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GLOBOSA: a homeotic gene which interacts with *DEFICIENS* in the control of *Antirrhinum* floral organogenesis

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***GLOBOSA* (*GLO*) is a homeotic gene whose mutants show sepaloid petals and carpelloid stamens. The similarity of Glo mutants to those of the *DEFICIENS* (*DEF*A) gene suggests that the two genes have comparable functions in floral morphogenesis. The *GLO* cDNA has been cloned by virtue of its homology to the MADS-box, a conserved DNA-binding domain also contained in the *DEF*A gene. We have determined the structure of the wild type *GLO* gene as well as of several *glo* mutant alleles which contain transposable element insertions responsible for somatic and germinal instability of Glo mutants. Analyses of the temporal and spatial expression patterns of the *DEF*A and *GLO* genes during development of wild type flowers and in flowers of various stable and unstable *def*A and *glo* alleles indicate independent induction of *DEF*A and *GLO* transcription. In contrast, organ-specific up-regulation of the two genes in petals and stamens depends on expression of both *DEF*A and *GLO*. *In vitro* DNA-binding studies were used to demonstrate that the DEFA and GLO proteins specifically bind, as a heterodimer, to motifs in the promoters of both genes. A model is presented which proposes both combinatorial and cross-regulatory interactions between the *DEF*A and *GLO* genes during petal and stamen organogenesis in the second and third whorls of the flower. The function of the two genes controlling determinate growth of the floral meristem is also discussed.**

Key words: development/DNA-binding/flower morphology/ *GLOBOSA* gene structure/MADS-box

Introduction

The sequential appearance of floral organs, and their type, number and position are governed by the spatially and temporally coordinated expression of a set of regulatory genes (Meyerowitz *et al.*, 1989; Schwarz-Sommer *et al.*, 1990; Bowman *et al.*, 1991; Coen, 1991). Mutations in these genes often confer a homeotic phenotype on the flower as revealed by development of organ types in the mutant at positions where they normally do not occur in the wild type flower (Meyer, 1966). In *Antirrhinum* and *Arabidopsis*, several homeotic genes have been found whose mutants display homeotic organ transformations, indicating that their function in the wild type flower is necessary for determination of floral organ identity. Morphological, genetical and in part molecular analyses of some of these genes support models predicting regulatory interactions between them (Haughn and Somerville, 1988; Carpenter and Coen, 1990; Schwarz-Sommer *et al.*, 1990; Bowman *et al.*, 1991; Coen and Meyerowitz, 1991; Lord, 1991). The precise molecular basis of these interactions is not yet understood.

In *Antirrhinum*, mutants of several loci show abnormalities of petal and stamen development in which the developmental fate of these organs is simultaneously altered to sepalody and carpellody, respectively. Two of these loci, *DEFICIENS* (*DEF*A; Klemm, 1927; Sommer *et al.*, 1990) and *GLOBOSA* (*GLO*; Baur, 1918, 1924) seem to interact intimately in this control as indicated by the high degree of phenotypic similarity of their mutants (Figure 1). Several kinds of interactions between the two genes could account for this. Firstly, the two genes could belong to a cascade of regulatory events in which the product of one gene positively controls the expression of the other. Alternatively, the two genes may function together to regulate downstream acting genes. Unfortunately, genetic analysis is not informative for determination of the hierarchical order of genes involved in one and the same process (Botstein and Maurer, 1982).

To gain insight into the molecular basis of the regulatory dependence and/or interaction of the *DEF*A and *GLO* functions we have cloned and characterized the *GLO* gene and studied its expression in flowers of plants carrying genetically stable and unstable *def*A and *glo* alleles. Comparison of *DEF*A and *GLO* expression in the respective mutants and data derived from *in vitro* DNA-binding studies with the DEFA and GLO proteins allow us to propose and discuss a model for interdependent co-regulation of expression of the two genes by heterodimer formation between the corresponding proteins.

Results

Flower morphology of Globosa mutants

The overall morphology of Glo mutant flowers is strikingly similar to that of Deficiens (DefA) mutants (Figure 1A). By morphological studies of Glo mutants we hoped to detect subtle differences between the spatial and temporal developmental patterns of organogenesis in mutants of these two genes as indications of their regulatory relationships. Because no major differences have been found and since altered morphology and organogenesis of flowers of the strong Deficiens^{globifera} (DefA-gli) mutant, as compared with wild type flowers, is described in detail elsewhere (Klemm, 1927; Sommer *et al.*, 1990), only those features of Glo and DefA mutants that are relevant to the Discussion are pointed out below.

