Epidermal control of floral organ identity by class B homeotic genes in *Antirrhinum* and *Arabidopsis*

Nadia Efremova, Marie-Christine Perbal, Alexander Yephremov, Winfried A. Hofmann, Heinz Saedler and Zsuzsanna Schwarz-Sommer*

Max-Planck Institut für Züchtungsforschung, Carl-von-Linné Weg 10, 50289 Köln, Germany

*Author for correspondence (schwarzs@mpiz-koeln.mpg.de)

Accepted 8 May 2001

**SUMMARY**

To assess the contribution of the epidermis to the control of petal and stamen organ identity, we have used transgenic *Antirrhinum* and *Arabidopsis* plants that expressed the *Antirrhinum* class B homeotic transcription factors DEFICIENS (DEF) and GLOBOSA (GLO) in the epidermis. Transgene expression was controlled by the *ANTIRRHINUM FIDDLEHEAD* (AFI) promoter, which directs gene expression to the L1 meristematic layer and, later, to the epidermis of differentiating organs. Transgenic epidermal DEF and GLO chimeras display similar phenotypes, suggesting similar epidermal contributions by the two class B genes in *Antirrhinum*. Epidermal B function autonomously controls the differentiation of *Antirrhinum* petal epidermal cell types, but cannot fully control the pattern of cell divisions and the specification of sub-epidermal petal cell-identity by epidermal signalling. This non-autonomous control is enhanced if the endogenous class B genes can be activated from the epidermis. The developmental influence of epidermal B function in *Antirrhinum* stamen development is very limited. In contrast, epidermal B function in *Arabidopsis* can control most if not all epidermal and sub-epidermal differentiation events in petals and stamens, without any contribution from the endogenous class B genes. Possible reasons for differences in the efficacy of B-function-mediated cell communication between the two species are discussed. Interestingly, our experiments uncovered partial incompatibility between class B functional homologues. Although the DEFICIENS/PISTILLATA heterodimer is functional in transgenic *Arabidopsis* plants, the APETALA3/GLOBOSA heterodimer is not.

**Key words:** Flower development, Epidermal chimeras, Cell communication, MADS-box protein, DEFICIENS, GLOBOSA, FIDDLEHEAD, Arabidopsis, Antirrhinum

**INTRODUCTION**

The organisation and developmental potential of meristems – the source of vegetative and reproductive organs in higher plants – has long been of great interest (for a recent review see Szymkowiak and Sussex, 1996). According to the generally accepted concept, floral meristems are organised in layers, and the three meristem layers termed L1, L2 and L3 contribute differentially to the formation of different tissues and to the development of different floral organs (Satina and Blakeslee, 1941). Organ identity becomes manifest in the number, shape, size and function of the cells that make it up. It follows that communication between neighbouring cells and cell layers is necessary to co-ordinate the pattern and rate of cell divisions and cell enlargement during organ development. Our interest is in elucidating the role of class B floral homeotic genes in this process.

In *Antirrhinum* two class B genes, DEFICIENS (DEF; Schwarz-Sommer et al., 1992) and GLOBOSA (GLO; Tröbner et al., 1992) control petal organ identity and, in combination with two class C genes, PLENA and FARINELLI (Davies et al., 1999), the development and function of stamens. In the absence of DEF or GLO expression in def and glo mutants, petals acquire a sepaloid developmental fate and stamens develop as carpels. DEF and GLO encode MADS-box transcription factors (Schwarz-Sommer et al., 1990) that are unstable when expressed alone and become stabilised by forming a heterodimer (Tröbner et al., 1992; Zachgo et al., 1995). Furthermore, DEF and GLO can form a ternary protein complex by interacting with other MADS-box proteins (Egea-Cortines et al., 1999). The genes are independently induced early during flower development, but their expression later becomes mutually interdependent. The major DEF and GLO expression domains overlap in whorl 2 (the position of petals) and whorl 3 (the position of stamens), where the genes are expressed during the entire period of organogenesis and differentiation (Zachgo et al., 1995). Most of the findings made with DEF and GLO have been confirmed for their *Arabidopsis* orthologues APETALA3 (AP3) and PISTILLATA (PI), although their expression patterns differ in detail (Riechmann and Meyerowitz, 1997).

The contribution of DEF and GLO to the control of cell and
organ identity and cell-cell communication between layers that differ in genotype has been studied in somatically stable def and glo periclinal chimeras, obtained using genetically unstable transposon-induced alleles (Carpenter and Coen, 1990; Perbal et al., 1996). In L1 periclinal chimeras the wild-type DEF allele is present in L1 whereas L2/L3 carry the def mutant allele. The genetic constitution of the layers in L2/L3 chimeras is the opposite of that in L1 chimeras: L2/L3 carry the wild-type DEF (or GLO) allele and L1 the def (or glo) mutant allele. Complete or partial rescue of wild-type cell types and cell division patterns in the layer carrying the wild-type allele in such chimeras reveals cell-autonomous (or autonomous) control of differentiation. The wild-type layer may also influence the number and identity of cells in the adjacent, genetically mutant layer. This is the hallmark of non-autonomous control and implies cell communication. The absence of autonomous control is also indicative of interdermal communication during wild-type development. Petal size and morphology in L1 and L2/L3 periclinal chimeras were found to be intermediate between wild type and mutant, revealing a mutual non-autonomous influence of layers on cell type and cell divisions. The wild-type genes autonomously controlled the differentiation of petal cell types in the layer in which they were expressed, and influenced the types of cells formed in the adjacent mutant layer. The moderate non-autonomous influence of DEF and GLO in L2/L3 on epidermal features of petals and stamens and on organ shape could be, in part, attributed to trafficking of the DEF and GLO proteins from L2 to L1, but no DEF protein movement from L1 into inner layer cells could be detected (Perbal et al., 1996). The absence of DEF-mediated lateral communication between cells within a layer is evident from the sharp boundary between wild-type and mutant cells in sectorial chimeras (Carpenter and Coen, 1990; Perbal et al., 1996). DEF and GLO behaved similarly in petals of sectorial chimeras and in L2/L3 periclinal chimeras; however, no glo L1 chimeras could be obtained to compare the influence of GLO and DEF directly.

AP3 and PI differ in the extent of their autonomous and non-autonomous contributions to Arabidopsis organ identity. PI can control petal and stamen development from the epidermis, as revealed by the wild-type phenotype of X-ray-induced chimeras (Bouhidel and Irish, 1996). In contrast, AP3 in L1 has little non-autonomous influence on sub-epidermal development in whorl 2, and no effect on the developmental fate of organs in whorl 3, according to more recent observations with transgenic Arabidopsis plants genetically engineered to mimic somatically stable transposon-induced alleles (Jenik and Irish, 2001). Whether the difference in the behaviour of class B genes in Antirrhinum and Arabidopsis and between the two class B proteins in Arabidopsis is real or is due to differences in the mode of production of chimeras is not known, and this question has been addressed in this report.

One disadvantage of somatic chimeras for the study of cell communication is that the genotypic differences between the cell layers are not heritable. This precludes genetic studies on the mechanisms underlying non-autonomous processes. They are also unsuitable for comparative studies of different genes in one species or orthologous genes in different species, because the genetic tools available for making chimeras (such as transposon-induced alleles for somatically stable chimeras, or genetic markers for X-ray-induced chimeras, or techniques for constructing graft chimeras and for vegetative maintenance of useful material) cannot always be applied to different genes and species. To overcome these problems we generated transgenic Antirrhinum and Arabidopsis plants that express DEF or GLO under the control of the epidermis-specific promoter of the Antirrhinum Fiddlehead (AFI) gene (A. Y. and N. Y., unpublished data). The AFI promoter is active over a long period of development, like the AtML1 promoter used in comparable experiments (Sessions et al., 2000). Its function depends on cell position and not on cell lineage: the AFI promoter is active in the L1 meristematic layer and in L1-derived and differentiated epidermal cells, but not in L1-derived sub-epidermal cells (A. Y., N. Y. and M.-C. P., unpublished data). In this report we first show that the phenotypes of somatically stable Antirrhinum chimeras that express DEF in L1 under the control of its own promoter are reconstituted in transgenic plants expressing DEF under the control of the AFI promoter. By expressing DEF or GLO in the epidermis of transgenic plants we then demonstrate that epidermal B function is sufficient to ensure near normal sub-epidermal differentiation in Arabidopsis, but is less effective in Antirrhinum.

MATERIALS AND METHODS

Plant material

Plants were either grown in the greenhouse at a daytime temperature of 18-25°C and with additional light during the winter, or in a climate chamber under standard conditions (16 hours light, 18-20°C).

The Arabidopsis thaliana mutant apetalala3-3 (referred to as the ap3 mutant in the text) was obtained from the Arabidopsis Biological Resource Centre (Ohio State University, Columbus) and the wild-type Antirrhinum line Sippe 50 from the Gatersleben collection. niv-98 (the progenitor of line 165E) and pal-rec2 were kindly provided by Rosemary Carpenter (John Innes Centre, Norwich, UK). The stable deficiens mutant def-B177 (referred to as the def mutant in the text) was obtained by transposon tagging (Schwarz-Sommer et al., 1992) and carried a Tum2-related insert (Z. Sch.-S., unpublished).

Binary vectors

Construction of the pHAF-XS and pBPF-XS vectors carrying a polylinker sequence (XS) and the AFI and FDH promoters, respectively, will be described in detail elsewhere (A. Y. et al., unpublished). The full-length cDNAs for DEF and AP3 and GLO, extending from the ATG translation start codon and specifying the 12-amino acid c-myc epitope as a C-terminal translational fusion, were inserted between the XhoI and XbaI sites in the polylinker of the vectors. The resulting AFI::DEF, AFI::GLO, FDH::AP3 and FDH::PI constructs were used for plant transformation.

Transgenic lines

Arabidopsis was transformed using the standard in planta vacuum infiltration protocol (modified after Bechtold et al., 1993) and Antirrhinum line 165E was transformed according to the method of Heidmann et al. (Heidmann et al., 1998). Transgenic plants and their progeny were selected on plates of solid MS medium containing hygromycin (15 μg/ml for Arabidopsis and 10 μg/ml for Antirrhinum). Subsequent generations were grown without selection and, if necessary, the presence of the transgene(s) was confirmed by PCR or by RT-PCR. For simplicity, transgenic plants are termed epidermal chimeras (or transgenic chimeras) in this report to indicate epidermal expression of genes controlled by the AFI promoter.

Transgenic Antirrhinum lines carried one to six copies of the transgene and transgenic Arabidopsis lines carried two to four copies,
as determined by Southern hybridisation in the T0 and T1 generations, respectively. Copy number did not influence the phenotype of Arabidopsis lines. In Antirrhinum only plants carrying one copy of the transgene, obtained in the primary transformants or after outcrossing the transgene copies, showed phenotypic effects. The findings described in this report derive from observations on two independent transgenic Antirrhinum lines that carried a single copy of the transgene in question. The presence of the c-myc epitope did not interfere with transgene expression as determined by observing the phenotype of transgenic plants transformed with an epitope-less variant of the transgenes. Unfortunately, attempts to detect the tagged proteins in situ, using antibodies directed against the epitope, were unsuccessful.

Microscopy and in situ hybridisation

Tissue preparation and in situ hybridisation with digoxigenin-labelled antisense RNA were carried out as previously described (Davies et al., 1996; Perbal et al., 1996). Cell walls of Antirrhinum sections were stained with Calcofluor or, for histological observations, with Toluidine Blue. Sections were viewed under fluorescent light or under bright-field illumination with a Zeiss Axiophot microscope.

For scanning electron microscopy (SEM) freshly harvested plant material was shock-frozen in liquid nitrogen, transferred to a cryochamber (Oxford Instruments), gold coated and examined in a Zeiss DSM 940 electron microscope at 5 kV.

All images were processed and assembled with the Adobe Photoshop and Canvas programmes.

RESULTS

Epidermal expression of DEF or GLO, separately or in combination did not affect the vegetative development of transgenic Antirrhinum or Arabidopsis plants. Floral phenotypes of transgenic plants in both species were variable within selfed and outcrossed populations, as well as within single plants. This was influenced slightly by the genetic background (stronger phenotypes were observed in Line 165E and weaker ones in the Pal-rec2 and Sippe 50 backgrounds in Antirrhinum and stronger phenotypes were found in the Ler compared to the Col background in Arabidopsis) and, more markedly, by growth temperature. Variability could not be correlated with the age of the plant or the position of the flower within the inflorescence. Most of the data presented refer to the commonly observed intermediate to strong phenotypes. Table 1 gives an overview of the phenotypic alterations associated with epidermal expression of the transgene(s), together with the phenotypes of plants examined.

Expression of the B function in the epidermis of def and glo mutant Antirrhinum plants reveals cell-cell communication during petal development

The AFI::DEF transgene was introduced into the def mutant background to test whether the AFI promoter is able to regulate DEF expression in the same manner as the endogenous DEF promoter and to reproduce the phenotype of somatically stable L1 chimeras (Perbal et al., 1996). In somatic chimeras L1-derived wild-type cells can invade the underlying L2 layer owing to rare periclinal cell divisions in L1; as a consequence, sub-epidermal cells derived this way express the DEF gene. This is not possible in def; AFI::DEF transgenic plants, because the epidermis-specific AFI promoter (in contrast to the endogenous DEF promoter) is inactive in sub-epidermal cells (see Introduction). def; AFI::DEF epidermal chimeras therefore provide a means of following more precisely the contribution of epidermal DEF to the control of organ identity. In addition, glo; AFI::GLO epidermal chimeras were obtained to determine whether epidermally expressed DEF and GLO differ in their developmental effects. However, in contrast to the differences observed between the phenotypes of PI and AP3 L1 chimeras in Arabidopsis (see Introduction), the

Table 1. Overview of genotypes and phenotypes of transgenic plants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Epidermal transgenes</th>
<th>First whorl</th>
<th>Second whorl</th>
<th>Third whorl</th>
<th>Fourth whorl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous genes</td>
<td>Antirrhinum</td>
<td>DEF; GLO</td>
<td>sepal</td>
<td>petal</td>
<td>stamen</td>
</tr>
<tr>
<td></td>
<td></td>
<td>def; GLO</td>
<td>sepal</td>
<td>sepal</td>
<td>carpel</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEF; glo</td>
<td>sepal</td>
<td>sepal</td>
<td>carpel</td>
</tr>
<tr>
<td></td>
<td></td>
<td>def; GLO</td>
<td>AFI::DEF</td>
<td>sepal</td>
<td>petaloid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEF; glo</td>
<td>AFI::GLO</td>
<td>sepal</td>
<td>petaloid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AFI::GLO</td>
<td>sepal</td>
<td>petal</td>
<td>stamen</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEF; GLO</td>
<td>AFI::DEF/GLO</td>
<td>petaloid</td>
<td>petal</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Arabidopsis</th>
<th>AP3; PI</th>
<th>sepal</th>
<th>petal</th>
<th>stamen</th>
<th>carpel</th>
</tr>
</thead>
<tbody>
<tr>
<td>ap3; PI</td>
<td>AFI::DEF</td>
<td>sepal</td>
<td>petal*</td>
<td>stamen*</td>
<td>stamenoid‡</td>
</tr>
<tr>
<td>AP3; PI</td>
<td>FDH::AP3</td>
<td>sepal</td>
<td>petal*</td>
<td>stamen*</td>
<td>stamenoid‡</td>
</tr>
<tr>
<td>AP3; PI</td>
<td>AFI::DEF</td>
<td>sepal</td>
<td>petal</td>
<td>stamen</td>
<td>carpel</td>
</tr>
<tr>
<td>AP3; PI</td>
<td>FDH::AP3</td>
<td>sepal</td>
<td>petal</td>
<td>stamen</td>
<td>stamenoid‡</td>
</tr>
<tr>
<td>AP3; PI</td>
<td>FDH::PI</td>
<td>petaloid</td>
<td>petal</td>
<td>stamen</td>
<td>carpel</td>
</tr>
<tr>
<td>AP3; PI</td>
<td>AFI::DEF/GLO</td>
<td>petal*</td>
<td>petal</td>
<td>stamen</td>
<td>stamenoid‡</td>
</tr>
<tr>
<td>AP3; PI</td>
<td>FDH::AP3</td>
<td>petal*</td>
<td>petal</td>
<td>stamen</td>
<td>stamenoid‡</td>
</tr>
<tr>
<td>AP3; PI</td>
<td>AFI::DEF/FDH::PI</td>
<td>petal*</td>
<td>petal</td>
<td>stamen</td>
<td>stamenoid‡</td>
</tr>
<tr>
<td>AP3; PI</td>
<td>FDH::AP3/AFI::GLO</td>
<td>petal</td>
<td>petal</td>
<td>stamen</td>
<td>stamenoid‡</td>
</tr>
</tbody>
</table>

*Immature organs; ‡Organs can proliferate inside whorl 3.
morphologies of glo; AFI::GLO and def; AFI::DEF chimeras were similar. In the following sections we therefore restrict our attention to the def; AFI::DEF chimeras.

Second whorl petaloid organs of def; AFI::DEF flowers are morphologically aberrant, forming a short tube at the base and a pigmented, partly unfolded lobe at the top (Fig. 1C). The three ventral organs display the typical curvature of wild-type ventral petals and include several cell types that are characteristic of wild-type ventral petals. The inner epidermal surface within the lobe region is made up of distinctive conical cells and, within the tube region, isometric flat cells are found, similar to wild-type petals and different from the second whorl sepals seen in def mutants (Fig. 1D-I).

The size of def; AFI::DEF petals is intermediate between that of wild-type petals and the second whorl sepals of the def mutant (Fig. 1A-C). Cross sections reveal that the sub-epidermal cells (as well as cells within the inner and outer epidermis) are smaller and more regular in shape than those of sepals or def second whorl organges (Fig. 1M-O). This shift towards wild-type morphology in the sub-epidermis suggests a limited degree of control of sub-epidermal cell division and cell shape by the epidermis, since the number of smaller cells has to increase in order to produce organs that are larger than in the def mutant. The intensity of the green colour in sub-epidermal cells in def; AFI::DEF petals is intermediate between mutant and wild type, indicating incomplete suppression of chlorophyll synthesis by a non-autonomous mechanism. Thus, as in somatic L1 chimeras (see Introduction), petal epidermal cell types and pigment synthesis are autonomously controlled by epidermal DEF, whereas epidermal DEF influences but cannot fully control petal cell identity in the sub-epidermal layers. As a consequence, epidermal B function is only partly sufficient to influence petal shape and size. These relationships are depicted schematically in Fig. 2.

To determine if epidermal signalling is achieved by trafficking of class B transcripts, the expression of DEF and GLO was monitored in def; AFI::DEF and glo; AFI::GLO flowers, respectively. As shown in Fig. 3, expression of the transgenes is confined to the outermost cell layer. The absence of detectable amount of sub-epidermal RNA in the transgenic chimeras corresponds to the absence of protein movement from L1 to L2 in somatic L1 chimeras (Perbal et al., 1996).

**Epidermal B function cannot direct stamen development**

The potential of AFI::DEF to restore stamen development in the def mutant is far less pronounced compared to petal restoration. The upper portion of the severely carpeloid def; AFI::DEF third whorl organs is often short and occasionally split (Fig. 1C). Their basal parts are frequently fused, in contrast to the absence of fusions between third whorl stamens in the wild type. This indicates defects in stamen initiation and early development. It is not possible to determine whether epidermal cell types correspond to that of a stamen or a carpel, because the style of a carpel and the filament of a stamen are superficially indistinguishable in terms of the type and arrangement of cells in the two organs. Development of stigmatic papillae at the tip of the carpeloid organs is frequently incomplete or abolished, revealing that this event is autonomously controlled. Epidermal expression of DEF is sufficient to suppress organ formation at
the position of the stamenodium (Fig. 1L), whose development is retarded in the third whorl of the wild type, but which develops fully as a fifth carpeloid organ in the third whorl of def mutants (Fig. 1K). In all these respects the morphology of third whorl organs in transgenic epidermal chimeras resembles that of somatic L1 chimeras. Interestingly, flowers carrying the AFI::DEF transgene in the temperature sensitive def-101 mutant background develop fertile stamens when grown at a moderate temperature (not shown). Under these growth conditions third whorl organs of def-101 mutant flowers initiate as separate organs, but they remain carpeloid during further development (Zachgo et al., 1995). This suggests that epidermal DEF can control stamen identity when supplemented with a very low level of endogenous B function. Temperature-shift experiments are in progress to define the developmental stage until which endogenous B function is essential for stamen development and after which stamen differentiation can be controlled by epidermal DEF alone.

Fourth whorl carpels do not initiate in def mutants (Fig. 1K; Schwarz-Sommer et al., 1992; Tröbner et al., 1992). Expression of DEF in L1 of somatic chimeras restores carpel initiation, indicating that it is under non-autonomous control, since organ

---

**Fig. 2.** Schematic summary of experimental observations on the influence of epidermal B function on petal development in Antirrhinum. Autonomous control is indicated by the solid arrows and non-autonomous control is shown by the broken arrows. The contribution of the endogenous DEF/GLO genes in wild-type sepals is indicated in red. All other control events are common to AFI::DEF/GLO wild-type sepals and def (or glo) mutant petals expressing AFI::DEF (or AFI::GLO). The dotted arrows on the right indicate an epidermal influence on petal shape and size, which are also affected by sub-epidermal events (Perbal et al., 1996), not discussed in this report.

**Fig. 3.** In situ analysis of DEF (A,B) and GLO (C,D) expression in longitudinal sections of def, AFI::DEF (A,B) and glo, AFI::GLO (C,D) flowers. The hybridisation signal is confined to the outermost cell layer in young flowers (A,C) and in older petals (B,D). The sections were stained with calcofluor and the hybridisation signal appears as a purple stain in fluorescent light. Bars, 100 μm. Numbers indicate whorls.

**Fig. 4.** Morphological features of Antirrhinum flowers that express the AFI::DEF/GLO transgenes in a wild-type background. (A-D) Mature flowers displaying various first whorl organ transformations. (A) Lateral view of flower with slightly aberrant petals (pet). (B-D) Frontal view of flowers, with some organs (all petals in B, all petals and stamens in C and all inner organs in D) removed. An anomalous carpel is shown in C. For comparison, B shows a wild-type carpel developing in a flower with a severe first whorl transformation. The position of the dorsal (d), lateral (l) and ventral (v) sepals are indicated. (E,F) SEM of inner epidermal cells in the lobe (E) and tube (F) regions of a petaloid sepal. Bars, 20 μm. (G,H) Expression pattern of DEF (G) and GLO (H) in sequential cross sections from a first whorl organ. The weak sub-epidermal hybridisation signal within the marginal region is most obvious around vascular bundles. Epidermal expression is present in this, and in the adjacent, region. Bars, 200 μm.
initiation involves cell divisions in inner layers (Perbal et al., 1996). In transgenic chimeras the flowers in an inflorescence may (Fig. 1C), or may not (Fig. 1L), develop fourth whorl carpels. This variability perhaps relates to fluctuations in the activity of the AFI promoter, which provides the DEF function, during early stages of development when DEF is known to be critical for carpel initiation (Zachgo et al., 1995).

**Epidermal signalling enhances first whorl petaloidy in flowers that simultaneously express DEF and GLO in the epidermis**

Mechanisms that non-autonomously activate endogenous class B genes can reinforce DEF/GLO expression in cells carrying the wild-type DEF and GLO genes, but have no influence on DEF and GLO expression in def or glo mutant cells. One question relevant to events occurring in the wild type therefore is whether the limitations on cell communication observed in def; AFI::DEF chimeras are valid when all cells carry wild-type DEF/GLO genes. This can be answered by ectopically expressing DEF/GLO in the epidermis of wild-type sepals and carpels. Although wild-type first and fourth whorl organs are not completely devoid of class B gene transcription (Tröbner et al., 1992; Zachgo et al., 1995), sepals and carpels of wild-type flowers carrying either AFI::GLO or AFI::DEF alone are phenotypically wild type. This suggests that levels of endogenous DEF and GLO expression in whorls 1 and 4 are too low to permit the formation of functional heterodimers with the DEF or GLO proteins expressed by the individual epidermal transgenes. Plants that were doubly transgenic for AFI::DEF and AFI::GLO (designated as AFI::DEF/GLO plants) were therefore generated and their first and fourth whorl organs were examined for morphological changes relative to def; AFI::DEF second and third whorl organs, respectively, to answer the question posed above.

Fourth whorl carpels in all wild-type backgrounds remained almost completely normal, only occasionally displaying a short style or a split stigma in flowers of double transgenic plants (Fig. 4C). Reduction or absence of stigmatic papillae at the tip of the stigma was the only indication of autonomous control of epidermal cell type by AFI::DEF/GLO in whorl 4. Thus, simultaneous, high-level epidermal expression of DEF and GLO in a wild-type background is essentially unable to cause any substantial changes in carpel morphology in whorl 4, reminiscent of the limited influence of epidermal DEF function on third whorl development in a def mutant background in transgenic or somatic L1 chimeras.

AFI::DEF/GLO flowers frequently formed petaloid first whorl organs in all genetic backgrounds tested. In colour, surface structure (Fig. 4E,F) and morphology (Fig. 4C), these resembled second whorl organs of def; AFI::DEF flowers (Fig. 1C), although overall their phenotype was more variable and less uniform, even within a flower. Petaloid development was more pronounced in the dorsal first whorl sepal and was least obvious in the lateral organs (Fig. 4A-D). Petaloid dorsal sepals were often split into two halves and ventral sepals appeared as composite structures displaying two or more ‘lobes’. Some of these ‘lobes’ remained sepal-like and green, while the adjacent ‘lobe’ resembled wild-type petals. However, even sepaloid regions were enlarged and modified in shape compared to genuine sepals, thus revealing non-autonomous control of sub-epidermal cell number and organ shape in whorl 1.

Analysis of DEF/GLO transcription by RT-PCR (which can distinguish between endogenous transcripts and transcripts originating from the transgene) indicated that the level of endogenous gene expression is enhanced in petaloid parts of the organs, relative to that in wild-type sepals (not shown). In situ studies detected weak sub-epidermal DEF/GLO expression in older petaloid first whorl organs, and also epidermal expression in regions where sub-epidermal DEF/GLO expression is absent (Fig. 4G,H). Thus, transcription of DEF/GLO in the epidermis does not always facilitate sub-epidermal DEF/GLO expression.

As mentioned above, sub-epidermal DEF/GLO expression in epidermal chimeras can result from invasions of wild-type L1 cells (Perbal et al., 1996). Such invasions are characterised in petals by the presence of a chlorophyll-less, fully petaloid margin made up of wild-type L1-derived sub-epidermal cells that express DEF/GLO. L2-derived def mutant sub-epidermal cells inside the margins are revealed by their light green colour, due to non-autonomous partial suppression of chlorophyll synthesis. The morphology conferred by sub-epidermal cell invasion in petaloid sepals of def; AFI::DEF flowers slightly resembles the phenotype of petals of somatic L1 chimeras, as illustrated by the dorsal organ in Fig. 4C. Since the AFI promoter is inactive in L1-derived sub-epidermal cells (see Introduction), DEF/GLO expression there has to be independent of AFI promoter activity. It is possible that AFI::DEF/GLO expression enhances epidermal transcription of the endogenous DEF/GLO genes, which then can be maintained by the autoregulatory mechanism even if epidermal cells acquire a sub-epidermal identity.

In many transgenic first whorl organs, however, the structure of petaloid sectors cannot be explained by invasions of cells from the L1 layer. For instance, dorsal first whorl organs of severely affected flowers are usually completely petaloid, and show no residual sub-epidermal sepaloidy (Fig. 4A,B), as neither do some of the petaloid sectors of lateral or ventral organs (Fig. 4B-D). For L1 invasions to account for such complete transformations, all L2 cells within fully petaloid sectors would have to be ‘expelled’ by L1 cells. This seems very unlikely. Even less likely are processes that reverse L1 invasion, to allow the formation of a sepaloid sector on top of a fully petaloid one (lateral organ in Fig. 4A). Enhancement of endogenous DEF/GLO transcription in L2 induced by signalling from the petaloid epidermal cells (Fig. 2) would seem to be the most probable explanation for these observations. Whether enhancement by signalling is achieved by transcriptional activation of otherwise silent endogenous genes, or by reinforcement and maintenance of low-level transcription cannot be decided.

Positional preferences for first whorl organ transformations in the double transgenic chimeras correspond to preferences observed in mutants in which spatial control of class B gene expression is impaired (Wilkinson et al., 2000). This coincidence suggests that the enhanced petaloidy of AFI::DEF/GLO first whorl organs is influenced by a corresponding distribution of endogenous factors that promote the manifestation of the B function. Apparently, these factors are crucial for wild-type petal development – in addition to class B genes – and are uniformly expressed in the second whorl, but less regularly distributed in the first whorl. Fluctuations in AFI promoter activity in the first whorl
may, in addition, cause variability in the size and phenotypic appearance of organs.

As summarised in Fig. 2, simultaneous epidermal expression of DEF and GLO in the first whorl autonomously controls cell shape and non-autonomously influences, to a limited extent, the petal cell identity and organ shape in the sub-epidermal layers. This is similar in overall effect to the influence of epidermal class B function on sub-epidermal cell fates in whorl 2. In addition, epidermal signalling can reinforce endogenous sub-epidermal DEF/GLO transcription and thereby enhance petaloid development. The variability of this event indicates that additional factors required for petal development are non-uniformly distributed.

Phenotypes of flowers expressing DEF in the epidermis of an ap3 mutant indicate a great degree of non-autonomy for the DEF function in Arabidopsis

That DEF is functional in Arabidopsis was demonstrated previously (Irish and Yamamoto, 1995; Samach et al., 1997). To test its ability to control petal and stamen organ identity by autonomous and non-autonomous processes when expressed in the epidermis, the AFI::DEF transgene was introduced into an ap3 mutant background. Restoration of wild-type petal and stamen features, as shown in Figs 5D,E and 6, indicates that sub-epidermal development can, in large part, be controlled by DEF-dependent signalling from the epidermis, in contrast to the more limited influence of epidermal DEF in Antirrhinum discussed above.

Second whorl petals of transgenic flowers have an essentially wild-type shape and are larger than true sepalas or ap3 second whorl organs (Fig. 5C,D). In surface morphology, as well as in size and shape of their sub-epidermal cells they are indistinguishable from wild-type petals (Fig. 6). The shape of stamens in the third whorl is close to wild type, and pollen production occurs in the theca (Fig. 6L). Since sub-epidermal cells in petals and pollen in stamens originate from L2, these findings indicate non-autonomous control of development by epidermal class B function in the transgenic chimeras. Epidermal DEF also conditions a mild stamenoid transformation of carpels within the third whorl (Fig. 6C), because PI is expressed in the centre of the flower and can form functional heterodimers with DEF (Samach et al., 1997).

However, petals and stamens in ap3; AFI::DEF flowers remain immature, in that elongation of petals and stamen filaments after stage 12 of development (Smyth et al., 1990), suppression of chlorophyll formation, and pollen maturation are incomplete. Since the floral phenotype of ap3; FDH::AP3 plants (not shown) did not differ from that of ap3; AFI::DEF epidermal chimeras we can exclude the possibility that inability of DEF to direct late developmental events in Arabidopsis is responsible for late defects. Possibly, AFI promoter activity decreases during later stages, and hence DEF (or AP3) activity might not reach the threshold necessary for wild-type function (see below). Incomplete late development may then relate to properties of the AFI promoter, rather than to temporal changes in cell communication. However, in spite of this potential limitation, the full developmental potential of epidermal PI observed with X-ray-induced chimeras (Bouhidel and Irish, 1996) could be reproduced in ap3; AFI::DEF epidermal chimeras.

Expression of DEF and GLO in the epidermis of wild-type Arabidopsis plants results in changes in organ identity at ectopic positions

The effect of epidermal DEF and GLO expression in a wild-type background was studied to see whether the autonomous and non-autonomous influence of epidermal B function on petal and stamen identity is whorl-specific.

Epidermal expression of DEF causes mild to severe stamenoid transformations in fourth whorl carpels, and proliferation of additional (usually 2+4) immature stamenoid organs within whorl 3 (Fig. 7B), reminiscent of the phenotype of superman mutants (Sakai et al., 1995). The homeotic changes are more pronounced in double transgenic lines carrying the AFI::DEF/GLO transgenes (Fig. 7D). This enhancement could be due to an insufficient level of endogenous PI expression in the centre of the flower, as was also observed on comparing transgenic lines expressing AP3 and AP3/PI under the control of the CaMV 35S promoter (Krizek and Meyerowitz, 1996). AFI::DEF/GLO flowers also display petaloid transformation of first whorl organs that resemble immature wild-type petals and whose shape, colour (Fig. 5B) and surface features (not shown) are very similar to those of second whorl organs of ap3; AFI::DEF flowers. Thus, unlike the case in Antirrhinum, endogenous class B genes do not seem to facilitate epidermal DEF/GLO non-autonomy in Arabidopsis. Furthermore, homeotic transformation of first whorl organs is more uniform in Arabidopsis, compared to the pronounced variability of Antirrhinum AFI::DEF/GLO first whorl organs (Figs 5B, 4A-D). This suggests that in Arabidopsis factors other than class B genes that promote the adoption of petal identity are similarly distributed in whorls 1 and 2. Experiments performed with FDH::AP3 and FDH::AP3/PI transgenes and not described in detail in this report resulted in phenotypes similar to those described for AFI::DEF and AFI::DEF/GLO (Table 1).

The APETALA3/GLOBOSA combination is non-functional in Arabidopsis

Surprisingly, epidermal expression of GLO alone has no phenotypic effect (Fig. 7E) although, as shown above, AFI::GLO is functional in Arabidopsis when combined with AFI::DEF. Endogenous AP3 expression is sufficient for mild homeotic first whorl transformation when combined with epidermal PI expression (Fig. 7F), but even enhancement of epidermal AP3 expression in FDH::AP3/AFI::GLO double transgenic plants does not result in phenotypic alterations in whorl 1 (Table 1). It seems then that the AP3/GLO heterodimer (in contrast to the DEF/PI heterodimer) either cannot form or is not functional in Arabidopsis. The reason why the AP3/GLO combination does not function in Arabidopsis was not pursued. It could either be due to impaired heterodimer formation and/or to inadequate interaction of the protein complex with ternary factors. Our inability to detect the role of GLO in Arabidopsis illustrates the limitations of using heterologous species as test systems to evaluate the function of proteins in their normal cellular environments.

In situ hybridisation studies with DEF and PI in Arabidopsis epidermal chimeras indicate the absence of mRNA trafficking

DEF expression was determined in AFI::DEF/GLO transgenic plants with an otherwise wild-type background to discover
whether RNA trafficking could account for the non-autonomous effects seen in *Arabidopsis*. As shown in Fig. 8A-C, this can be excluded, since DEF expression during early and late developmental stages is only detectable in the outermost cell layer. The in situ hybridisation pattern for DEF also shows that AFI promoter activity decreases during late development of floral organs, as indicated by the weak hybridisation signal in older sepals compared to (developmentally younger) petals or (stamenoid) carpels of the same flower (Fig. 8B). Furthermore, epidermal expression of DEF in stamens is reduced compared to petals or (stamenoid) carpels.

AP3 and PI are not expressed in AFI::DEF/GLO first whorl petaloid sepals (Fig. 8D,E) suggesting that epidermal expression of the endogenous class B genes cannot be influenced by DEF/GLO. In addition, hybridisation of sections from a FDH::AP3/PI flower with a PI probe (Fig. 8F) demonstrates the absence of a sub-epidermal hybridisation signal in whorl 1. Thus, in contrast to Antirrhinum, where the endogenous class B genes were shown to be inducible by the epidermally expressed functions (Fig. 4G,H), B function provided by the AFI::DEF/GLO or FDH::AP3/PI epidermal transgenes does not influence transcription of the endogenous PI or AP3 genes in *Arabidopsis*.

**DISCUSSION**

**Communication between cell layers during acquisition of identity by floral organs in Antirrhinum**

The identity of floral organs becomes manifest as specific combinations of cell and tissue types derived from different layers. The number, shape and size of the different cells that arise during development must be co-ordinated, in order to produce complex mature structures with distinct functions. Our observations on transgenic *Antirrhinum* flowers expressing the DEF and GLO functions, which control organ identity, in the epidermis show that cell-cell communication indeed contributes to petal development, as revealed by alterations in cell shape and number as well as suppression of chlorophyll synthesis in the sub-epidermal tissue. In *Antirrhinum*, the developmental influences of epidermally expressed DEF and GLO are comparable, indicating that the two proteins do not differ in their ability to affect the differentiation of sub-epidermal cells.

However, non-autonomous control of the fate of L2 cells by L1 cells in def or glo mutant petals is incomplete, suggesting that the DEF/GLO proteins themselves must be present in sub-epidermal cells to ensure normal development. This finding implies that autonomous control of a subset of target genes in
L2 is necessary for sub-epidermal development. Indeed, wild-type sepals that carry the wild-type DEF/GLO genes in all layers (but are devoid of B function) can develop fully wild-type petal sectors when DEF/GLO are functional in the epidermis. Endogenous DEF/GLO transcription is enhanced in sub-epidermal cells of such transgenic first whorl organs and this is most probably achieved by DEF/GLO-controlled signalling from the epidermis (Fig. 2). This non-autonomous mechanism would result in a balance of B function in adjacent layers, which is perhaps necessary for normal petal development.

The influence of L1 or epidermis on stamen development is very limited, and this limitation is valid in the third whorl of L1 periclinal chimeras (Perbal et al., 1996) and def mutant AFI::DEF transgenic flowers, as well as in the fourth whorl of the wild-type AFI::DEF/GLO double transgenic plants. This suggests that stamen development depends on the concerted action of the B function in all cell layers. However, it is difficult to determine the extent to which the mechanisms of cell communication in petals and stamens differ, because stamen development (but not petal development) is very sensitive to changes in DEF/GLO function during organ initiation and at early stages in the differentiation process (Zachgo et al., 1995). Such early defects then irreparably affect organ identity in the third whorl, precluding study of cell communication during later development. Ongoing experiments with plants expressing the AFI::DEF transgene in the temperature sensitive def-101 mutant background will allow us to study the epidermal contribution of the B function during later stages of stamen development.

Communication between layers during specification of identity in floral organs of Arabidopsis: conflicting observations in different experiments

In Arabidopsis epidermal B function provided by the AFI::DEF or FDH::AP3 transgenes is sufficient to control petal and stamen epidermal cell identity and all sub-epidermal developmental events. Epidermal control is equally efficient in the second and third whorls of ap3 mutant flowers and in the first and fourth whorls of wild-type flowers. This agrees well with the developmental potential of epidermally expressed PI (Bouhidel and Irish, 1996), but is at variance with the whorl-specific differences in the response of layers to the B function and the absence of epidermal control of stamen development.
observed with transgenic AP3 epidermal chimeras (Jenik and Irish, 2001). To explain this contradiction we assume that the 927-bp AP3 promoter fragment used to control expression of the AP3 transgene in the latter experiments is insufficient to achieve wild-type AP3 expression levels in individual layers in whorl 3, although it seems to be capable of directing wild-type stamen development when used to express AP3 in all layers simultaneously. This assumption is supported by the non-autonomy of PI in directing stamen development, observed with X-ray induced sectorial chimeras – and hence under conditions where a class B gene is controlled by its endogenous promoter (Bouhidel and Irish, 1996). According to this interpretation wild-type organ identity in Arabidopsis can be governed by wild-type epidermal B function via signalling to sub-epidermal cells, or by a direct transcriptional control of class B target genes by sub-optimal levels of B function in all layers. Furthermore, as in Antirrhinum, the two class B genes in Arabidopsis do not differ with respect to their potential to control organ identity by cell communication.

We would like to note that signal intensity in the epidermal layer appears to be crucial for detection (or exclusion) of non-autonomy of the function controlling production of that signal. For instance, as recently reported, epidermal APETALA1 (API) function in transgenic Arabidopsis flowers expressing API under the control of the L1-specific AtML1 promoter in an ap1 mutant background revealed two different classes of mutant phenotypes (Sessions et al., 2000). The majority of plants displayed second whorl organs (which fail to develop in the ap1 mutant) with petal epidermal cell types, but without influence on sub-epidermal cell identity. The stronger class (representing a minority of transgenic lines) revealed a more pronounced rescue of petaloid identity of sub-epidermal cells, together with a ‘gain of API function’ phenotype and ectopic activation of AP3. Whether the level of API expression provided by the ML1::API transgene exceeds the wild-type level in the restored petals, or whether it is more wild-type-like in the weaker plants is not known. It is therefore difficult to decide whether the non-autonomous features of API are conferred by enhanced signal intensity due to over-expression, or, vice versa, low signal intensity due to insufficient API expression prohibits its detection of non-autonomous functions of API. To conclusively determine the role of signalling between layers in wild-type development for any given gene it will be important to compare transgenic chimeras (possibly representing enhanced or decreased transcript levels) with genetic chimeras (representing the endogenous level of transcription), as exemplified by the class B genes in this report.

**Differences in the non-autonomous control of organ identity in Arabidopsis and Antirrhinum**

The difference between the two species with regard to the extent of non-autonomy of the B function in Arabidopsis compared with Antirrhinum reported here is not due to intrinsic properties of the AP3/PI or DEF/GLO proteins themselves, because we used the same pair of genes in both species. It seems rather that the mechanisms involved in sending/receiving a B-function-dependent ‘signal’ between layers differ. Possibly, factors are present in Arabidopsis that reinforce the ability of the DEF/GLO (and AP3/PI) proteins to control epidermal production and transmission, or sub-epidermal reception of the signal. The nature of the epidermal ‘signal’ remains unknown, although DEF/GLO RNA trafficking most likely can be excluded. Similarly, protein movement from L1 to L2 was not observed in previous studies with DEF in Antirrhinum (Perbal et al., 1996) or AP3 in Arabidopsis (Jenik and Irish, 2001). At the site of reception it appears that a subset of the sub-epidermal target genes controlled by the B function in Antirrhinum requires the physical presence of class B proteins in these cells, while these targets can be activated indirectly, by signalling, in Arabidopsis. It is also possible that, at least in petals, epidermal signalling in both species can control most if not all sub-epidermal target genes, but in Antirrhinum the threshold for this control is high and cannot be fully satisfied by the epidermal signal. According to this scenario, the transcriptional control of DEF/GLO target genes and petal L2 identity is facilitated by, but does not completely depend on, the physical presence of the DEF/GLO proteins. This can now be tested by comparing gene expression in petals of wild type and transgenic epidermal chimeras; one would expect to find only quantitative differences if this idea is correct.

The epidermal contributions to stamen development in Arabidopsis and Antirrhinum differ dramatically. This appears to be due to differences in early mechanisms that operate during organ initiation in whorl 3, which can be regulated by signalling from the epidermis in Arabidopsis, but depend on a concerted and B-dependent control in all layers in Antirrhinum. In fact, stamen-specific cell division patterns in whorl 3 are independent of the presence of class B gene expression before stage 6 of development in Arabidopsis (Hill and Lord, 1989), but stamen development is irreversibly affected in Antirrhinum by reducing the level of B function long before flowers reach a comparable developmental stage (Zachgo et al., 1995).

Interestingly, a less prominent role for cell-cell communication between layers in Antirrhinum compared to Arabidopsis was also observed for FLORICULA (FLO; Coen et al., 1990) and LEAFY (LFY; Weigel et al., 1992), a pair of orthologues that control floral meristem identity in Antirrhinum and Arabidopsis, respectively. While LFY is fully non-autonomous in each layer (Sessions et al., 2000), this is true for FLO only when it is sub-epidermally expressed (Hanetke et al., 1995). The difference in the relative contribution of cell communication to cell identity in Antirrhinum and Arabidopsis, observed in the case of class B genes and also for genes that control meristem identity, is intriguing, because it demonstrates that plants can use different strategies to realise their wild-type morphologies. The availability of genetically stable transgenic plants will allow us now to search for the genes responsible for conferring the observed restrictions on cell communication in Antirrhinum.

We are grateful to Drs Sabine Zachgo, Peter Huijser and Brendan Davies for discussions during preparation of this manuscript, Dr Paul Hardy for proof-reading the text and Sybille Richter and Manfred Pohe for the maintenance of plants. This work was in part supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 243) to Z. Sch.-S.

**REFERENCES**

Bechtold, N., Ellis, J. and Pelletier, G. (1993). In-planta Agrobacterium-


