1978).

Elaboration of the different types of organs requires co-ordination between the three cell layers. Studies on periclinal chimeras in which the genetic constitution of one layer differs by one or more characters from the others showed the ability of one or two layers to influence the behaviour of the other(s). For example, the L3 layer appeared to control the size of the meristem and the number of carpels in tomato intraspecific chimeras (Szymkowiak and Sussex, 1992). In another experiment, the restoration of Lateral Suppressor (LS) activity in the L2 and L3 layers of tomato (Szymkowiak and Sussex, 1993) was sufficient to direct the formation of petals in the second whorl, even though L1 cells still carried the *ls* mutation. The co-ordination of growth patterns within the three cell layers suggests the transmission of signals between layers. In the above-mentioned examples, the nature of the signal has not been further investigated, and it is not known whether communication between cells involves a ligand and a corresponding membrane-bound receptor or whether it involves other mechanisms.

Plant cells are interconnected by plasmodesmata which potentiate the symplastic transfer of molecules between cells (Lucas et al., 1993; Mezitt and Lucas, 1996). Primary plasmodesmata are formed during cytokinesis and consequently establish con-

nections between daughter cells within the L1, L2 or L3 layers. Communication between cell layers is subsequently established by the formation of secondary plasmodesmata. Plasmodesmata have been traditionally considered as static entities mediating the transport of small molecules. However, the novel concept of dynamic plasmodesmata has now emerged. The plasmodesmal size exclusion limit can be modified under stress and physiological conditions leading to the obstruction, restriction or enhancement of the trafficking of molecules. Furthermore, it is becoming established that plasmodesmata allow the trafficking of macromolecules such as proteins and nucleic acids (Lucas et al., 1995, 1993). The first evidence for the trafficking of an endogenous protein was provided by studies performed on a maize transcription factor, KNOTTED1 (KN1), whose trafficking via plasmodesmata was demonstrated by microinjection experiments (Lucas et al., 1995). The ability of the KN1 protein to move from cell-to-cell would explain its localisation in the nuclei of both L2 and L1 cells, while *KN1* gene expression is restricted to L2 cells of the apical meristem. KN1 was also shown to mediate the selective transport of its own transcript, although the biological relevance of these observations remains unclear. These data raise the question whether trafficking of macromolecules, including transcription factors, could play an important role during plant development by synchronising gene expression in cells in different layers. Recent studies with *floricaula* (*flo*) (Carpenter and Coen, 1995; Hantke et al., 1995) and *pistillata (pi)* (Bouhidel and Irish, 1996) periclinal chimeras in *Antirrhinum* and *Arabidopsis*, respectively, have shown that both transcription factors have non-cell-autonomous functions in the flower. It will be important to determine whether the FLO and PI proteins can also traffic from cell-to-cell via plasmodesmata.

In *Antirrhinum*, the *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*) genes encode MADS box transcription factors that interact to control petal and stamen organ identity in the flower (Schwarz-Sommer et al., 1992; Sommer et al., 1990; Tröbner et al., 1992). The DEF and GLO proteins form a heterodimer which binds specifically to DNA not only to control the activation of potential target genes but also to maintain the level of their own transcription (Schwarz-Sommer et al., 1992; Tröbner et al., 1992). This autoregulatory control is established once all the organ primordia have been initiated (Zachgo et al., 1995). The heterodimerisation between DEF and GLO was shown to be important for the stability of both partners (Zachgo et al., 1995).

The *def*-gli (Schwarz-Sommer et al., 1992; Sommer et al., 1990) and *glo-1* (Tröbner et al., 1992) mutant alleles described in previous reports are due to the insertion of a transposon. These mutations result in the homeotic transformation of petals to sepaloid organs and stamens to carpelloid organs. In plants carrying such genetically unstable genes, reversion events may occur, thus restoring wild-type *DEF* or *GLO* gene function in a few cells, phenotypically revealed by the presence of petaloid sectors (Carpenter and Coen, 1990; Sommer et al., 1990, 1991). The sharp boundaries delimiting these sectors may suggest that DEF and GLO act cell-autonomously.

In this report we show, using somatically stable periclinal chimeras, that two floral regulators, DEF and GLO, can control organ identity by acting interdermally in a non-cell-autonomous fashion. We also provide evidence for DEF and GLO protein trafficking which, at least for the DEF protein, is polar and occurs from the inner layer cells to the epidermis. The analysis of these chimeras also highlights the role of DEF in the stimulation of cell division and/or in the control of cell shape when expressed in the L1 cell layer.

MATERIALS AND METHODS

Plant material

Seeds of the *def*-gli and *glo-1* genetic stocks were obtained from the Gatersleben seed collection. The temperature sensitive *def-101* mutant used to confirm the genotype of plants in this report was obtained in a transposon mutagenesis experiment (Schwarz-Sommer et al., 1992).

Flowers heterozygous for *def*-gli or *glo-1* alleles were selfed and homozygous mutant plants were propagated vegetatively. Several plants of the mutant progenies showed revertant sectors of variable size. In some plants, branches appeared which displayed an unusual and uniform floral phenotype. Vegetatively propagated cuttings of these branches maintained, despite slight variations, their uniform phenotype over seven years.

In situ mRNA hybridisation

Tissue preparation and in situ hybridisation experiments were carried out as previously described (Huijser et al., 1992) with the following modifications: digoxigenin-labelled MADS-box-less RNA probes were prepared using the Boehringer Mannheim nucleic acid labelling kit, following the manufacturer's instructions. Detection was performed using a secondary anti-digoxigenin antibody conjugated to alkaline phosphatase, and with nitro blue tetrazolium chloride and 5-bromo-4-chloride-3-indolyl-phosphate as substrates giving rise to a dark blue precipitate. Cell walls were stained with calcofluor.

In situ immunolocalisation experiments with affinity-purified polyclonal antibodies directed against the MADS-box-less DEF and GLO proteins were carried out according to the method of Zachgo et al. (1995).

Photographs in Figs 2 to 5 were scanned with the FotoLook programme, and were processed and assembled using Adobe Photoshop.

RESULTS

Antirrhinum wild-type flowers comprise four whorls. From the outermost to the innermost whorls, the flowers are composed of five sepals, a corolla made of five petals, four stamens and a stamenodium, and two fused carpels (Fig. 1A).

The *def*-gli allele is a null allele due to the insertion of the *Tam*7 transposable element in the third intron of the *def* gene (Sommer et al., 1990, 1991). As a result of the absence of DEF functions, the identity of the second and third whorls is affected. In the second whorl, five sepaloid organs develop instead of five petals. In the third whorl, five fused carpelloid organs, tipped with stigmatic tissue develop, representing the four feminised stamens and the stamenodium. In addition, the fourth whorl carpels do not develop (Fig. 1B). Due to excision of the transposon, wild-type sectors of variable size can be observed on the second whorl organs (Fig. 1B).

On branches of some of the *def*-gli plants, flowers appeared with uniform phenotypes which displayed features intermediate between the *def*-gli and wild-type flowers. Branches of two plants displayed an irregularly formed corolla with otherwise wild-type-like petals (Fig. 1C) and five plants gave rise to branches with small flowers whose second whorl organs were rimmed by a green sepaloid tissue (Fig. 1D). During vegetative

propagation slight variations in the phenotype could be observed, mainly affecting the phenotypic appearance of the third whorl organs. One representative plant for each of these two types of somatically stable revertants was used for the studies presented in the following sections.

The genetic constitution of these plants as periclinal chimeras was determined by the genetic and molecular studies described below. For clarity we designate them as L1 (Fig. 1C) and L2L3 (Fig. 1D) chimeras throughout the entire text, thereby indicating the layer(s) which carries the wild-type allele.

Genetic analysis of *def* chimeras

For genetic analysis of the vegetatively stable *def* chimeras, flowers were crossed with flowers carrying the genetically stable *def*-101 allele. The progenies were then grown at 25°C, a temperature at which *def*-101 flowers display a phenotype similar to that of *def*-gli flowers.

The mutant phenotype of the L1 and L2L3 chimeras was not heritable, suggesting that the aberrant morphology of the flowers was not related to a structural alteration other than excision of the Tam7 element within the *DEF* gene. Indeed, PCR amplification of the genomic DNA of *def* chimeras produced PCR products characteristic of the *def*-gli and the wild-type alleles for both types of chimeras, demonstrating that the wild-type gene was restored in some cells (not shown).

Inheritance of the reversion events in the germinal progeny of genetic chimeras is observed only when excision of the transposon occurs in cells of the L2 layer. The progeny resulting from the cross between *def*-101 and L2L3 chimeras whose second whorl organs displayed green rims (Fig. 1D) segregated wild type to mutants in an approximately 1:1 ratio (13:28, 8:16) suggesting that at least the germinal cells were heterozygous for *def*-gli and the restored wild-type *DEF* allele. In contrast, no wild-type plants were obtained in the progeny from the cross with *def* chimeras showing petaloid type revertant flowers (L1 chimeras, see Fig. 1C) indicating that cells in the L2 layer were genetically mutant. We would like to mention here that segregation results obtained with a vegetative progenitor of the chimeras and a heterozygote *def*-gli/*DEF* male parent were in agreement with the data shown above.

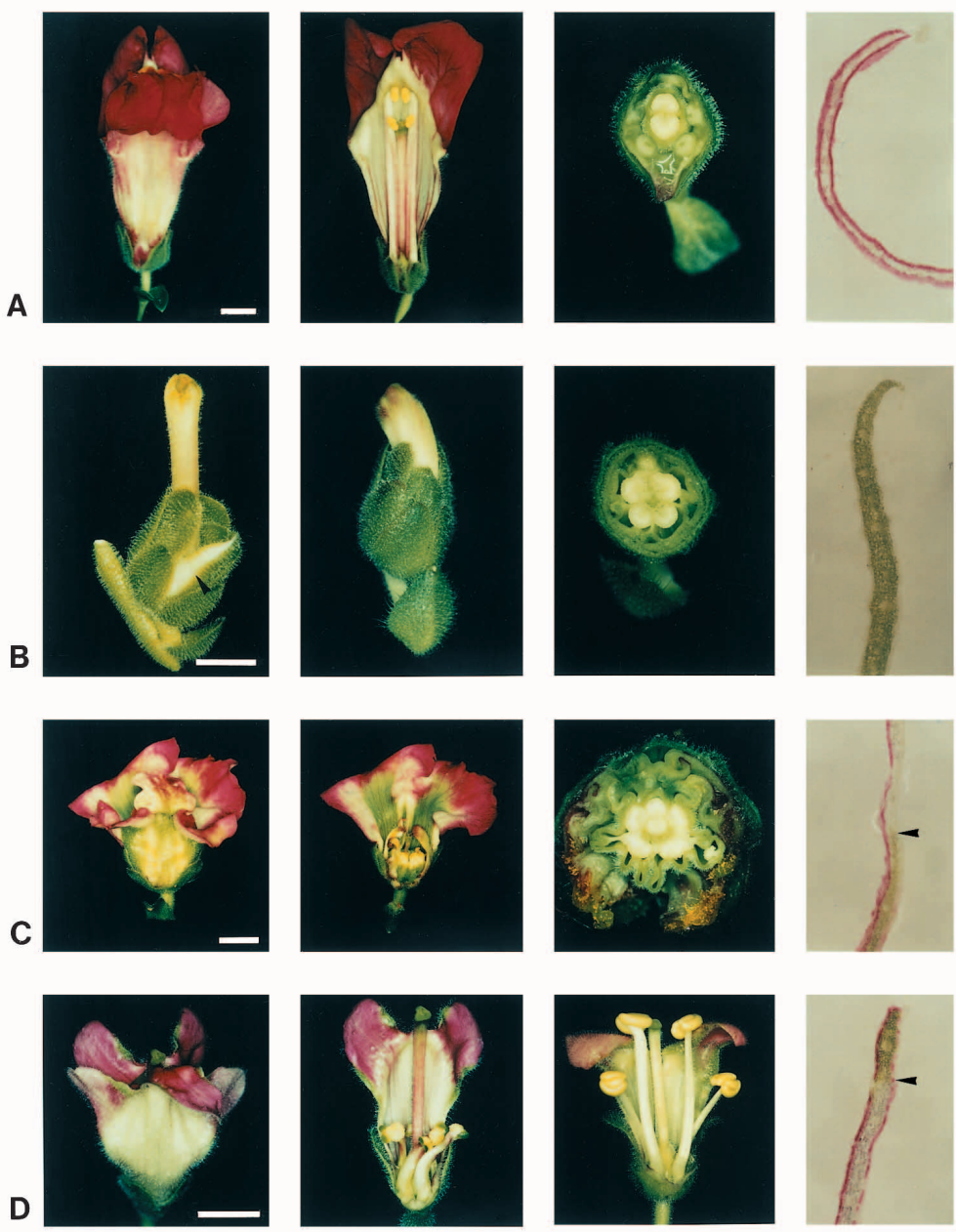

Fig. 1. Phenotypes of mature flowers of wild-type (A), *def*-gli mutant (B), L1 *def* chimera (C) and L2L3 *def* chimera (D) *Antirrhinum majus* plants. The first panel in each row shows intact flowers in front (A,C,D) or side (B) view. In B the somatically restored petaloid sector in the second whorl of a *def*-gli flower is indicated by an arrowhead, while the second panel shows a stable *def*-gli mutant flower in front view. The photographs in the second panel of A, C and D show internal parts of the flowers after removal of the lower first and second whorl organs. For comparison with the morphology of the third whorl organs of *def* L2L3 chimeras a putative L2L3 chimera of a *glo*-1 mutant flower is depicted in the third panel of D. In A-C the third panels show cross sections of flowers uncovering the central female structures. The right panels in A-D depict free-hand cross sections prepared from the second whorl organs of flowers of the respective genotypes. Black arrowheads in C and D (right panels) indicate the beginning of the margin regions (upper parts) which are derived from the L1 layer. Bars represent 5 mm and none of the photographs in this figure reflect real size differences. For further explanations see Results.

Unfortunately, segregation data with *glo-1* chimeras were obscured by the high germinal instability of this allele. Selfing of wild-type revertants as well as crosses or selfing of suspected chimeras resulted in over-representation of wild-type plants in the analysed progenies. Genetic analysis therefore could not be used to confirm the genetic constitution of the different cell layers in these plants.

Morphology of *def* chimeras

In the L1 chimera (Fig. 1C), five petals developed in the second whorl. The petal lobes were broader than wild type, their shape was distorted and the individual organs were not, or only partially, fused. Occasionally, the number of petal lobes was increased. In the central region of individual petals green tissue underlying the pigmented epidermis could be discerned. Free-hand cross sections from this region of the petals confirmed the presence of chlorophyll-containing mesophyll cells beneath the anthocyanin-expressing epidermal cells (Fig. 1C right panel). These observations suggested that DEF activity was restored in the L1 but not in the L2 and L3 cell layers. The central region was rimmed by broad margins with chlorophyll-less mesophyll cells. Inside the corolla a variable number of petaloid protrusions developed (Fig. 1C second panel), some of which were fused to the petals or to the base of the third whorl organs. The third whorl comprised stylar structures tipped with stigmatic tissue. These laterally open organs could be fused to their neighbours. The cross section of a flower shows four loculi of irregular shape filled with ovules (Fig. 1C third panel). The fourth whorl gynoecium developed as in wild-type flowers although the style was often shorter and flatter.

In L2L3 chimeras, the corolla was partially restored in the second whorl (Fig. 1D). Petal lobes were narrower and smaller than wild-type and adaxial petals were only partially united. In addition, petal lobes were rimmed by green sepaloid

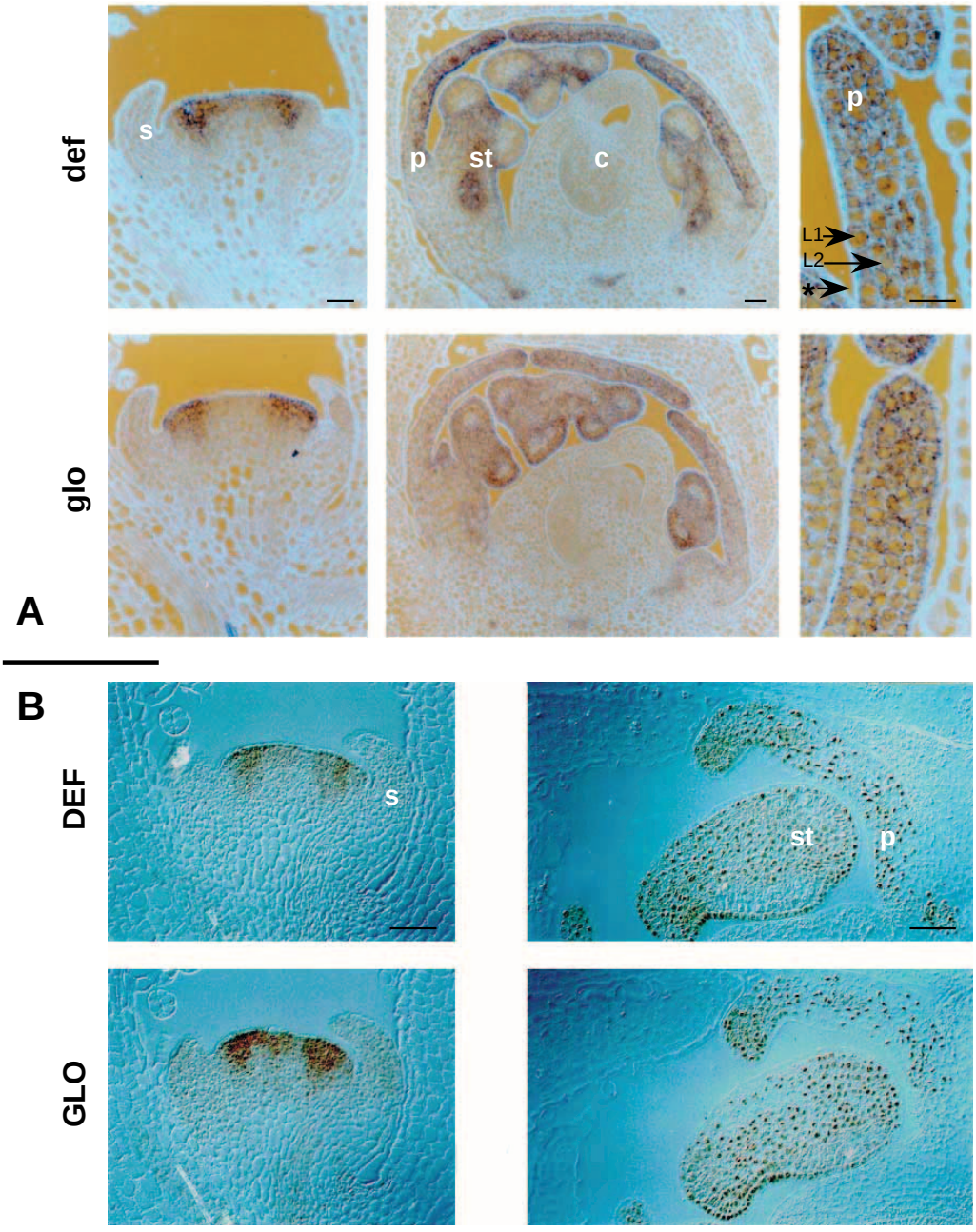

Fig. 2. Expression patterns of the *DEF* and *GLO* genes and DEF and GLO proteins in developing wild-type flowers. Serial longitudinal sections of wild-type inflorescences were probed with DIG-labelled *def* and *glo* antisense RNA transcripts (A) or DEF and GLO affinity-purified antibodies (B). In A, cell walls were counter stained by calcofluor, and in B, in situ immunolocalisation pictures were visualised using Nomarski optics. The presence of def and glo mRNA is revealed by the formation of a dark blue precipitate (A) and expression of the DEF and GLO proteins results in a brown signal (B). We should mention that the def or glo mRNAs are not detectable in sections derived from *def* or *glo* null mutants, respectively (Tröbner et al., 1992) and both proteins are absent in both mutants (Zachgo et al., 1995 and unpublished observations). The left panels in A and B show flowers at an early stage of development when petal and stamen primordia just begin to emerge. Older flowers with differentiating fourth whorl organs are shown in the middle panels of A. The right panels in A show higher magnifications of differentiating second whorl organs. The arrows point to the position of the L1 and L2 layers and the cuticle surrounding the epidermis (highlighted by the calcofluor stain) is indicated by an arrow with asterisk. s, sepal; p, petal; st, stamen; c, carpel. Size bars represent 50 μm.

tissue, also present in small patches at their tips. Free-hand cross sections of second whorl organs showed the absence of chlorophyll in mesophyll cells (Fig. 1D right panel) indicating that the *DEF* gene is functional in the L2 layer. The L1-derived marginal region of the tip of the petal, however, contained green cells suggesting that the L1 layer was genetically mutant for the *def* gene. This assumption was confirmed by in situ hybridisation experiments as described below. Interestingly, the epidermal cells were pigmented, as if the DEF protein was functional there (Fig. 1D right panel). We interpret this observation to be the result of the non-cell-autonomous function of the DEF protein. Pigmentation of mutant epidermal cells surrounding the L1-derived tip of the petals may hence result from anthocyanin diffusion or indirect activation of its synthesis (Vincent et al., 1995). The third whorl comprised organs with a stamen-like shape (Fig. 1D second panel). The filaments of these organs were broader and shorter than those of wild-type stamens. The adaxial surface of the anther-like structures was partially or completely covered with ovules, whereas their abaxial side was composed of carpelloid tissue tipped with stigmatic papillae. During propagation, occasionally sterile but morphologically almost wild-type stamens bearing just a few ovules at their adaxial lobes were observed, but this property was not observed in subsequent cuttings suggesting that this phenotype is not heritable. The fourth whorl carpels developed as in wild-type flowers.

Flowers of somatically stable *glo* chimeras revealed a petal morphology similar to the phenotype of *def* L2L3 chimeras. However, stamen identity was generally restored to a higher degree than the identity of third whorl organs in flowers of *def* chimeras and occasionally fertile wild-type stamens were observed (Fig. 1D third panel). Free-hand cross sections of second whorl organs revealed similar properties to *def* L2L3 chimeras (not shown).

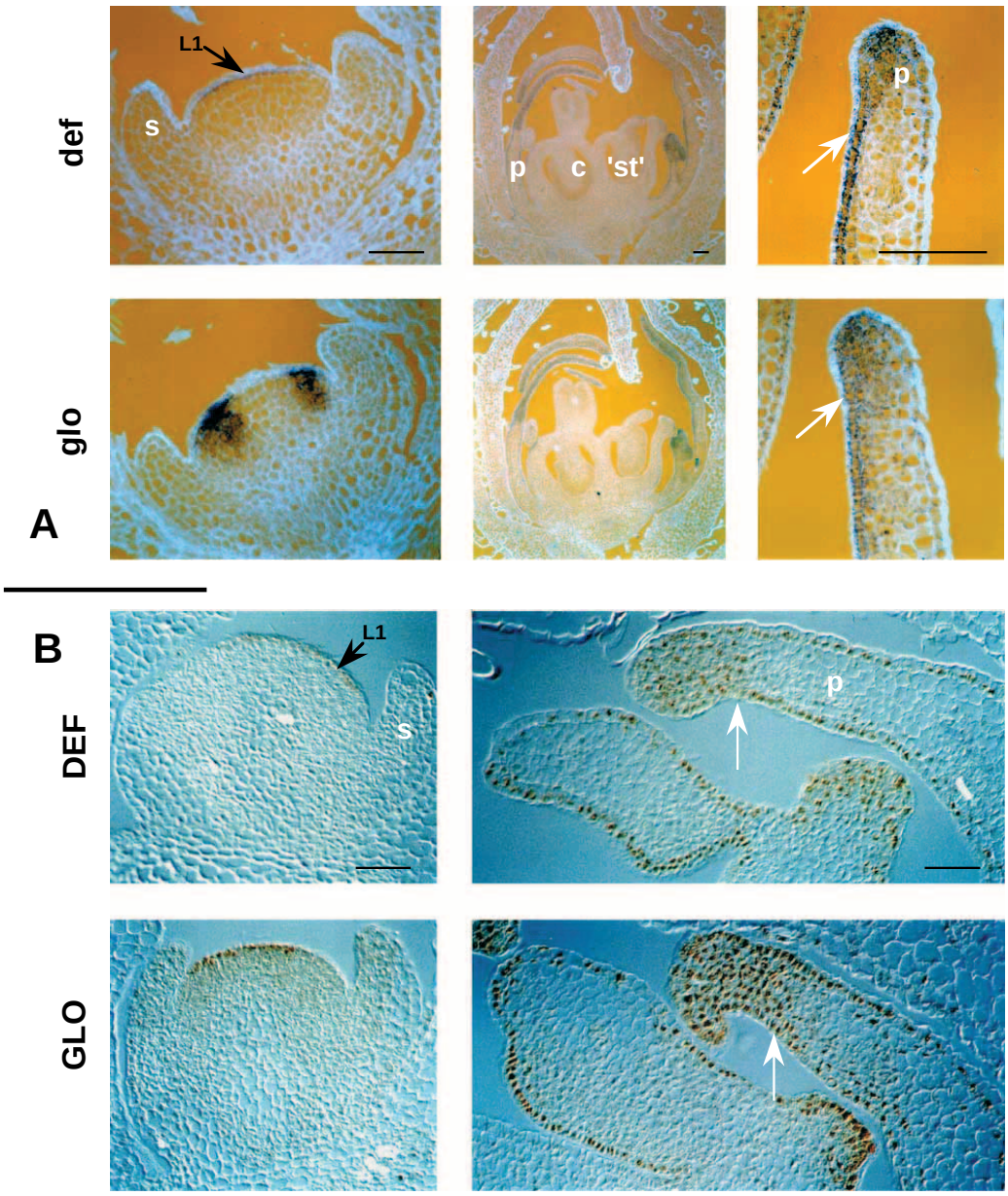

Fig. 3. Expression patterns of def and glo mRNA and DEF and GLO proteins in *def* L1 chimeras. Serial longitudinal sections of developing flowers of *def* L1 chimeras were probed and photographed as described in the legend of Fig. 2. Young flowers at the stage of initiating second whorl organ primordia are shown at the left and older flowers with differentiating fourth whorl organs are shown in the middle (A) or at the right (B). The right panels in A show enlarged sections of a differentiating second whorl organ. Note that def mRNA (A) and protein (B) remain confined to the epidermis or to L1-derived cells forming the petal margins (indicated by white arrow in the figures), whereas glo mRNA is initially expressed in all three layers of the meristem (left panel in A) and only later becomes confined to the L1 layer. Notice that, depending on the plane of sectioning, some L1 cells constituting a broad marginal region and expressing GLO protein may appear inside the rim of L1 cells delimiting the sections. The DEF and GLO protein expression patterns are identical in all stages of development. The sections presented at the left of part B are slightly off the median of the flower, thereby revealing signals along the entire dome (see Zachgo et al., 1995). The organ beneath the petal shown in the right panels is one of the petaloid structures flanking the third whorl organs (Fig. 1C). s, sepal; p, petal; 'st', carpelloid third whorl organ; c, carpel. Size bars represent 100 μm.

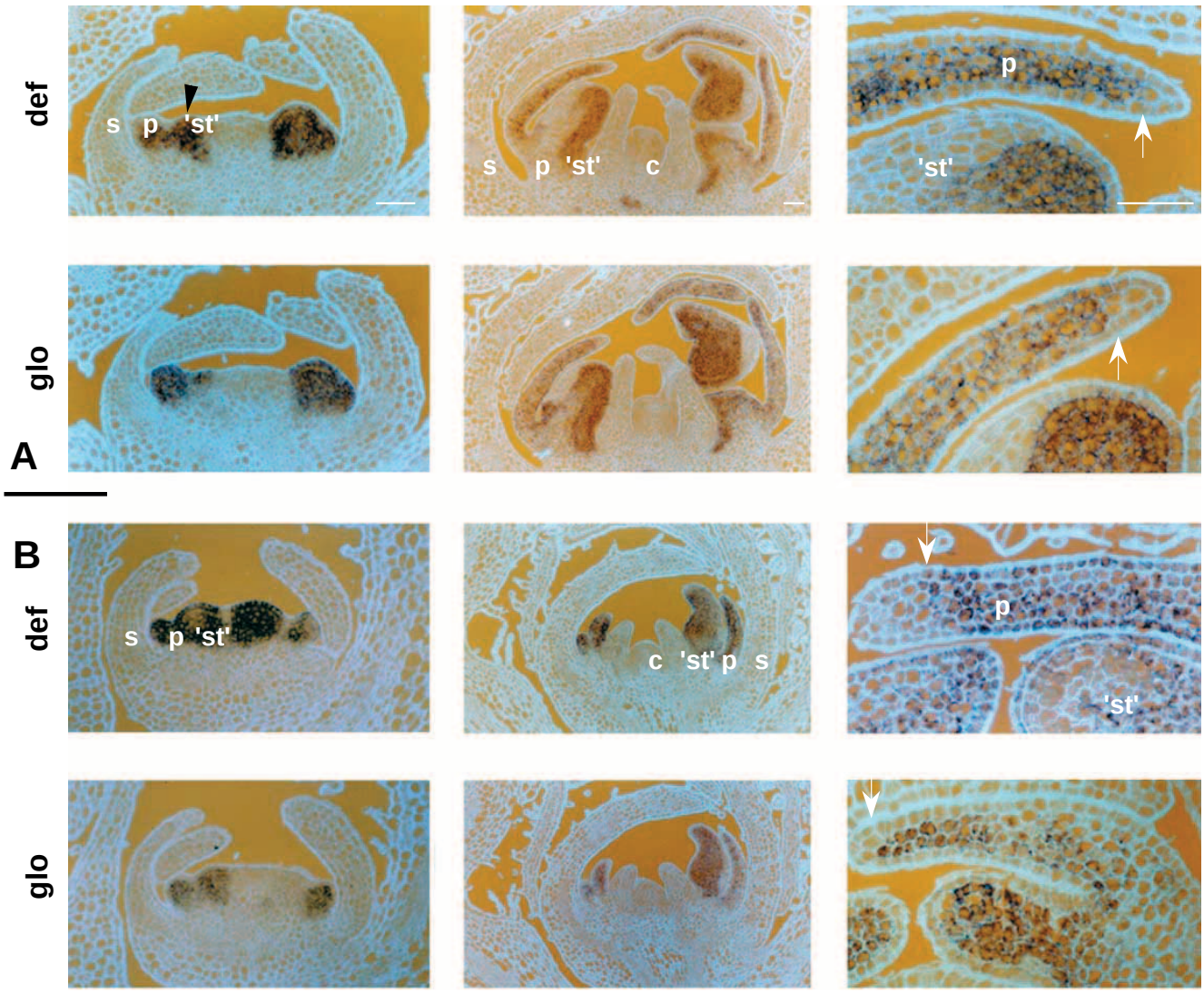

Fig. 4. Expression patterns of the *DEF* and *GLO* genes in *def* L2L3 chimeras (A) and in *glo* chimeras (B). Serial longitudinal sections of developing flowers of *def* (A) and *glo* (B) L2L3 chimeras were probed with DIG-labelled def and glo antisense transcripts as described in the legends to Figs 1 and 2. Young flowers with emerging second and third whorl organ primordia are shown at the left. The middle panels show older flowers with differentiating fourth whorl organs and the right panels depict enlarged sections of second whorl organs. Note that in *def* L2L3 chimeras def mRNA is not expressed in the epidermis at early stage, although occasionally a weak signal could be observed in young stamen primordia (indicated by a black arrowhead in the left panel of A). The *DEF* gene is also not expressed in the L1 layer and in the L1-derived margins of older petals (white arrow in the right panel of the upper row in A). glo mRNA is expressed in all three layers of second and third whorl organ primordia at early stages (left panel). At later stages its expression is maintained in the epidermis, except for the petal margins (white arrow). In the putative *glo* L2L3 chimeras (B) the *DEF* gene is expressed in all three layers of respective regions of the floral meristem except for the margins of older second whorl organs (white arrow), whereas *GLO* gene expression remains confined to L2- and L3-derived cells. s, sepal; p, petal; 'st', stamenoid carpelloid third whorl organs; c, carpel. Size bars represent 50 μm.

Transcription of the *DEF* and *GLO* genes and expression of the DEF and GLO proteins in different layers of *def* and *glo* chimeras

The spatial and temporal expression patterns of *DEF* and *GLO* mRNAs and proteins were determined by in situ hybridisation and in situ immunolocalisation in order to identify more precisely the type of chimeras and to investigate whether DEF could exert its non-cell-autonomous function by trafficking itself interdermally. Inflorescences of *def* chimeras as well as wild-type plants were sectioned and probed with digoxigenin-labelled def and glo antisense RNA or affinity-purified DEF and GLO antibodies.

At early developmental stages, def and glo mRNAs and proteins are expressed in all three cell layers of wild-type meristems in cells giving rise to petals and stamens and their expression is maintained throughout flower development (Fig. 2A,B).

In meristems of L1 chimeras, def mRNA was only detectable in the epidermal cells (Fig. 3A), confirming that these were L1 periclinal chimeras. At young developmental stages, before any visible sign of initiation of second and third whorl organ primordia, the signal extended over the central dome (Fig. 3A left panel). Later it was restricted to the epidermal cells of petals

and of the protrusions developing next to the petals (Fig. 3A middle panel). In addition, underlying layers in some regions of petals were labelled (Fig. 3A right panel). These area may correspond to the margins of petals where epidermal cells divide periclinally and form the subepidermal tissue (Satina, 1944). The abaxial epidermis of third whorl carpelloid organs was also labelled. At early stages, glo mRNA was expressed in all three cell layers of the meristem as in the wild type (Fig. 3A). Once all the organs had been initiated, its expression was primarily confined to the epidermal cells and to the subepidermal cells derived from the L1 layer (Fig. 3A right panel). This suggested the absence of DEF protein in the inner layer cells and consequently the absence of GLO since both proteins need each other to be stabilised. The weak signal observed in other layers probably resulted from the basal transcription of the *GLO* gene which occurs in the absence of the autoregulatory control exerted by DEF and GLO proteins. The results obtained by in situ immunolocalisation agreed with these predictions. During the entire period of development the DEF and GLO proteins were only detected in the epidermal cells or in subepidermal cells derived from the L1 layer (Fig. 3B). These data were consistent with the morphological observations showing that only the epidermal cells and L1-derived subepidermal cells had recovered a petaloid identity (Fig. 1C right panel).

Floral meristems from *def* L2L3 chimeras showed no *DEF* gene transcription in the L1 cell layer, but presence of def mRNA in the L3 layer and also in the L2 layer (Fig. 4A). Therefore we concluded that these plants were L2 L3 periclinal chimeras. A weak signal was occasionally observed in L1 cells of initiating third whorl organ primordia (as an example, see arrow in Fig. 4A, left panel). The glo mRNA expression pattern was similar to wild type at early stages (Fig. 4A). Interestingly, after initiation of all organs, its expression level remained high in all three cell layers. In some sections no expression was detected in any layer at the tip of the petals (Fig. 4A right panel). The observations made previously with the L1 chimeras suggested that these regions corresponded to the petal margins where cells of L1 origin constituted the subepidermal tissue. This was consistent with the presence of green sepaloid tissue observed at the petal margins of mature flowers (Fig. 1D), suggesting that there was no restoration of DEF functions in these cells. Thus, the presence of anthocyanin in the epidermal cells of the margins (Fig. 1D, right panel) is probably due to diffusion or to indirect activation of the synthetic pathway. In epidermal cells in other regions of the petal, a high level of *GLO* expression was maintained indicating that the autoregulation of *GLO* expression in the presence of DEF function was established. Indeed, by in situ immunolocalisation both DEF and GLO proteins were detected in all three cell layers of mature second and third whorl organs, except for the margin of petals (Fig. 5A). At early stages, the two proteins were not detectable in L1-derived cells except occasionally in initiating third whorl organ primordia. During the course of subsequent flower development the two proteins became progressively detectable in these cells.

For comparison, *glo* chimeras with floral phenotypes similar to those of the *def* L2L3 chimeras were also analysed. *GLO*

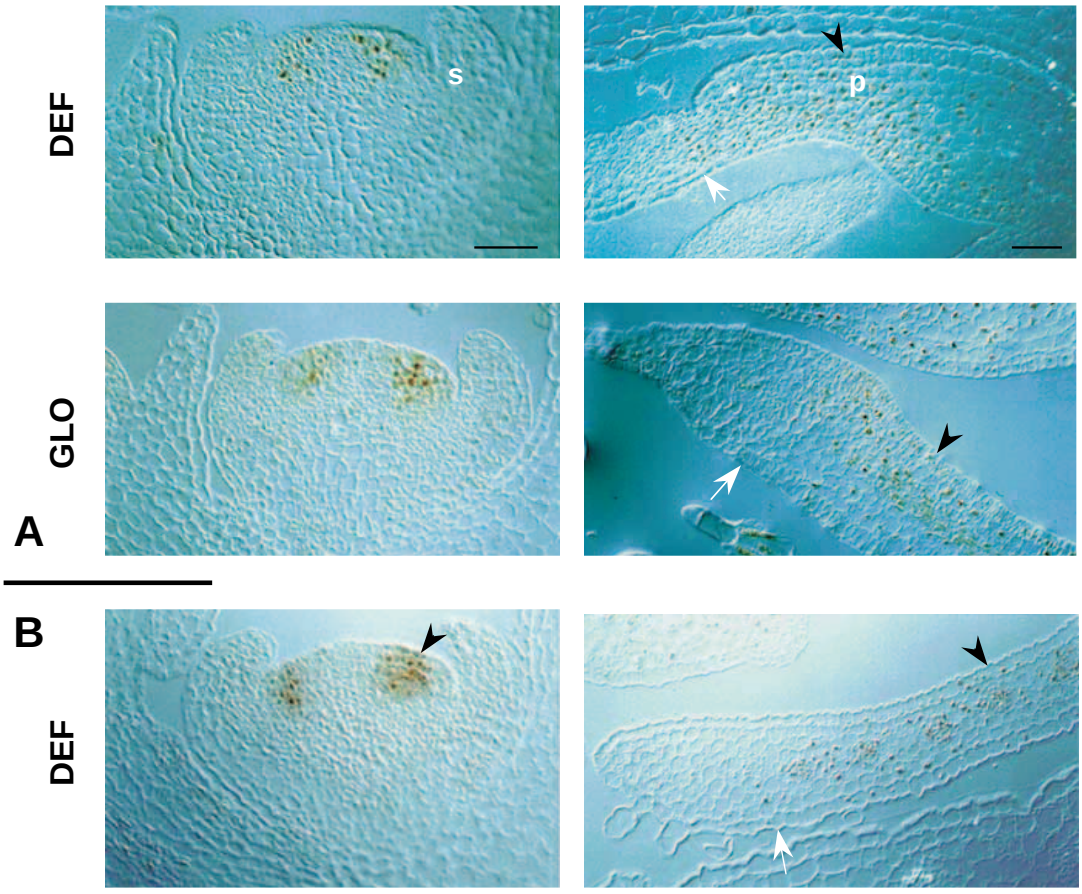

Fig. 5. Expression patterns of the DEF and GLO proteins in *def* and *glo* L2L3 chimeras. Longitudinal sections of developing flowers of *def* L2L3 chimeras (A) were probed with DEF and GLO antibodies and photographed as described in Figs 1 and 2. For both early (left panels) and late developmental stages (right panels) identical DEF and GLO protein expression patterns were obtained. Note that the DEF and GLO proteins are only detectable in L2- and L3-derived cells at early stages (left panels in A), while later both proteins are expressed in the epidermis of mature organs (indicated by black arrowheads), except for the petal margins (white arrows). In *glo* chimeras (B) the DEF protein (and similarly GLO, not shown) was typically detected in the epidermis of young stamen primordia, indicated by the arrowhead at the left. s, sepal; p, petal. Size bars represent 50 μm.

expression, due to somatic restoration of the wild-type *GLO* gene, was primarily restricted to the L2 and L3 layers (Fig. 4B). A weak signal could be occasionally observed in epidermal cells of initiating stamen primordia. In the absence of genetic evidence these data indicated that not only the phenotype, but also the genetic constitution of the cell layers of the glo chimeras was related in type to the L2 L3 chimeras of *def*. The def mRNA was expressed in all cell layers except for the margins of the petals (Fig. 4B right panel). Similar to *def* chimeras, the DEF and GLO proteins were detected in inner layer and epidermal cells in mature second and third whorl organs, except for the petal margins (Fig. 5 or not shown). In contrast to *def* chimeras, the DEF and GLO proteins were consistently present in epidermal cells of initiating third whorl organs. This could account for the higher degree of stamen restoration observed in *glo* chimeras compared to that of *def* chimeras.

DISCUSSION

Until recently, it was considered that if transcription factors acted non-cell-autonomously, then they were controlling the production of a diffusible molecule (Bouhidel and Irish, 1996; Hantke et al., 1995; Huala and Sussex, 1993). However, recent studies have demonstrated the cell-to-cell trafficking of viral nucleic acids and proteins via plasmodesmata (Lucas et al., 1993) and also the movement of endogenous factors, like the transcription factor KN1 (Lucas et al., 1995). In this report, we provide evidence that two MADS box transcription factors, DEF and GLO can be transported in this way. Microinjection experiments suggest that this movement is governed by plasmodesmata (Mezitt and Lucas, 1996). It appears that cell-to-cell communication by the transport of proteins via plasmodesmata is a broadly applied mechanism in plants, and that trafficking of transcription factors plays a more general role in their non-cell-autonomous functions than expected. Since low levels of def mRNA or glo mRNAs could occasionally be detected in the L1 layer of L2L3 chimeras, the trafficking of def and glo mRNAs cannot be ruled out. In the following sections specific aspects of the movement of the DEF and GLO proteins as well as the biological relevance of this movement in the control of floral organogenesis is discussed.

Polar trafficking of DEF from inner layer cells to epidermal cells

The trafficking of DEF and GLO proteins is not temporally regulated. The occasional detection of the DEF and GLO proteins in the epidermal cells in L2 L3 chimeras in young floral primordia (Fig. 5) shows that protein trafficking already occurs at early stages of development. During later stages the proteins are not always detectable by immunolocalisation, but their function, and hence their presence, is revealed by the establishment of the autoregulatory control of *DEF* or *GLO* expression, as well as by the phenotypically wild-type appearance of the epidermis. In mature floral organs, the proteins are consistently detected in the epidermis. Their presence in amounts higher than in the L1 cell layer of early stages may result from the higher level of overall expression of the DEF and GLO proteins, and/or may reflect their progressive accumulation.

Interestingly, the transport of DEF is directionally regulated in that it can only occur from the inner layer cells to the epidermal cells. Such a control of polarity has not been reported so far. It suggests that trafficking of endogenous macromolecules is subject to regulation and thus points to the importance of this transport. A possible reason for the trafficking of these transcription factors could be to secure the synchronisation of gene expression in all cells sharing the same fate. The polarity of DEF trafficking may reflect potential differences or fluctuations of inducing factors between layers during the activation process of these genes. For example, a 'signal' from internal tissues could spread to the periphery and thus progressively induce the expression of these organ identity genes; the trafficking of DEF in the same direction could reinforce this process. However, such an explanation seems to contradict the results obtained with *flo* periclinal chimeras which indicate a reverse direction of non-cell autonomy of the *FLO* function (or of an intermediate molecule), which controls early *DEF* expression (Hantke et al., 1995).

In contrast to the (polar) trafficking of DEF and GLO between cell layers, their transport between cells of the same layer seems to be prohibited, or limited, as suggested by the sharp boundary delimiting revertant sectors of unstable *def* and *glo* mutants or by the mutant margins in the L2L3 chimeras where DEF or GLO may move into the cells at the boundary between L2 and L1-derived subepidermal tissue but not any further. It seems then that the control of protein trafficking via primary plasmodesmata, connecting cells within a layer is different from the trafficking via secondary plasmodesmata which connect cells between layers. This level of regulation of protein movement may be required to avoid violating the boundaries of the expression domains of the DEF and GLO proteins. It will be interesting to learn in the future whether genes which negatively control the spreading of DEF and GLO into the first whorl of *Antirrhinum* (*CHORIPETALA*, E. de Andrade Silva and Z. Schwarz-Sommer, unpublished) or that of APETALA 3 (Jack et al., 1992), the homologue of DEF, towards the fourth whorl of *Arabidopsis* (*SUPERMAN/FLO*10, Sakai et al., 1995) act by affecting the movement of the respective proteins through primary plasmodesmata.

DEF expression in the L1 cell layer promotes the growth of petal lobes

In the absence of *DEF* gene expression, petal development is abolished as indicated by the presence of small sepaloid organs in the second whorl of *def*-gli null mutants. *DEF* gene expression in the L1 cell layer of L1 chimeras or trafficking of DEF to the L1 in L2L3 chimeras rescues the petaloid identity of epidermal cells and also in part the characteristic curvature of the organs. The striking difference in the morphology, in particular in the size of petals comparing L1 and L2L3 chimeras suggests that the activity of DEF in the L2L3 chimeras is insufficient to rescue the expansion of petal lobes. It seems, that high level of DEF expression in the L1 favours the growth of petal lobes by stimulating L1 cell division and/or cell shape and elongation and thus the development of broader margins. The presence of anthocyanin-producing L1 cells and the lack of chloroplast-containing L2-derived mesophyll cells in large regions at the periphery of the petal lobes in L1 chimeras support this hypothesis.

Differential effects of DEF trafficking in third and fourth whorl organs

A role as activator of cell division, outlined above, has already been attributed to *DEF* during the initiation of the fourth whorl. In fact, initiation of fourth whorl organogenesis in L1 chimeras

indicates that *DEF* activity in epidermal cells is sufficient to promote cell proliferation in the centre of the meristem. The re-establishment of carpel initiation in L2L3 chimeras could be attributed to the fact that DEF and GLO are moved to the L1, although it is still possible that expression of DEF in either of the three layers in the centre of the meristem has the same effect.

Expression of DEF in L1 cells alone is not sufficient to restore stamen identity, although the structure of third whorl organs is less aberrant than in flowers carrying a *def* null allele. Restoration of DEF and GLO activity in L2 and L3 layers allows the development of stamenoid features, but the organs are still feminised. It seems, therefore, that for wild-type stamen development high level of DEF expression in all cell layers is necessary and that trafficking from the L2L3 to the L1 layer in *def* chimeras is insufficient to control stamen development. Previous studies have shown that the threshold level of DEF and GLO gene function for the establishment and maintenance of stamen identity is high (Zachgo et al., 1995). These crucial conditions are obviously not always fulfilled in the *def* L2L3 chimeras. In contrast, restoration of stamens in the third whorl of *glo* L2L3 chimeras is complete, suggesting that in these flowers the DEF and GLO functions reach the threshold level for wild-type function. Based on the observation that during initiation of third whorl organs the DEF and GLO proteins were detectable in the L1 layer of *glo* L2L3 chimeras, but were rarely detectable in *def* L2L3 chimeras, we conclude that the trafficking of DEF is under higher constraints during early stages of development than the trafficking of GLO.

Recently, expression of the *PISTILLATA* gene, the structural and functional homologue of *GLO* (Goto and Meyerowitz, 1994), in the L1 cell layer of *Arabidopsis* periclinal chimeras was reported to direct the formation of wild-type organs in both the second and third whorls (Bouhidel and Irish, 1996). Unfortunately, *glo* chimeras of this type were not detected in our population, where wild-type-looking plants were genetically confirmed as germinal revertants (see Results). Nevertheless, since *def* L1 chimeras do not display overall wild-type morphology and trafficking of DEF from L1 cells has not been observed, one may speculate that the trafficking properties of the heterodimerisation partners DEF and GLO from the L1 into the L2L3 cell layers may differ. This would be in agreement with our previous speculations on the existence of different levels of constraints exerted on the trafficking of DEF and GLO. Alternatively, there may be substantial differences in the mechanism of cell-to-cell communication between *Arabidopsis* and *Antirrhinum*.

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