Early morphological events were followed using scanning electron microscopy (not shown). These studies indicated

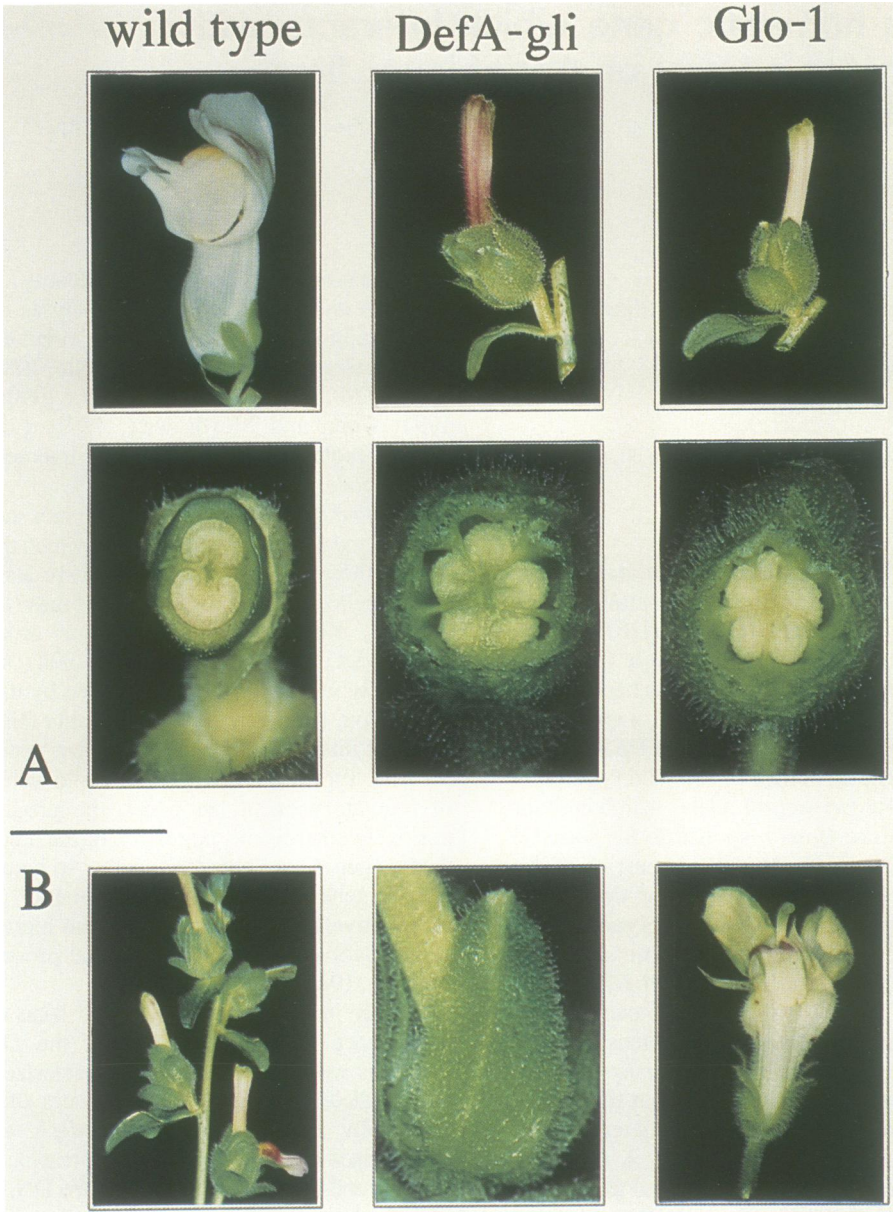

Fig. 1. Phenotype of wild type, Deficiens and Globosa flowers. (**A**) Mature flowers of plants carrying either the wild type (left) or mutant alleles of the *DEFICIENS* (*def*A-gli/*def*A-gli; middle) and *GLOBOSA* (*glo*-1/*glo*-1; right) genes, as indicated above each panel. In the second row, the upper part of the flowers shown at the top was cut off to reveal the cross-sectional structure of the central female organ. Flowers of stable mutants (natural length ≈2 cm) are enlarged twice as much as the wild type. (**B**) Somatic instability of the *glo*-1 allele. The inflorescence on the left carries flowers displaying sectorial restoration of petal morphology. A second whorl organ of the second flower from the top of the inflorescence is enlarged (centre), showing a narrow petaloid sector. At the right, petals in the second whorl of the flower are almost completely restored, except for their altered morphology and for the presence of a green sepaloid rim.

that the temporal pattern of appearance of organ primordia in Glo and DefA-gli mutants is similar to that of wild type flowers. The time of appearance of morphological alterations in the two mutants is indistinguishable.

The outermost whorl of five sepals is unaffected in Glo-1 flowers (Figure 1A). In the second whorl of the mutant five large sepaloid organs develop, the upper two (adaxial) of which are larger than the lower (abaxial) three. The position of second whorl organs in relation to the first whorl sepals and their basal developmental pattern resemble those of genuine petals. The third whorl of the mutant is occupied by a syncarpous and pentalocular gynoecium. The five loculi contain ovules which give rise to viable seeds after fertilization. As judged by their alternate position with respect to the second whorl organs and by their number, these female organs represent five transformed third whorl stamens. It should be noted, however, that development of the fifth organ (the staminodium in the wild type) is not suppressed in Glo or DefA-gli mutants. Due to their fusion the upper part of the transformed third whorl organs resembles the style of the gynoecium. This central chimney-like structure is often composed of two tubes, each of which is tipped with stigmatic tissue. The length of the inner tube is variable. We cannot relate this structure to any particular part of the third whorl organs and we also cannot rule out the possibility that it is a remnant of the fourth whorl. Most probably the genuine wild type gynoecium of the innermost whorl is missing in the Glo-1 mutant, since ovule bearing placentas

do not develop internal to the third whorl. Flowers of plants with the *glo*-75 and *glo*-3D alleles exhibit virtually the same features as Glo-1 flowers.

These observations suggest that a mutation in the *GLO* gene does not interfere with initiation, position and number of floral organ primordia in the second and third whorls and that the *DEF*A and *GLO* genes act in concert in the control of wild type petal and stamen organogenesis.

Genetic instability of the glo-1 mutation

The *glo*-1 allele displays somatic and germinal instabilities. In a *glo*-1 population, somatic reversions often led to restoration of petals or to appearance of petaloid tissue in the second whorl (Figure 1B). Sometimes only half of the organ was restored and the other half was still sepaloid, or petaloid revertant sectors appeared within the sepaloid tissue. Restoration of the male organ was often incomplete and resulted in feminized stamens, but occasionally anthers producing fertile pollen were formed.

Some of the *glo*-1 plants carried flowers with wild type morphology. Selfing of such revertant flowers from six independent plants resulted in 288 plants with wild type flowers and 81 plants with the Glo-1 phenotype, approximating a 3:1 ratio. These results suggest that instability of the *glo*-1 allele is due to the excision of a transposable element which results in restoration of the wild type genotype and, as a consequence, the wild type phenotype (see below).

Molecular cloning of the GLOBOSA gene

The conserved MADS-box of the *DEF*A gene was used to screen a flower-specific cDNA library of *Antirrhinum* (Schwarz-Sommer *et al.*, 1990; Sommer *et al.*, 1990). One of the *def*iciens-*h*omologous (defH) clones selected, defH46, when used as a probe in Southern blot experiments, revealed restriction fragment length polymorphisms (RFLP) between genomic DNA of the wild type line T53 and of the three mutant *glo* alleles.

In order to confirm that defH46 represents a cDNA corresponding to the *GLO* gene product, we utilized the germinal instability of the *glo*-1 allele in a similar manner as described for cloning of the *DEF*A gene (Sommer *et al.*, 1990). Genomic DNAs of two wild type and two mutant plants from the progeny of six selfed revertant flowers were subjected to Southern blot analysis (not shown). In all plants with mutant flowers a 13 kb *Eco*RI fragment was detected by the defH46 probe, corresponding to the fragment size of the *glo*-1 allele. In plants with wild type flowers a 5.7 kb *Eco*RI fragment appeared which corresponded to the genuine wild type fragment. This indicates excision of the transposable element and restoration of the wild type genomic fragment. The correlation between phenotypic reversions and excision events provides the evidence that the defH46 cDNA is derived from the *GLOBOSA* gene.

DefH46 is 848 bp long, contains an open reading frame of 215 amino acids and includes 5′ and 3′ untranslated sequences (Figure 2). Since defH46 does not contain a poly(A) tail, we sequenced another cDNA, defH22. defH22 is identical to defH46, except that it is 9 bp shorter at the 5′ end and 10 bp shorter at the 3′ end, and that it terminates in a 15 bp long poly(A) stretch. For all further hybridization experiments the defH46 cDNA insert (henceforth designated glo) was used as a probe.

The GLOBOSA coding region and the GLO protein

After its MADS-box coding region had been removed, the glo cDNA was used as probe to isolate homologous genomic clones from the wild type line T53 and from the three mutant alleles *glo*-1, *glo*-75 and *glo*-3D, which were characterized by DNA sequencing. Of the genomic wild type clone, ~6 kb was sequenced, comprising 3.3 kb of coding region and 2.8 kb of upstream region.

The structure of the *GLOBOSA* transcription unit (Figure 2) was determined by comparing genomic and cDNA sequences. Consensus sequences such as the start of translation, exon−intron boundaries and the high AT content of introns are in agreement with the rules established for other eukaryotic genes (Joshi, 1987a,b). The location of the presumed polyadenylation signal AAATATTT 58 bases from the poly(A) site does not fit the rule (27 ± 9 bases) found for other plant genes (Joshi, 1987b), but the signal may be functional, since one cDNA carried a poly(A) tail.

The *GLOBOSA* gene consists of seven exons separated by introns of different length. A similar structural organization was also found in the *DEF*A and *SQUAMOSA* genes (Schwarz-Sommer *et al.*, 1992; Huijser *et al.*, 1992). The predicted protein obtained by translating the coding sequence contains 215 amino acids. The 206 bp first exon codes for the MADS-box whose features [a conserved stretch of 58 amino acids with a hydrophilic domain at the amino-terminal end, a hydrophobic putative dimerization domain and a putative phosphorylation site (Sommer *et al.*, 1990; Yanofsky *et al.*, 1990; Schwarz-Sommer *et al.*, 1992)] are characteristic of floral MADS-box proteins and the transcription factors MCM1 of yeast (Passmore *et al.*, 1988) and SRF of mammals (Norman *et al.*, 1988).

The small exons 3, 4, 5 and 6 code for the K-box (Ma *et al.*, 1991), a region with low but significant homology to keratin-like proteins which exhibit a coiled-coil structure due to the propensity of certain regions to form α-helices. Computer analysis of the secondary structure indicated that the K-box region of GLO is likely to form α-helices interrupted either by turns or by β-sheets, and helical wheel analysis revealed the possibility of three such amphipathic helices (indicated in Figure 2). The spacing between the three putative helices is nearly identical (12 and 11 aa, respectively) and corresponds to that found in other floral MADS-box proteins. However, for the majority of MADS-box proteins only two possible amphipathic helices have been proposed (Ma *et al.*, 1991; Pnueli *et al.*, 1991; Jack *et al.*, 1992).

Genomic structure of the three mutant glo alleles

Genomic sequences of *glo* mutant alleles have been cloned and the sites of alterations, relative to the wild type sequence, were determined (Figure 2). These analyses indicate that all three mutations are due either to insertion of transposons that possess characteristic features of the CACTA-type elements (for review see Gierl *et al.*, 1989; Sommer *et al.*, 1988), or to their imprecise excision (see Materials and methods).

Temporal and spatial expression pattern of GLOBOSA in developing wild type flowers

The transcription of *GLO* was studied by *in situ* mRNA hybridization to longitudinal sections of developing wild type flower buds. The earliest stage at which *GLO* mRNA is

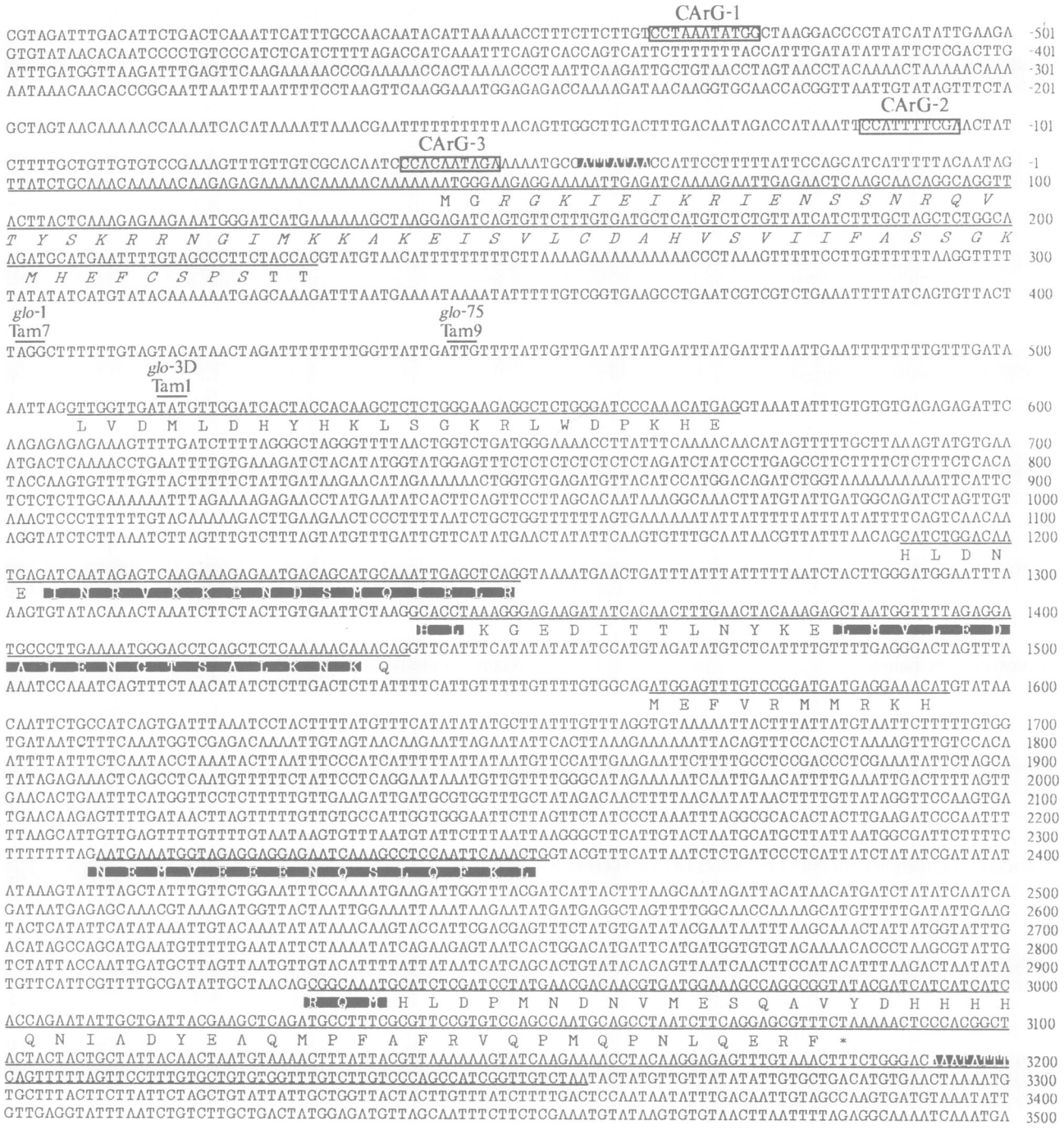

Fig. 2. Structure of the *GLOBOSA* transcription unit. The sequence represents part of a 6.1 kb stretch of DNA containing the entire wild type *GLO* gene (additional upstream sequences deposited in the EMBL data library under the accession number X68831). Exons are underlined and the encoded amino acids (the MADS-box in italics) are shown below the DNA sequence. Amino acids forming the three putative amphipathic helices are in inverted boxes. Consensus sequences such as TATA-box and polyadenylation signal are in inverted boxes. S1 mapping (data not shown) revealed at least two transcription initiation sites. The strongest signal, although not experimentally proven to represent the genuine start of transcription, was arbitrarily designated as position +1. