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Functional analysis of the *Antirrhinum* floral homeotic *DEFICIENS* gene in vivo and in vitro by using a temperature-sensitive mutant

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SUMMARY

Flowers of the temperature-sensitive *DEFICIENS* (*DEF*) mutant, *def*-101, display sepaloid petals and carpelloid stamens when grown at 26°C, the non-permissive temperature. In contrast, when cultivated under permissive conditions at 15°C, the morphology of *def*-101 flowers resembles that of the wild type. Temperature shift experiments during early and late phases of flower development revealed that second and third whorl organ development is differentially sensitive to changes in *DEF* expression. In addition, early *DEF* expression seems to control the spatially correct initiation of fourth whorl organ development. Reduction of the *def*-101 gene dosage differentially affects organogenesis in adjacent whorls: at the lower temperature development of petals in the second whorl and initiation of carpels in the centre of the flower is not affected while third whorl organogenesis follows the mutant (carpelloid) pattern. The possible contribution of accessory factors to organ-specific DEF functions is discussed. In situ analyses of mRNA and protein expression patterns during *def*-101 flower development at 15°C and at 26°C support previously proposed combinatorial regulatory interactions between the MADS-box proteins DEF and GLOBOSA (GLO), and provide evidence that the autoregulatory control of *DEF* and *GLO* expression by the DEF/GLO heterodimer starts after initiation of all organ primordia. Immunolocalisation revealed that both proteins are located in the nucleus. Interestingly, higher growth temperature affects the stability of both the DEF-101 and GLO proteins in vivo. In vitro DNA binding studies suggest that the temperature sensitivity of the *def*-101 mutant is due to an altered heterodimerisation/DNA-binding capability of the DEF-101 protein, conditioned by the deletion of one amino acid within the K-box, a protein region thought to be involved in protein-protein interaction. In addition, we introduce a mutant allele of *GLO*, *glo*-confusa, where insertion of one amino acid impairs the hydrophobic carboxy-terminal region of the MADS-box, but which confers no strong phenotypic changes to the flower. The strong mutant phenotype of flowers of *def*-101/*glo*-conf double mutants when grown in the cold represents genetic evidence for heterodimerisation between DEF and GLO in vivo. The potential to dissect structural and functional domains of MADS-box transcription factors is discussed.

Key words: flower development, gene expression, MADS-box, in vitro DNA binding, *Antirrhinum*, *DEFICIENS*

INTRODUCTION

Mature flowers of *Antirrhinum* are composed of a whorl of sepals, the first organs initiated, followed by a whorl of petals, then stamens and, initiated lastly, an internal whorl of carpels. The distinct structural and cellular identity of organs within each whorl is controlled by a group of homeotic genes (for reviews see Coen and Carpenter, 1993; Coen and Meyerowitz, 1991; Schwarz-Sommer et al., 1990). Altered expression of these regulatory genes results in homeotic organ transformations, in which an organ type develops at a position where it would not occur in the wild-type flower. *DEFICIENS* (*DEF*; Schwarz-Sommer et al., 1992; Sommer et al., 1990) and *GLOBOSA* (*GLO*; Tröbner et al., 1992) are flower-specific homeotic genes in *Antirrhinum* which control petal organogenesis in the second, and stamen organogenesis in the third whorl. In the absence of either of these functions sepaloid organs form instead of petals and carpelloid organs form instead of stamens. In addition, initiation of carpel organogenesis in the centre of the flower is abolished. The spatial and temporal requirements of DEF and GLO proteins for wild-type organogenesis are currently unknown.

The DEF and GLO proteins are transcription factors containing the MADS-box, a DNA-binding domain (Schwarz-Sommer et al., 1990), and the K-box, a potential dimerisation domain (Ma et al., 1991). Molecular cloning of these genes allowed the study of their transcriptional regulation and interaction during flower development. Using these studies as a basis we have previously suggested that (1) in vitro, the two proteins interact to form a heterodimer and that the DEF/GLO protein complex binds to DNA, and (2) maintenance of transcription of the two genes in petals and stamens, but not the establishment of their initial expression, is controlled by an autoregulatory mechanism (Schwarz-Sommer et al., 1992;

Tröbner et al., 1992). Homologous floral MADS-box genes in various species, for example *APETALA3* (*AP3;* Jack et al., 1992) and *PISTILLATA* (*PI*; Goto and Meyerowitz, 1994) in *Arabidopsis* or *pMADS1* and *FBP1* in *Petunia* (Angenent et al., 1992, 1993; van der Krol et al., 1993) share several aspects of regulation, structure and function with the *DEF* and *GLO* genes of *Antirrhinum*. However, striking differences exist as well, for example, the functional interdependence between *DEF* and *GLO* in the maintenance of transcription of either gene operates in both second and third whorl organs (Tröbner et al., 1992), while transcriptional regulation of *AP3* in petals is independent of *PI* gene function (Jack et al., 1992). Furthermore, whereas maintenance of *PI* transcription requires AP3 function in both whorls, pMADS1 in petunia only controls expression of *FBP*1 in petals (van der Krol et al., 1993). The molecular basis of these differences is not known.

The *def*-101 mutation, which causes a deletion of a lysine at position 93 of the DEF protein at the beginning of the first putative amphipathic helix within the K-box, confers temperature sensitivity to flower development (Schwarz-Sommer et al., 1992). This made possible the manipulation of the amount of functional DEF protein during floral organogenesis and hence revealed the specific developmental stages during which *DEF* function is necessary for petal and stamen organogenesis. In addition, the mode of interaction between the DEF and GLO proteins in vitro and in vivo was studied.

MATERIALS AND METHODS

Plant material

The origins of the *def*-101 mutant and of the wild-type line T53 were described previously (Schwarz-Sommer et al., 1992). The *glo*-confusa mutant of *Antirrhinum* was obtained by R. Carpenter in Norwich as a heterozygote in the F_2 of a cross between the stable *glo*-26 allele and the *cycloidea*-25 allele. The homozygote was subsequently established by a series of crossings and self-pollinations.

Germination and subsequent growth of plants took place in the greenhouse at 18-22°C. After formation of 2 to 3 internodes, young plants were transferred into climate chambers where they were grown at 15°C (permissive temperature) or 26°C (non-permissive temperature). The light intensities and day-night regimes (16 hours light and 8 hours dark) were identical, as was the humidity (60%). Further increase or decrease in temperature did not result in more extreme phenotypes of *def*-101 flowers, but affected the overall development of the plants.

SEM and in situ hybridisation

Scanning electron microscopy (SEM) and in situ hybridisation followed previously described protocols (Huijser et al., 1992; Sommer et al., 1990).

Immunolocalisation

The def and glo cDNAs (without the MADS-box region) were cloned into the pQE 16 vector (Qiagen). For immunisation the DEF and GLO proteins were expressed in *E. coli* as fusion proteins with mouse dihydrofolate reductase and purified on a Ni-affinity column (Qiagen) according to the manufacturer's protocol. Immunisation of rabbits was carried out by EUROGENTECH (Belgium) using 200 μg protein per injection. After 4 boosts the sera were tested for specificity in western blots using the bacterial proteins and proteins obtained by in vitro translation of the cRNAs (see below). For further experiments only sera, which did not react with unrelated proteins, were used.

The antibodies were affinity purified by using a western blot purification procedure (Burke et al., 1982). Flowers were fixed, embedded and sectioned similarly to tissue prepared for in situ hybridisation, except that fixation was at 15°C to avoid protein degradation. After incubation with the affinity purified first antibodies, bound antibodies were detected using the VECTASTAIN ABC elite kit (VECTOR Laboratories, Burlingame, California). The detection system uses a biotinylated secondary antibody, which is incubated with an avidin/biotinylated-peroxidase complex. Detection of the amplified signal was carried out with diaminobenzidine (ready tabs, Sigma). The sections were counter-stained with 4′, 6-diamidine-2′-phenylindole dihydrochloride (DAPI; 1 μg/ml, dissolved in PBS) to determine subcellular localisation.

In vitro DNA-binding assay

In vitro transcription, in vitro translation and gel retardation analysis of DNA/protein complexes were performed as described previously (Schwarz-Sommer et al., 1992; Tröbner et al., 1992).

In vitro binding assays were carried out using the oligonucleotide GGCAACTCTTT**CCTTTTTAGG**TCGCATATGG derived from the *DEF*-CArG1 box within the *DEF* promoter sequence (Schwarz-Sommer et al., 1992; Tröbner et al., 1992). The double stranded oligonucleotide contained at each end a 5′ protruding G nucleotide and was labelled using the Klenow polymerase. 0.3 ng of the ^{32}P-labelled DNA was added per assay to the in vitro co-translated DEF/GLO proteins.

Deletion derivatives

All deletion constructs are derived from the pT7β DEF and the pT7β GLO plasmids (Schwarz-Sommer et al., 1992), which contain the leader-less full-size cDNAs inserted between the *Nco*I and *Eco*RI sites (pT7β DEF) or the *Acc*I and *Eco*RI sites (pT7β GLO) of the pT7β Sal vector (Norman et al., 1988). The structure of deletion derivatives and the position of the deleted amino acids within the DEF and GLO protein sequences are presented in Fig. 10B. Arrowheads show the internal restriction sites which were used either to linearise the plasmids for in vitro transcription or for the plasmid constructions. For generation of some of the constructs PCR amplification with primer sequences derived from the DEF and GLO cDNAs was carried out. The DNA sequences of all constructs were confirmed.

RESULTS

Morphology of mature *def*-101 mutant flowers

Morphological features of wild-type and *def*-101 mutant flowers were described previously in detail (Schwarz-Sommer et al., 1992; Sommer et al., 1990). In the following section only features relevant to the interpretation of data presented in this report will be considered.

The perianth of wild-type flowers contains five green sepals in the first whorl and five petals of a distinct shape in the second whorl. The third and fourth whorls comprise the sex organs, four stamens and one stamenodium and a bilocular gynoecium, respectively. Flowers of *def*-101 plants display different phenotypes depending on the growing temperature (Fig. 1). At low temperature (15°C, the permissive condition), wild-type-like flowers develop (Fig. 1A). The petals of *def*-101 flowers are slightly smaller than the wild-type organs, and the curvature of the corolla is reduced. The third whorl contains four fertile stamens and an aborted stamenodium at the uppermost position. The filaments of the stamens may appear normal or as a broadened structure and they can partially fuse at their base with sepaloid and petaloid protrusions. The gynoecium develops as in the wild type and contains two (less frequently three) locules with placentas and fertile ovules.

Plants cultured at a higher temperature (26°C, the non-per-

missive condition), display flowers similar to those of *def*-gli, a null mutant of the *DEF* gene (Schwarz-Sommer et al., 1990; Sommer et al., 1990). The small, green second whorl organs resemble sepals rather than petals and the centre of the flower is occupied by a tube-shaped female structure, tipped with stigmatic papillae (Fig. 1B). Cross-sections through the central organ reveal two groups of central structures which differ with respect to the number of locules (Fig. 1C,D). The central organs of one third of the 67 flowers investigated resembled the central organs of *DEF* or *GLO* null mutants, in that they formed five ovule-filled locules (Schwarz-Sommer et al., 1992; Tröbner et al., 1992). The fifth locule is located at the position of the stamenoid in the wild-type flowers (Fig. 1C, and floral diagram at the right). In two thirds of the flowers this upper fifth locule was empty as it was in somatically unstable DEF and GLO mutants upon somatic restoration of the DEF function (Schwarz-Sommer et al., 1992). This indicates that the *def*-101 function is not fully absent in the mutant. The remaining four locules contained a variable number of placentas (Fig. 1D). To explain the developmental origin of the placentas one should recall that they are composed of two halves which develop at the margins of the individual organs and appear as a single structure when the margins fuse. Incomplete fusion of the margins can result in locules with two and occasionally three non-fused placentas (as an example of incomplete fusion see cross-section in Fig. 4C,D). As the *DEF* function does not seem to be completely abolished at higher temperature, carpelloidy of the third whorl organs (and hence the fusion of the margins) is not always complete in the flowers, resulting in a higher number of placentas. The two lower locules more frequently display such incomplete transformation than the two upper ones. A central gynoecium composed of an upper and lower placenta, as present in the wild type or in *def*-101 flowers at 15°C, has never been observed in *def*-101 flowers when cultured at high temperature.

Early morphological abnormalities of *def*-101 flowers cultured at 15°C and at 26°C

In order to define the stages in which the developmental fates of organs deviate, the flower morphology of *def*-101 plants grown at the lower and at higher temperatures was studied by SEM. To assist in description of these deviations and also for interpretation of morphological data obtained by temperature shift experiments, early events during wild-type floral development were divided into stages (Fig. 2A) which correspond to those defined in more detail and named by R. Carpenter et al. (unpublished data). The appearance of organ primordia of a new organ type was used to recognise and define stages relevant for observations in this report. The sizes of primordia at each stage and the time schedules of their development were not considered, because these parameters vary at different growth temperatures.

In stage 0 the bract primordium is initiated as a bulge on the flank of the inflorescence apical meristem. Stage 1 begins with the initiation of the (eye-shaped) floral primordium in the axil of the bract, once the tip of the bract primordium starts to surpass the height of the apex. During further growth the primordium adopts a rectangular shape (loaf), characterising stage 2. During stage 3 the symmetry of the primordium changes to pentameric (pentagon) and the stage ends with the initiation of two lower sepal primordia and slightly later by initiation of the three upper sepal primordia. During stage 4 (the floritypic stage) sepal primordia become separated from the floral apex and the organs start to overgrow it. Stage 5 starts with the initiation of five petal primordia in the second whorl, at alternate positions with respect to the sepals in the outermost whorl. The initiation of five stamen primordia in the third whorl occurs shortly after initiation of the petal primordia. After their initiation the stamens grow faster than the petals. Once the internal floral whorls are fully enclosed by sepals, stage 6 begins with the initiation of the gynoecium. A groove is formed in the centre of the floral primordium, where the two c-shaped carpel primordia will develop in a linear arrangement

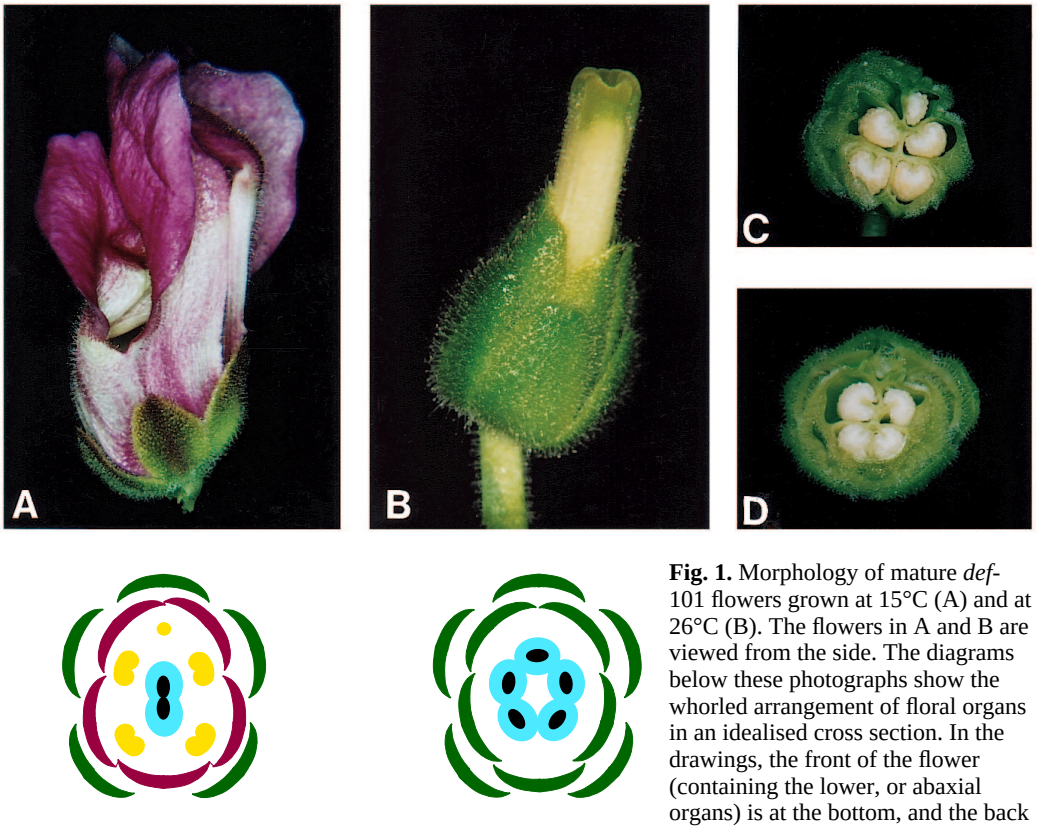


Fig. 1. Morphology of mature *def*-101 flowers grown at 15°C (A) and at 26°C (B). The flowers in A and B are viewed from the side. The diagrams below these photographs show the whorled arrangement of floral organs in an idealised cross section. In the drawings, the front of the flower (containing the lower, or abaxial organs) is at the bottom, and the back of the flower (containing the upper or adaxial organs) is at the top. The colour code and the schematic form indicates organ identity under different growth conditions. The aborted stamen (stamenoid) at the uppermost position is shown by a yellow dot. For the photographs in C and D, flowers grown at 26°C were dissected to show the two prevailing phenotypes of the central female organ.

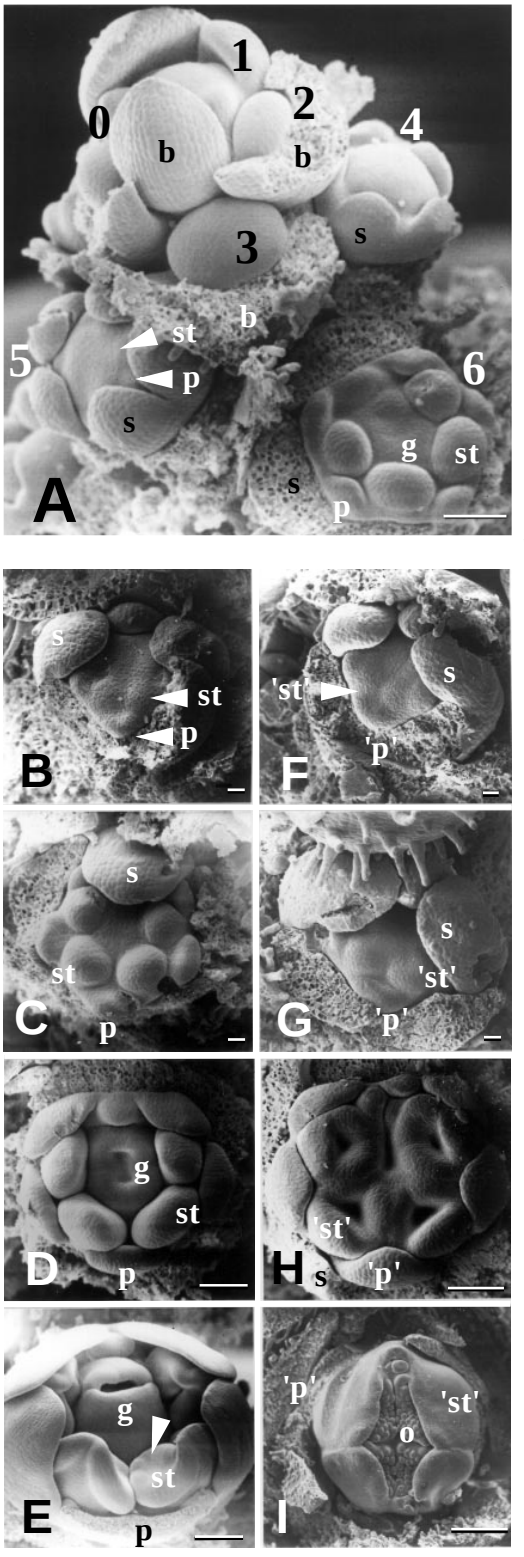


Fig. 2. Early development in wild-type *Antirrhinum* flowers and the effect of temperature on organ differentiation of *def*-101 mutant flowers. The SEM photograph in A shows the tip of a young wild-type inflorescence. Developmental stages are defined in the text and are shown here by numbers. The SEM photographs below (B-I) show side by side individual buds at a similar stage of development obtained from *def*-101 inflorescences grown at 15°C (left panel B,C,D and E) and at 26°C (right panel F,G,H and I). The youngest buds shown in B and F are in early stage 5 and maturity of buds increases from the top to the bottom. The arrow in E points to an irregularly formed *def*-101 anther, marking the first visible deviation of *def*-101 from wild-type development at 15°C. Bracts (b) and sepals (s) were partially removed to reveal covered organ primordia. p, petal; st, stamen; g, gynoecium; 'st', feminised third whorl organ; o, ovules. Bars, 100 μm.

along the upper-lower floral axis. The subsequent stages which lead to flower maturation are not considered here.

At 15°C, the pattern of *def*-101 floral organ initiation and early organ differentiation (Fig. 2B-D) is similar to that of wild-type flowers. The first small deviation from wild-type development becomes visible when differentiation of the anthers starts. On the ventral pollen sacs small additional globular protrusions can be observed, which resemble ovule primordia (Fig. 2E).

At 26°C, overall *def*-101 development is faster than at 15°C, but organ initiation is not affected up to early stage 5 (Fig. 2B,F). However, at late stage 5, second whorl organ development seems to be retarded and the five third whorl stamen primordia cease their development into globular structures (Fig. 2C,G). During stage 6, third whorl primordia display a 'horse-shoe' like structure in the centre of which holes develop (Fig. 2H). The outer parts of these 'horse-shoe' structures fuse and give rise to the outer wall of the complex tube-like organ that occupies the centre of the flower. Inside each of these holes placentas develop, which produce fertile ovules (Fig. 2I).

Sensitivity of petals and stamens to decrease of *DEF* function during early stages of development

Temperature shifts of *def*-101 mutant plants were conducted in order to define the developmental stages during which DEF must be functional for normal organogenesis of stamens and petals. Plants were cultured at 26°C until the inflorescences reached a length between 4 and 8 mm and were then shifted to 15°C ('restoration shift'). When flower maturation was completed the degree of floral organ restoration was investigated.

In order to define the stage of the floral buds at the time of the temperature shift, wild-type and mutant inflorescences were analysed by SEM. The bracts of inflorescences that were visible with the naked eye were counted and the developmental stage of the floral bud shielded by the youngest visible bract was determined. Analysis of about forty inflorescences showed that independent of the total number of visible bracts, the youngest visible bract of inflorescences with a size between 4 to 8 mm always shielded a floral primordium that was in stage 5 (sepal, petal and stamen primordia initiated). The next youngest bud was in an early stage 5, followed by one bud in late stage 4 (sepals start to overgrow the primordium), one at stage 4 and one late stage 3 primordium (sepals are just initiated). Primordia further towards the top of the inflorescence are in stage 2 (no floral organs initiated), stage 1 and stage 0 (bract primordia visible). As buds develop in a predictable order towards the tip of the inflorescence, counting the visible bracts before shifting plants was sufficient to determine the developmental stage of younger primordia prior to the shift.

Fig. 3A shows that after a 'restoration shift' all mature flowers along the axis revealed apparently petaloid second

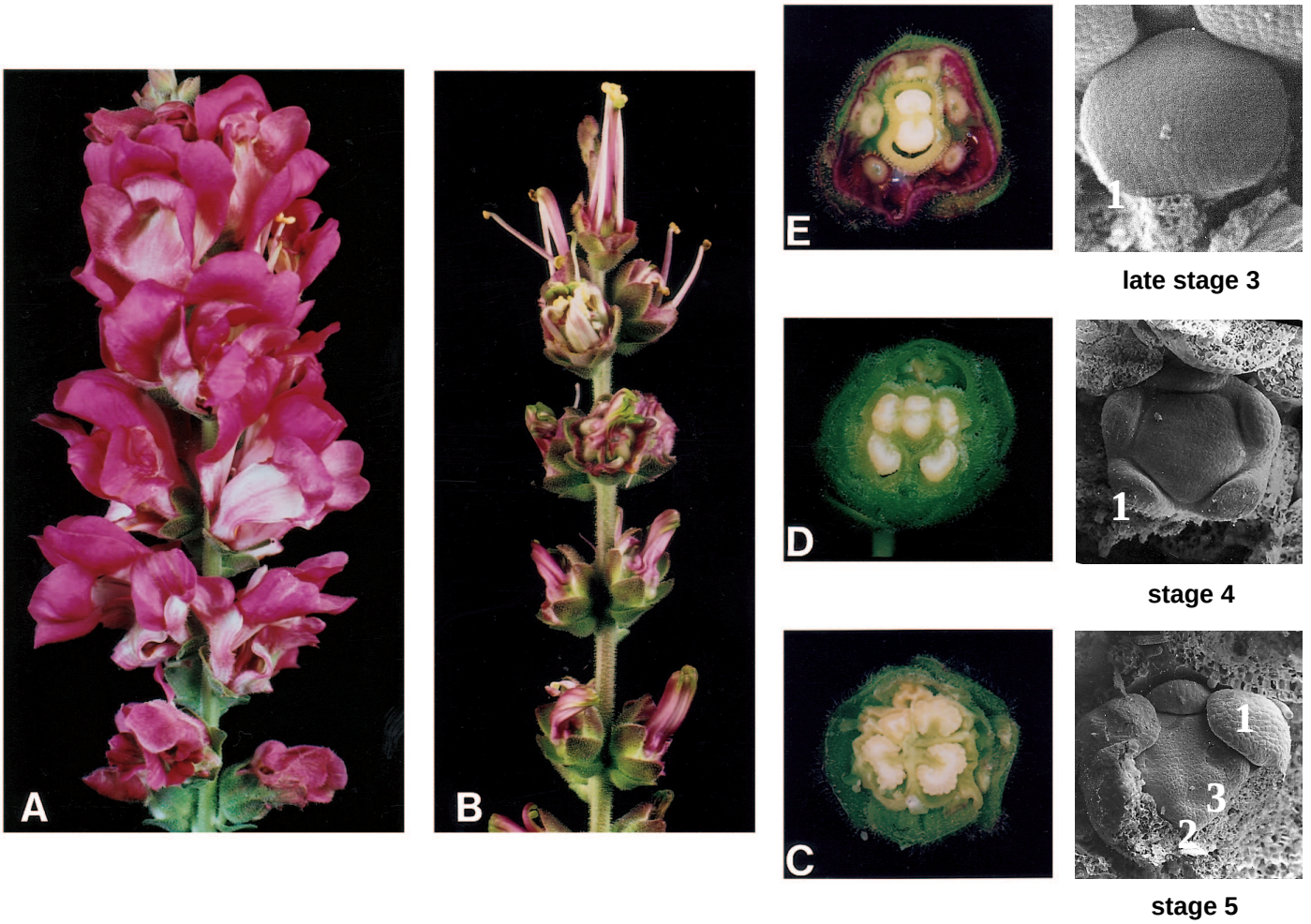


Fig. 3. Effects of shifting *def*-101 plants from 26°C to 15°C during early stages of flower development. A shows mature flowers of a *def*-101 plant which was cultivated during early stages of flower development at 26°C and was then shifted to 15°C ('restoration shift'). In B the petals were removed and C,D,E show cross sections of flowers at the bottom, in the middle and at the top of the same inflorescence. The flowers were of three different phenotypes with respect to their third and fourth whorl morphology: a 'warm-phenotype' (C), an 'intermediate-phenotype' (D) and a 'cold-phenotype' (E). The developmental stage of the corresponding buds at the time when they were shifted was determined by SEM analysis of buds from another plant and is shown at the right of panel C, D and E (for details see text).

whorl organs. However, the degree of sepalody gradually increased the more advanced the development of the bud was prior to the temperature-shift. For example, corolla tube fusion was not restored in flowers that had passed stage 6 at higher temperature. The sensitivity of the two upper and three lower petals to an increase in *DEF* function differed. The morphology of the two upper petals was almost completely restored, while lower petals contained large sepaloid sectors at their base when shifted at later stages of development.

With respect to the extent of stamen restoration and carpel initiation three phenotypes can be observed along the inflorescence axis (Fig. 3B-E). The oldest flowers at the bottom of the inflorescence (Fig. 3B) reveal the 'warm-phenotype': the shift occurred at a developmental stage in which no stamens or genuine carpels could be restored (Fig. 3C). The number of flowers with this phenotype on an inflorescence equalled the number of counted visible bracts. Thus, all flowers with the 'warm-phenotype' had reached or passed stage 5 by the time they were shifted. The next two to three flowers displayed an 'intermediate-phenotype' where separate, strongly feminised stamens are formed instead of the fused central female organ. The youngest of such 'intermediate-phenotype' flowers developed a normal central carpel with two locules and two placentas with ovules (Fig. 3D). According to the developmental scheme outlined above the two to three 'intermediate-phenotype' flowers were in late stage 4 and those containing a central female organ in stage 4 (Fig. 3D) when the temperature-shift was carried out. At the top of the inflorescence, flowers developed with restored third and fourth whorl organs ('cold-phenotype'), similar to flowers of plants cultured at lower temperature (Fig. 3E). It follows that for complete restoration of stamens, buds must have been in late stage 3, where sepal primordia were just initiated, or earlier, before organ primordia were initiated.

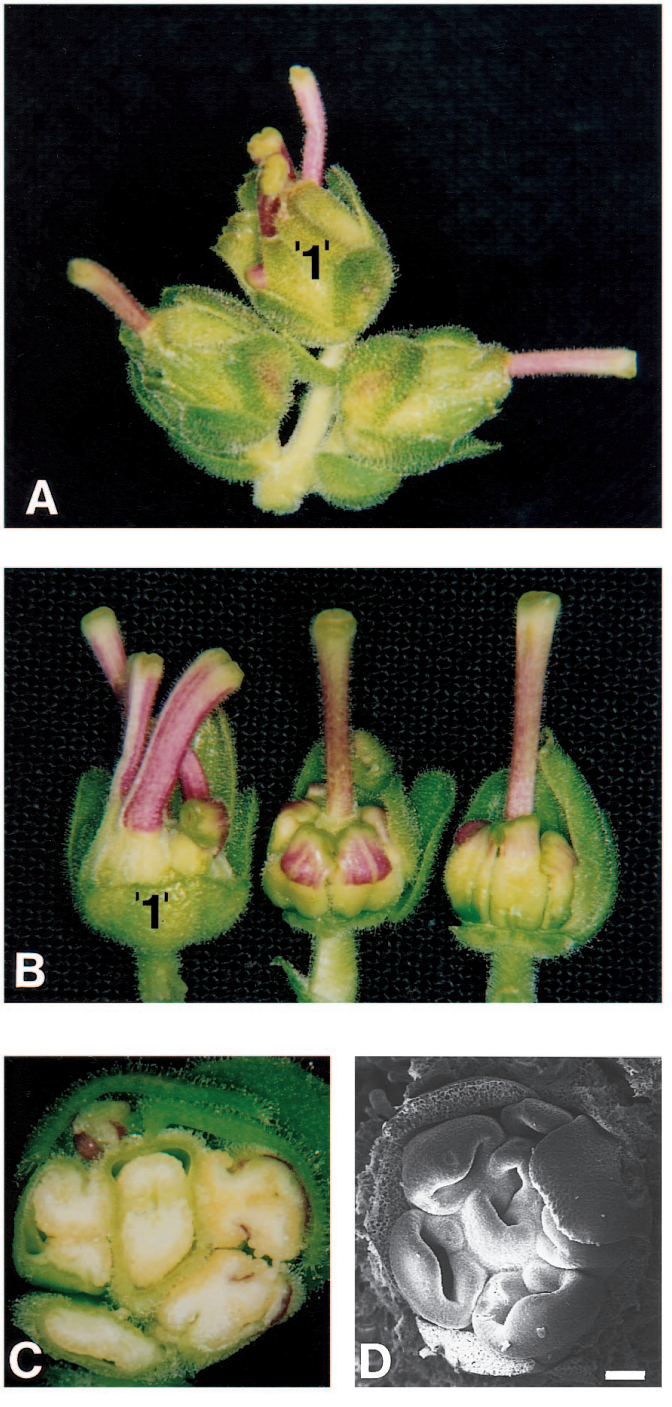
Early *DEF* function is sufficient for carpel initiation in the centre of the flower, but is insufficient to maintain the correct developmental pattern in other whorls

The 'restoration shift' experiment described above indicated that carpel development in the centre of the flower depends on

early *DEF* function. The following ‘decapitation shift’ experiments were conducted to determine more precisely the time schedule of this function.

Control experiments with decapitated wild-type flowers showed that the tip of an inflorescence could be removed without disturbing the development of older buds that remain on the inflorescence. *Def*-101 plants were grown at 15°C and were decapitated before being shifted to the higher temperature. The tip of the inflorescence that was shielded by bracts of older buds was carefully removed and the stage of the oldest bud on the removed tip was determined by light microscopy. It was then possible to predict the developmental stage of the youngest bud on the remaining inflorescence. The oldest bud on the removed tip of the inflorescence shown in Fig. 4 was in stage 4, the earliest stage that could be removed without destroying the older tissue underneath. Thus the youngest bud on the remaining inflorescence was in late stage 4 (see Fig. 11).

Morphological analysis of mature flowers showed that fourth whorl development was normal, if a primordium was shifted from 15°C to 26°C at late stage 4 (Fig. 4B-D). In contrast, the developmental fate of second and third whorl organs of the three youngest buds was severely altered. As *DEF* was functional up to stage 4, third whorl organs were initiated as four separate structures. However, reduction of the *DEF* function after stage 4 resulted in strong feminisation of third whorl organs rather than stamen development. Cross sections of the youngest bud on the remaining inflorescence showed that each of the feminised ‘stamens’ formed a locule with two non-fused placentas (Fig. 4C,D) and a style with stigmatic tissue at the tip (Fig. 4A,B). The styles of the feminised ‘stamens’ of older flowers were shorter and the ovules were no longer shielded in closed locules. Thus, the more advanced the development of the flower buds at the time of the shift to permissive conditions, the less feminisation of stamens occurred.



Effect of early transient or late changes in *DEF* function on organogenesis

‘Double shift’ experiments were used to determine the shortest time period in which reduced early *DEF* function could affect stamen morphology. *Def*-101 plants were cultured at 15°C, shifted for different periods to 26°C and then shifted back to 15°C until maturation. Flowers which had just initiated sepals (late stage 3) at the time they were shifted to the higher temperature revealed an altered morphology after exposure to the higher temperature for only four hours (not shown). The upper stamens were feminised, their filaments were bent and carried ovules. This short temperature pulse at the higher temperature did not result in an altered morphology of the second whorl organs. Increasing the time at the higher temperature up to 24 hours resulted in strong feminisation of both lower and upper third whorl ‘stamens’ (not shown).

To investigate how long *DEF* function must be maintained for normal development of stamens and petals, *def*-101 flowers grown at 15°C until microsporogenesis was complete were shifted to 26°C. Reduction of *DEF* function at this late stage still resulted in an altered morphology of mature stamens and

Fig. 4. Effects of shifting *def*-101 plants from 15°C to 26°C during early stages of flower development. For the ‘decapitation shift’ experiment (see text) *def*-101 plants were cultured at 15°C until formation of a 5 mm long inflorescence. After removing the tip of the inflorescence the plant was shifted to 26°C. The developmental stage of the youngest remaining bud (marked with ‘1’) was late stage 4 (see SEM photographs showing this stage in Figs 2 and 10). A-C show morphological features of mature flowers of a decapitated inflorescence. Notice the sepalody of second whorl organs in A and that all flowers contain a genuine gynoecium in the centre of the flower. In B sepals were partially removed from the flowers shown in A to demonstrate that a decrease in stamen feminisation correlates with longer period of development at the lower temperature. C shows the central bilocular gynoecium in a cross section of the youngest remaining bud. The SEM photograph in D was taken of an immature flower which developed at the same position in an independent ‘decapitation shift’ experiment. Bar, 50 μm.

petals (not shown). Development of petals was slightly affected in that green sectors lined up at the edges of the organs. In the third whorl, ovules formed close to the base of the filaments of both upper stamens, but not on the lower stamens, indicating that organs at different positions in the third whorl show a differential sensitivity to a decrease in *DEF* function.

Expression patterns of *DEF* and *GLO* mRNAs in *def*-101 mutant flowers

Previous studies suggested that the DEF/GLO heterodimer controls the transcription of the *DEF* and *GLO* genes in petals and stamens by an autoregulatory mechanism (Tröbner et al., 1992). In the *def*-101 mutant the structure and function of *DEF* is altered, therefore the study of mRNA and protein expression patterns of the two genes promised an insight into the temporal establishment of this autoregulatory control. For this purpose serial sections prepared from inflorescences of *def*-101 plants grown either at 15°C or at 26°C were probed with in vitro generated ^{35}S-labelled antisense *DEF* and *GLO* transcripts. As in the wild type (not shown), both mRNAs were first detectable in mutant flower primordia grown at both temperatures, at late stage 3 of development, when sepal primordia had just initiated at the flanks of the floral primordium (Fig. 5A,B,E,F).

The earliest signal detected with the *def* probe in inflorescences from plants grown at the lower or at the higher temperature (and also in the wild type, not shown) is located in the centre of the flower, where it occupies about one third of the primordium (Fig. 5A,E). *GLO* mRNA at this stage of development appears as a bipartite signal in the region between the emerging sepals and the central portion expressing *DEF* mRNA (Fig.

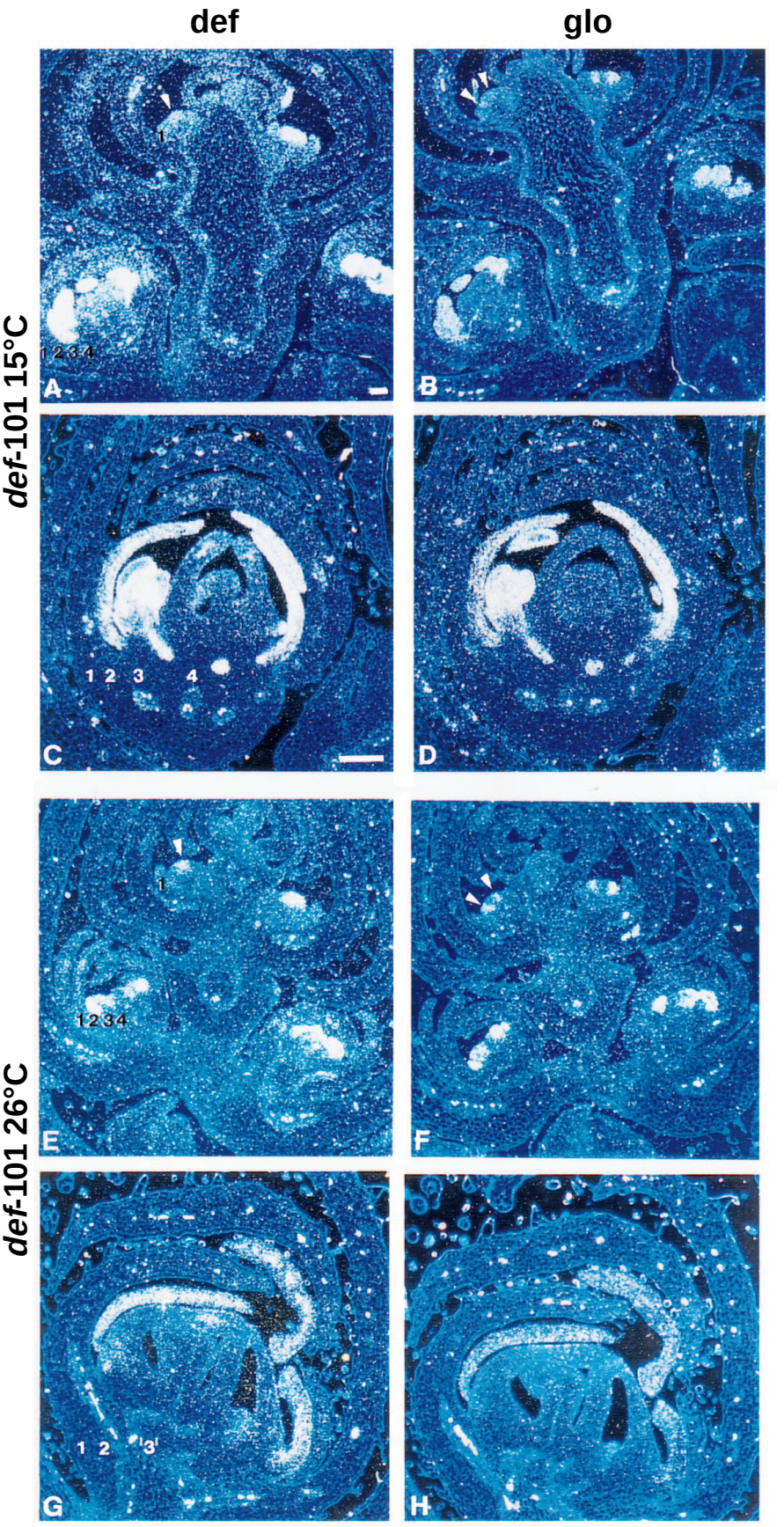


Fig. 5. In situ detection of the mRNA expression pattern of the *DEF* and *GLO* genes in developing *def*-101 flowers. Longitudinal, serial sections of inflorescences were hybridised with ^{35}S-labelled antisense RNA derived from the 3′ end of the *def* (left panel) and *glo* (right panel) cDNAs, not containing the MADS-box. (A,B) Oldest buds in early stage 6, (E,F) oldest buds in late stage 5 and individual buds in late stage 6 (C,D,G,H) of *def*-101 plants cultured at 15°C (A-D) and at 26°C (E-H). The earliest detectable signals are marked with arrowheads. The dark-field exposure, detecting the silver grains, is superimposed on an epifluorescence image to visualise the underlying tissue. 1, 2, 3 and 4 designate the four floral whorls, and '3' designates the central female organ of *def*-101 flowers grown at the higher temperature.

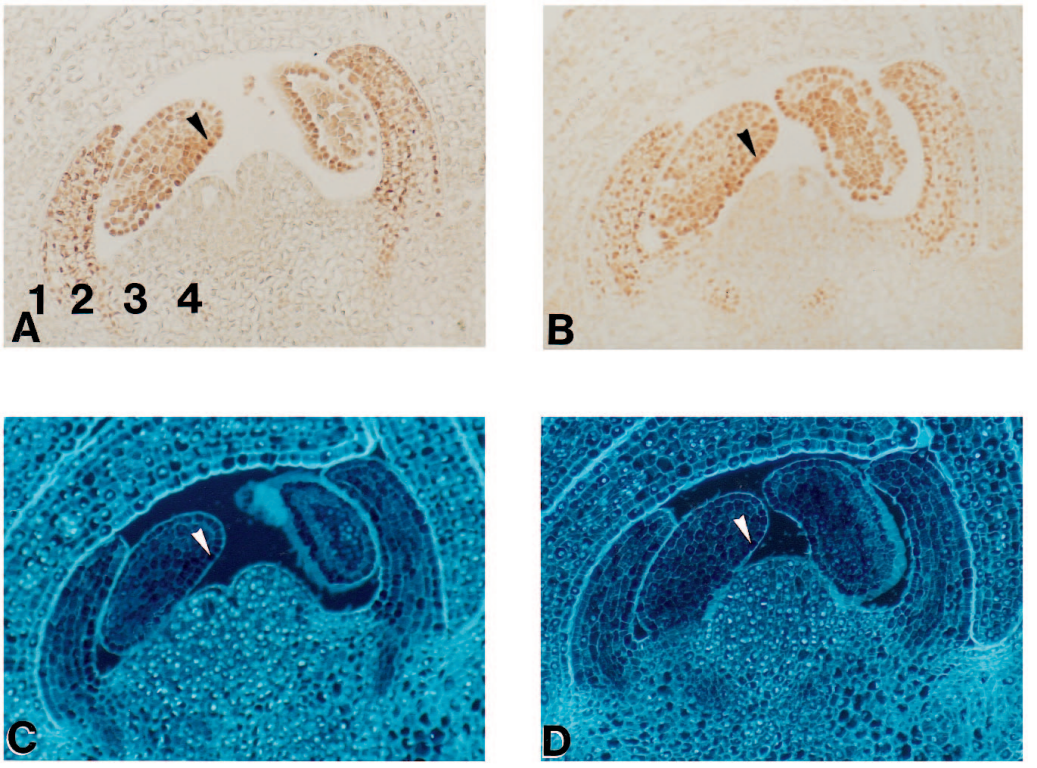


Fig. 6. Subcellular localisation of the DEF and GLO proteins in wild-type flowers. Longitudinal serial sections of developing wild-type flowers (around stage 6) were immunohistochemically stained with affinity purified anti-DEF (A,C) and anti-GLO (B,D) antibodies and subsequently with DAPI (for details see Methods). The sections in A and B were viewed under light-field optics, where DEF and GLO protein expression in petals and stamens appears as a dark brown signal (black arrowhead). The same sections viewed under dark-field optics using fluorescence light reveal DAPI-stained nuclei as white fluorescent structures in a blue background. C and D show that the DAPI fluorescence is quenched only in nuclei that reveal immunolocalisation signals in A and B (white arrowhead). 1, 2, 3 and 4 designate the four floral whorls.

5B,F). This pattern is maintained until stage 4 when the sepals start to overgrow the primordium. After this stage *DEF* mRNA disappears from the central region and also displays a bipartite expression pattern which overlaps with the *GLO* mRNA pattern in the region where the second and third whorl organ primordia will be initiated. The quantity and spatial distribution of *DEF* and *GLO* mRNA is independent of the culturing conditions. Thus the *def*-101 mutation does not affect the initial level of mRNA expression nor the mRNA stability.

The effect of growth at a higher temperature on *DEF* and *GLO* mRNA expression is evident from stage 6 on. During later developmental stages *DEF* and *GLO* mRNA in the second whorl organs is low compared to that in flowers grown in the cold and only very weak expression of both mRNAs is observed in the feminised third whorl organs (Fig. 5C,D,G,H).

Subcellular localisation of the DEF and GLO proteins and their expression patterns in wild-type and *def*-101 mutant flowers

Since no difference in *DEF* and *GLO* mRNA expression patterns was observed between wild-type and *def*-101 flowers at early stages, we examined the corresponding protein expression patterns (Figs 6, 7). Immunolocalisation experiments with the affinity purified antibodies showed that the DEF and GLO proteins are expressed in the petals and stamens of wild-type flowers (Fig. 6A,B). Nuclear location of the proteins was confirmed by counter-staining of the immunohistochemically stained sections with DAPI, a DNA-specific dye. DAPI fluorescence is present in all nuclei except in petal and stamen cells, where it is quenched by the coloured precipitate obtained during immunolocalisation (Fig. 6C,D).

Immunolocalisation of the DEF protein on longitudinal sections of young wild-type inflorescences revealed a strong bipartite signal in buds at stage 4 (Fig. 7A). The signal is visible in those areas of the meristem where the second and third whorl organs will be initiated. When cultured in the cold, *def*-101 mutant flowers display wild-type-like, strong DEF-101 protein expression during early stages of development (Fig. 7B). During later stages, when all organs are initiated, the spatial distribution of DEF expression is still similar to that observed in wild-type buds (Fig. 7D,E) and the amount of DEF-101 protein is only slightly decreased.

Increased temperature does not affect the spatial pattern of DEF protein expression during early developmental stages, although the level of expression is strongly decreased (Fig. 7C). Interestingly, exposure to 26°C for just 30 minutes is sufficient to reduce DEF and GLO protein levels in the nuclei of young *def*-101 flowers grown in the cold (not shown). When the female structures start to form in the third whorl, a small amount of DEF protein is still detectable in the second whorl but only barely visible in the third whorl (Fig. 7F). The amount and spatial expression pattern of the GLO protein follows that of the DEF-101 protein during early and later stages of flower development (Fig. 7G-L).

Sensitivity of organs towards quantitative changes of *DEF* gene expression differs

Def-101 flowers display minor morphological abnormalities of their petals and stamens when cultured at 15°C (Fig. 1A), and both the DNA-binding capability (see later) and the amount of DEF-101 protein (Fig. 7E) are reduced compared to the wild type. Thus the (reduced) *DEF* function provided by the mutant protein in the cold seems to represent a threshold level for the correct control of organ identity in the second and the third whorls. Reduction of the *DEF* function in heterozygous plants, carrying a *def*-gli (null) allele in combination with the wild-type allele, does not result in an altered flower morphology (Schwarz-Sommer et al., 1992). However, further reduction of

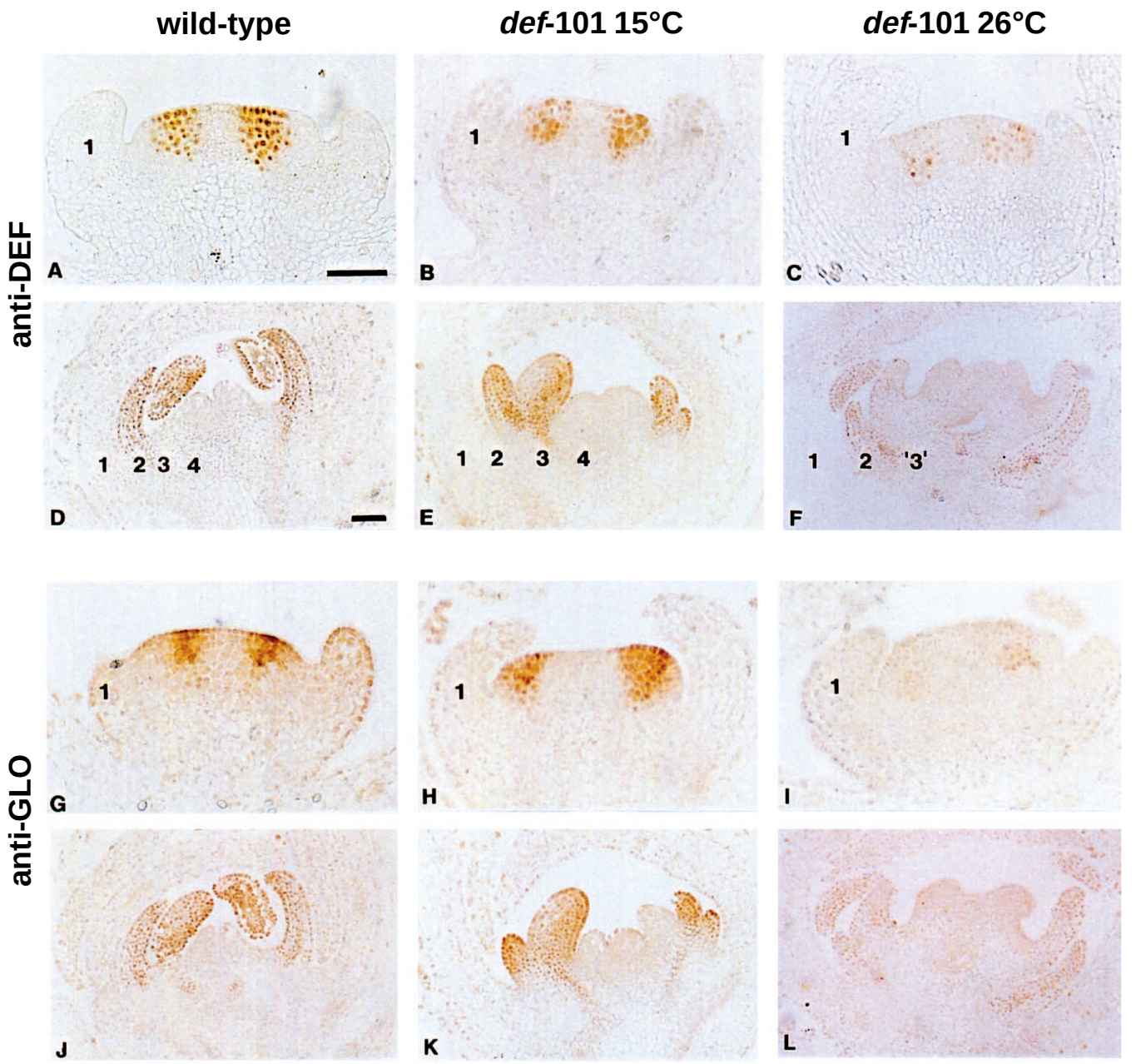


Fig. 7. In situ detection of the expression pattern of the DEF and GLO proteins in developing wild-type and *def*-101 flowers. Longitudinal sections of wild-type (A,D,G,J) and *def*-101 flowers, cultured at 15°C (B,E,H,K) and at 26°C (C,F,I,L) were immunohistochemically stained with affinity purified anti-DEF (A-F) and anti-GLO antibodies (G-L). The developmental stage of floral buds used for immunolocalisation was either stage 4 (A-C, G-I) or around stage 6 (D-F, J-L). 1, 2, 3 and 4 designate the four floral whorls, and '3' the central female organ of *def*-101 flowers grown at the higher temperature.

the *DEF* function in heterozygous plants, carrying a *def*-gli null allele together with the *def*-101 allele, grown at 15°C, induces severe feminisation of third whorl organs (Fig. 8) which are morphologically indistinguishable from the carpelloid organs of a null mutant, or of *def*-101 homozygotes at 26°C (Fig. 1B). In contrast, when grown in the cold, decreased dosage of *def*-101 has no effect on carpel development in the fourth whorl and has only a slight, if any, effect on petal development in the second whorl. Thus, in addition to temporal differences in their requirements for *DEF* function, the control of organogenesis and organ identity by *DEF* in the second, third and fourth whorls is also determined by their different threshold levels.

The *def*-101 mutation confers temperature sensitivity to the DNA-binding capability of the DEF/GLO heterodimer in vitro

To analyse the molecular nature of the temperature-sensitivity of the *def*-101 mutant, the effect of the temperature on the DNA-binding capability of the DEF-101 protein was studied in vitro by gel retardation assays. Neither the temperature of in

vitro translation nor the binding temperature had substantial effects on formation of the wild-type DEF/GLO-DNA complex (Fig. 9). However, only weak bands were detectable when the DEF-101/GLO proteins were incubated at 15°C for the binding assays, whereas no band shifts were obtained at a binding temperature of 25°C (Fig. 9). Decreasing the temperature to 15°C during in vitro translation increased the intensity of the shifted band after binding at 15°C, but did not improve DNA binding at 25°C. The time allowed for binding at different temperatures had no influence on the results (not shown). These studies indicate that the in vitro DNA-binding capability of the DEF-101 protein decreases at the higher temperature and may cause the temperature-dependent loss of *DEF* function observed in flowers of *def*-101 plants grown in the warm.

To test whether the *DEF*-101 mutation affects dimerisation or DNA binding of the heterodimer, we performed experiments to compare the capability of the full size wild-type DEF and mutant DEF-101 proteins to compete with a truncated wild-type DEF protein for dimerisation with GLO ('dimerisation interference' or 'binding competition' assay; Norman et al., 1988; Sharrocks et al., 1993). Increasing the amount of competitor protein in control experiments reproducibly showed increasing competition between the full-size and truncated wild-type protein. However, experiments with the DEF-101 protein yielded variable results, when using different reticulocyte lysate batches for in vitro co-translation (not shown). This technical problem may be related to the presence (or absence) of components in the lysates which may stabilise (or destabilise) the labile dimers. Thus we were not able to distinguish clearly between dimerisation or DNA binding being impaired by the mutation.

The K-boxes of DEF and GLO are involved in DNA/protein complex formation in vitro

Gel retardation assays using various truncated versions of the DEF protein were carried out in order to investigate whether the first amphipathic helix of the K-box (helix 1), the region that is altered by deletion of Lys 93 in the DEF-101 protein, is essential for in vitro heterodimerisation and DNA binding (Fig. 10A,B). These experiments showed that elimination of the second helix (helix 2) either alone, by an internal deletion (ΔDEF3, not shown), or together with the carboxy terminus (ΔDEF4), still allowed formation of a DNA/protein complex. But DNA binding was abolished when half of helix 1 of the K-box, in addition to helix 2 and the carboxy terminus, were removed (ΔDEF5; Fig. 10A,B). Similarly, a truncation in the AP3 protein of *Arabidopsis*, caused by a mutationally introduced stop codon carboxy-terminal to the MADS-box and N-terminal to the K-box, resulted in a null phenotype (Jack et al., 1992).

The K-box of the GLO protein contains three putative amphipathic helices [(Tröbner et al., 1992), see Fig. 9B] and in vitro binding studies showed that at least one third of these is dispensable for the interaction of GLO with the full size DEF protein (ΔGLO3 in Fig. 10B). Interestingly, combinations of truncated DEF and GLO proteins, such as ΔDEF3 and ΔGLO3, resulted in the loss of detectable DNA-protein complexes in gel shifts (not shown), although either of these versions was still able to bind to DNA when combined with the full size partner (Fig. 10B).

Genetic studies indicate interaction between DEF and GLO in vivo

Interaction between the DEF and GLO proteins was also demonstrated in vivo using a weak allele of the *GLO* gene, *globosa*-confusa (*glo*-con). Sequence analysis showed that the mutation is due to three additional nucleotides at position 183 of the *GLO* gene (Tröbner et al., 1992), thereby inserting a phenylalanine residue at position 49 into the hydrophobic carboxy-terminal region of the MADS-box. Interestingly, the GLO-con protein is not able to bind to DNA when combined with the wild-type DEF protein in vitro and it is also not able to compete with the wild-type GLO protein for dimerisation (not shown).

The phenotype of *glo*-con flowers is almost indistinguishable from that of wild-type flowers, in spite of the mutationally altered GLO-con protein. However, reduction of the *glo*-con dosage by combining it with a null allele (*glo*-75; see Tröbner et al., 1992) does confer weak morphological abnormalities to the petals (displaying sepaloid streaks) and stamens (slightly feminised without affecting fertility and flanked by additional protrusions). This suggests that the function of the GLO-con protein in vivo is impaired and represents a threshold value in the homozygous mutant. The most striking morphological effect of *glo*-con on flower development was observed

Fig. 8. Phenotype of *def*-101/*def*-gli flowers grown at 15°C. The photograph on the left shows that petal development was not affected and that the third whorl organs resembled the feminised structure of *def*-101 flowers grown at 26°C (see Fig. 1). On the right is a cross section of third and fourth whorl carpels.

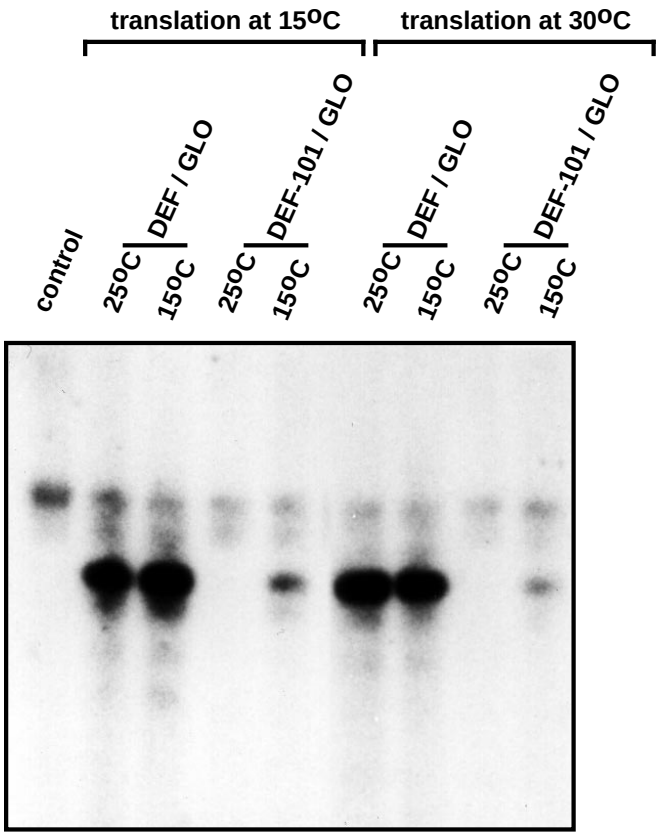


Fig. 9. In vitro binding studies with the mutationally altered DEF-101 protein. For gel retardation assays the *def/glo* and *def*-101/*glo* cRNAs were co-translated either at 15°C for 3 hours or at 30°C for 1 hour as indicated above the lanes. Nearly equal amounts of the translation products were subjected to DNA binding by incubation with ^{32}P-labelled *DEF*-CArG1 probe for 1 hour. The temperature during the binding assays and the combination of the tested proteins are indicated above the lanes. In the control experiment no template was added to the reticulocyte lysate. The unbound labelled probe runs at the bottom of the gel and is not shown in the photograph.

in double mutants homozygous for *glo*-con and *def*-101. Already at low temperature such double mutants displayed, instead of the wild-type like morphology of the single mutants, a phenotype characteristic of plants that carry null alleles of either the *DEF* or *GLO* genes. The effect of *glo*-con on the degree of homeotic alterations in double mutant combinations with other mutant *def* alleles, carrying a mutation either in the *DEF* gene promoter (*def*-chl) or within the MADS box (*def*-nic; Schwarz-Sommer et al., 1992) was distinct, but far less pronounced (not shown).

DISCUSSION

Sensitivity and restoration potential of floral organs in response to decreased and increased *DEF* functions during flower development

Temporal aspects of morphological responses of different organs towards changes in *DEF* functions are schematically compiled in Fig. 11 and will be discussed for each organ separately.

DEF function in second whorl organogenesis

Petal development, including corolla tube formation, is still possible when *DEF* function is established subsequent to initiation of all floral organ primordia (stage 6). Except for corolla shape, which is slightly affected in the mutant flower even at the lower temperature, several other morphological features of petals could be restored by activating *DEF*-101 function during a very long developmental period (Fig. 3). This is in agreement with an earlier observation of late restoration of *DEF* function, due to excision of a transposon, conferring petaloid morphological features to a small sector of a sepaloid second whorl organ in a genetically unstable *def* mutant (Carpenter and Coen, 1990). However, the possibility of restoration of petal features decreased the more advanced sepaloid development was at 26°C. Interestingly, restoration of the upper two petals was more pronounced than restoration of the three lower organs when *DEF* function was activated during late developmental stages. This differential sensitivity of organs within a whorl perhaps reflects the more complex form of the lower organs and hence implicates *DEF* in the establishment of petal shape. Alternatively, or in addition it may reflect a difference in the timing of development of the upper and lower petals.

Transient DEF expression during initiation of petal development in the second whorl is insufficient to maintain the wild-type pattern of petal organogenesis (Fig. 4). Thus it seems that continual control by DEF during second whorl organogenesis is necessary to establish and maintain wild-type petal organ identity, despite the broad partial restoration potential of petals during development.

DEF function during third whorl organogenesis

For complete stamen restoration DEF must be functional from late stage 3 onwards, at which time sepal primordia are just initiating on the flanks of the floral primordium. This is the stage at which *DEF* mRNA first becomes detectable in wild-type plants, although DEF protein is first detectable at a somewhat later stage. Onset of *DEF* function before initiation of organ primordia in the second and the third whorl during stage 4 conditions an ‘intermediate’, strongly feminised stamen phenotype and even a short and limited reduction of *DEF* function in a bud at stage 3 for 4 hours can alter stamen morphology. Establishing *DEF* function subsequent to initiation of organ primordia in the second and the third whorl (during late stage 4 to early stage 5) is insufficient to change the (homeotically altered) carpelloid developmental programme of third whorl organs. Thus, in contrast to the long, slowly decreasing restoration potential of *DEF* function during second whorl organogenesis, the potential to restore normal stamen organogenesis in the third whorl is limited to a very short early developmental phase.

The temperature-independent period of stamen development in the temperature-sensitive *ap3*-1 mutant of *Arabidopsis* extends to stages subsequent to initiation of organ primordia (Bowman et al., 1989). Interestingly, this corresponds to the developmental stage when a change from the stamenoid to a carpelloid pattern of early cell divisions in the third whorl organ primordia of the (morphologically very similar) *pistillata* mutant of *Arabidopsis* is detectable (Crone and Lord, 1994; Lord et al., 1994). This suggests that restoration of

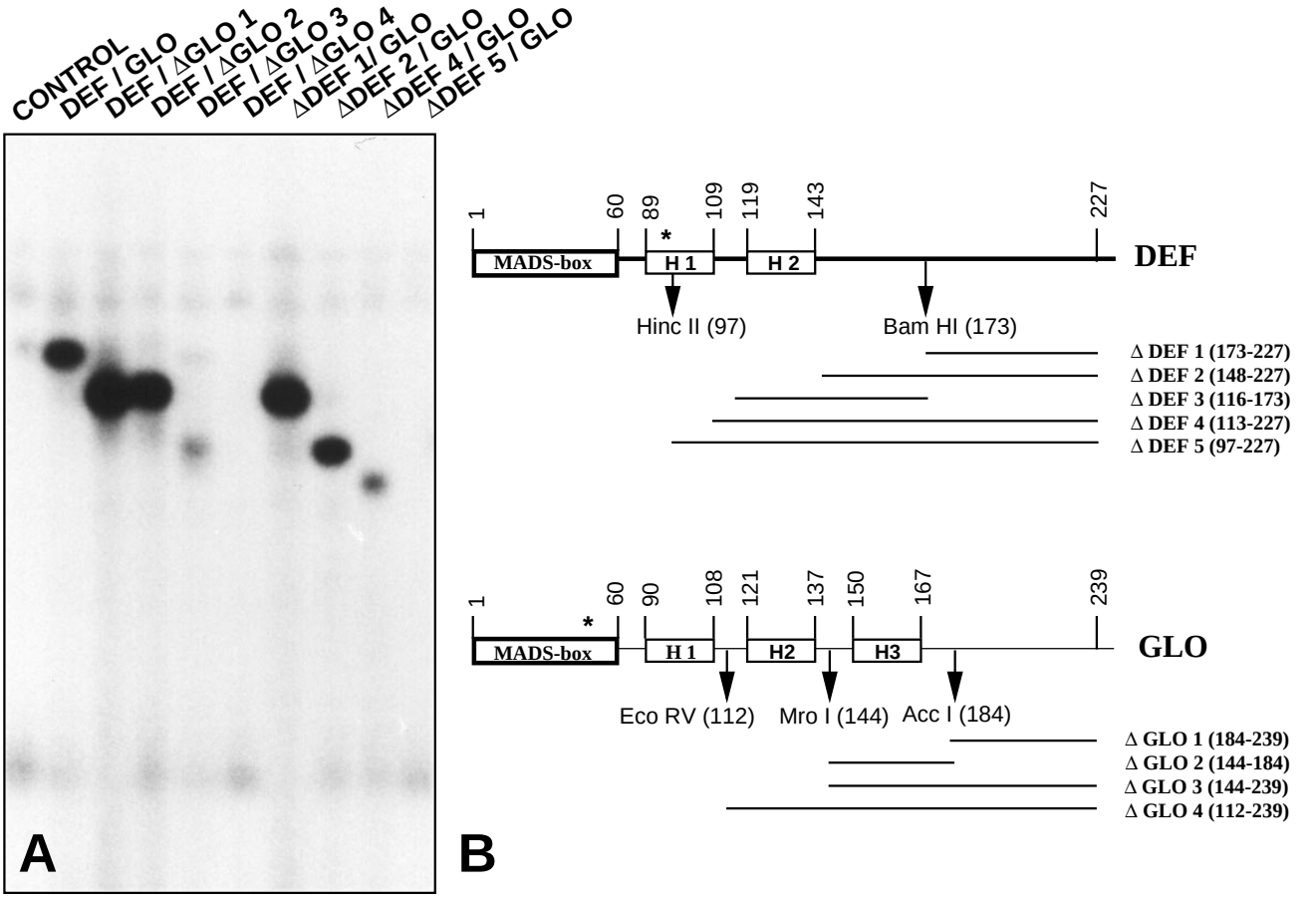


Fig. 10. In vitro DNA-binding assays with deletion derivatives of the DEF and GLO proteins. (A) For the gel retardation assays the in vitro translated full-size and truncated DEF and GLO cRNAs were co-translated (combinations shown above the lanes). The structure of the truncated proteins is depicted in B. DNA binding was performed with the *DEF*-CArG1-oligonucleotide for 30 minutes at 25°C. In the control experiment on the left no template was added to the reticulocyte lysate. (B) Schematic presentation of the DEF and GLO proteins and structure of the carboxy-terminal and internal deletion derivatives. The numbers above each cartoon indicate the position of amino acids with respect to the MADS-box and of the putative α-helices (H 1,2,3) within the K-box. Positions of amino acids at the deletion end points are shown in brackets. ΔGLO 1, ΔGLO 3, ΔGLO 4, ΔDEF 1 and ΔDEF 5 were generated by digestion of the cDNA templates at the indicated restriction sites (position within the protein indicated in brackets). The other derivatives were constructed by insertion of PCR-derived DNA fragments and using the indicated restriction sites. Asterisks show the position of amino acid alteration generating the mutation in the DEF-101 and GLO-con proteins.

stamen development in the third whorl is limited to those stages at which the differentiation programme of the organs is independent of the homeotic gene functions. Apparently the *DEF* function to control stamen identity is required earlier in *Antirrhinum*, conditioning the earlier response of stamens to altered *DEF* function in the *def*-101 mutant.

Besides the early and indispensable expression of DEF for normal stamen organogenesis, DEF also has a permanent maintenance function during later development. Reduction of *DEF* function during stages after microsporogenesis still results in ovule formation at the base of the upper stamen filaments. As observed in the petals, lower and upper organs in the third whorl are differently sensitive for a late decrease in *DEF* function.

DEF function in fourth whorl organ initiation

DEF function is not necessary for carpel organogenesis, because in the null mutant *def*-gli, and also in the *def*-101 mutant flowers at the higher temperature fertile female organs develop, although at the wrong position. Once fourth whorl organ development is initiated, decrease in *DEF* expression can no longer affect the development of carpels in this whorl (Fig. 4). In contrast, primordia in the second and the third whorl are initiated at correct positions, but maintenance of *DEF* function is necessary to control their subsequent organogenesis.

Initiation of carpel development in the centre of the primordium may depend on *DEF* and *GLO* function, as indicated by the presence of genuine carpels in flowers in which the structure and function of the genetically unstable *def*-gli or *glo*-1 allele is somatically restored (Tröbner et al., 1992 and Schwarz-Sommer, unpublished observations). The 'decapitation shift' experiments (Fig. 4) confirmed that *DEF* gene expression prior to late stage 4 of flower development promotes initiation of female organs inside homeotically altered third whorl organs. As also indicated by the development of genuine fourth whorl carpels inside carpelloid third whorl organs of the *def*-101/*def*-gli heterozygote (Fig. 8),

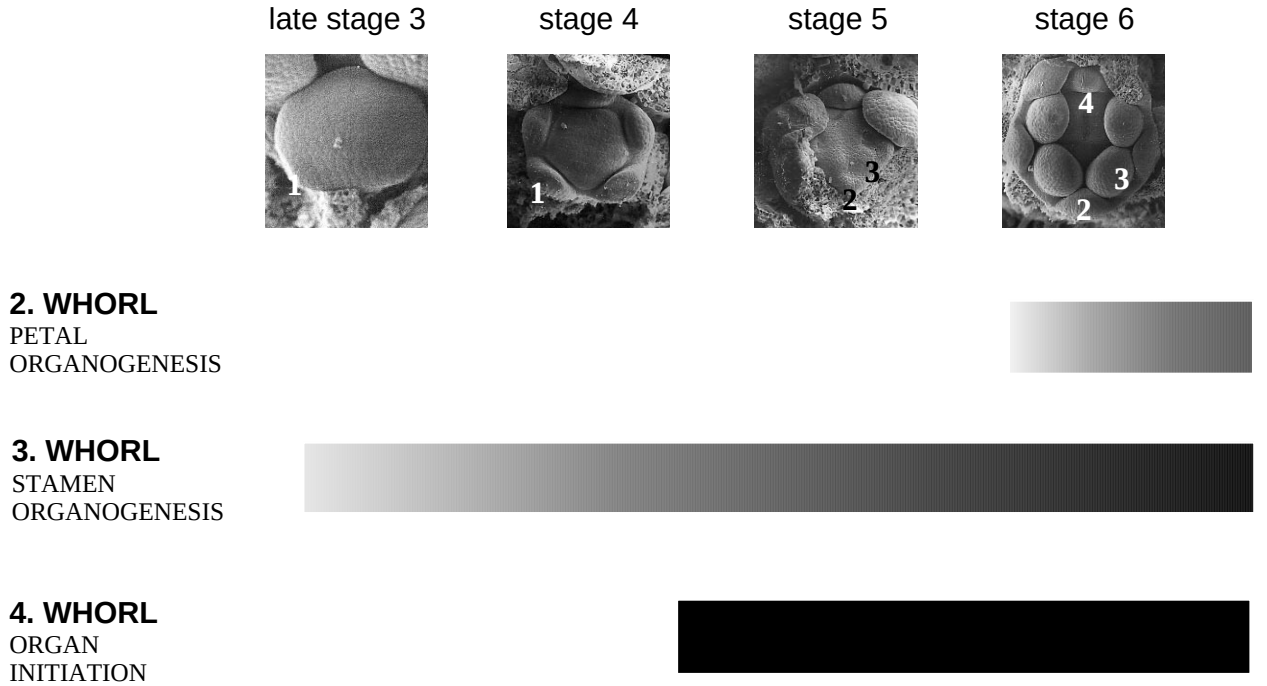


Fig. 11. Sensitivity of second, third and fourth whorl organs towards changes in *DEFICIENS* function during early developmental stages. Data from morphological analyses are summarised schematically to show stages during early flower development (stage 3 to stage 6, see SEM photographs at the top) when DEF function is necessary for correct organogenesis in each whorl. The left border of the bars indicates the stage before which absence of *DEF* function does not affect organogenesis. The degree of inability to restore wild-type organogenesis by activating DEF after this stage is indicated by shading.

initiation of carpel development in the centre of the flower seems to be independent of the developmental pattern of the third whorl organs.

DEF mRNA but no detectable DEF protein is expressed in the centre of the flower primordium at late stage 3 [Fig. 5, also see Bradley et al., (1993)]. It is possible that the mRNA is not translated and therefore the role of DEF in initiation of carpels is indirect, as discussed in detail in a previous report (Tröbner et al., 1992). Alternatively, it may be that the fourth whorl is composed of two types of cells with respect to expression of the DEF protein; an outer region composed of few cells located at the inner boundary of the bipartite DEF/GLO protein expression region, and a central region in which DEF protein is not expressed. While DEF and GLO in the former cells may actively participate in carpel initiation, the absence of early DEF and GLO functions in the centre of the flower could be a prerequisite to terminate floral organ initiation inside of the carpel.

Maintenance of DEF expression by autoregulation starts after initiation of all floral organ primordia

Previous observations showed that *DEF* and *GLO* transcription is maintained by an autoregulatory mechanism which follows independent initiation of their transcription (Schwarz-Sommer et al., 1992; Tröbner et al., 1992). Comparison of the *DEF* and *GLO* transcription patterns in *def*-101 flowers grown at the lower and higher temperature showed no temporal or quantitative differences until stage 6. In contrast, the amount of DEF-101 protein in the warm is strongly reduced in all these stages. It is unlikely that at the higher temperature this residual and mutationally impaired DEF-101 function accounts for the maintenance of *DEF* and *GLO* transcription. We consider it more likely that the transcriptional control of *DEF* and *GLO* mRNA expression is independent of the *DEF* function until stage 6 and that the autoreguloratory transcriptional control starts when all organ primordia have initiated and have begun to differentiate.

Posttranslational control of DEF/GLO heterodimer formation

During early stages of flower development at higher temperature the level of DEF-101 protein was decreased. Transcription of the gene during these stages is independent of the growing temperature suggesting that the mutationally induced structural alteration (deletion of Lys 93 in the K-box of the DEF-101 protein) promotes DEF-101 protein degradation. Concomitantly also the amount of the GLO protein was reduced. This indicates that interaction between DEF and GLO in the wild-type stabilises both proteins. Similarly, in *Arabidopsis, AP3* transcription but no AP3 protein was detectable in the absence of the PI protein (Jack et al., 1994). It seems, therefore, that stabilisation by heterodimerisation of the respective proteins is a common feature of these pairs of orthologues. In *Petunia,* however, stabilisation of FBP1 and pMADS2, the two PI/GLO orthologues, in the third whorl may depend on other proteins, because in a deletion mutant of the *pMADS1* gene (the AP3/*DEF* homologue), FBP1 and function in stamen development is not affected (van der Krol et al., 1993).

It is likely that in addition to heterodimer formation accessory factors contribute to the stability of the DEF/GLO protein complex. For example, *glo*-con mutants display a wild-type like phenotype, suggesting that the in vivo function of the

protein is only weakly affected by the mutation. In contrast, the GLO-con protein is not able to form the expected DNA/protein complex with DEF in vitro. This apparent contradiction was also observed with mutants of other transcription factors (Aukerman et al., 1991) and can be resolved by assuming that accessory proteins, not present in the in vitro assay, stabilise the DEF/GLO-con dimer (or their complex with DNA) in vivo and rescue its function in the plant. The severe mutant phenotype of *def*-101/*glo-con* double mutants may then reflect that this accessory protein-binding is strongly affected when both partners in the DEF/GLO complex carry the respective mutations. Alternatively, or in addition, dimerisation of GLO-con with DEF-101 may be less efficient than with DEF.

Structural domains involved in heterodimerisation and DNA binding of MADS-box proteins

MADS-box transcription factors are composed of structurally and functionally distinct regions. As shown for SRF, a human MADS-box protein, the MADS-box itself contains an N-terminal α-helix involved in DNA binding and a carboxy-terminal β-sheet region with a conserved group of hydrophobic amino acids (the hydrophobic patch) proposed to be involved in homo- and heterodimerisation (Sharrocks et al., 1993; Shore and Sharrocks, 1995). Mutations such as that in GLO-con, which alter the protein sequence within the hydrophobic patch of the MADS-box, suggest that the integrity of this region is required for in vivo functions.

The K-box of plant MADS-box proteins has the potential to form amphipathic helices which can be involved in protein-protein interactions (Landschulz et al., 1988). Mutants like *def*-101 or *ap3*-1, with sequence alterations within the K-box, suggest such a function for the K-box in vivo. In vitro studies with carboxy-terminal deletions showed that the presence of helix 1 of DEF and the helices 1 and 2 of the GLO protein are sufficient for interaction with the (full-size) GLO and DEF proteins, respectively. Interestingly, based on a comparison with studies of AP3 and PI, the *Arabidopsis* homologues of DEF and GLO, it seems that different parts of the K-box are functionally exchangeable. For example, the AP3-1 protein is mutationally altered within helix 2 of the K-box, which seems to be dispensable for interaction of DEF with the full size GLO protein in vitro (Fig. 10). Also, an internal deletion which removed helix 2 of the PI protein did not affect its dimerisation with AP3 (Goto and Meyerowitz, 1994), similar to our observation that two neighbouring helices in the GLO proteins are sufficient for the interaction with DEF.

It is, however, difficult to determine the specific contribution of distinct protein regions to DNA/protein and protein/protein interactions for the following reasons. Firstly, the value of negative results from in vitro DNA-binding experiments is limited. As argued before, the presence or absence of accessory factors in vitro and in vivo may lead to contradictory observations. Second, mutations may alter the overall conformation of the protein and observed alterations (in vivo or in vitro) in any of the monitored properties may only be a secondary consequence of these. In the case of the DEF-101 and GLO-con proteins the mutations are an amino acid deletion and an insertion, respectively, which may, for example, alter the spacing between domains rather than affecting the function of either domain. Third, it is possible that functionally distinct domains structurally overlap with each other. Such overlap has been demonstrated for the DNA-binding and dimerisation domain of SRF (Norman et al., 1988; Shore and Sharrocks, 1995).

Separation of DEF function between different whorls

The phenotype of heterozygous plants at 15°C carrying one temperature-sensitive and one null allele of DEF (*def*-101/*def*-gli) is striking: the stamens are feminised, as in a null mutant, whereas the petals appear normal, and fourth whorl carpels are also normally positioned in the centre of the flower, as in *def*-101 homozygotes (Fig. 8). A similar functional separation in two adjacent whorls was observed before with the *def*-23 allele, where the carboxy-terminal end of the DEF protein is altered due to insertion of a transposon in the gene (Schwarz-Sommer et al., 1992). Thus, it seems that for each organ type a functional 'threshold' exists, conditioning the lower sensitivity of petal development towards decrease of the DEF function compared to that of stamens. This difference in functional threshold may also correlate with the observation that at the non-permissive temperature a higher level of DEF-101 protein is maintained in the second whorl of the *def*-101 mutant than in the third whorl (Fig. 7F,L). The molecular nature of such differences in threshold levels is not clear, but possibly accessory factors, discussed above, contribute to the stability of the DEF/GLO protein complex in an organ-specific manner.

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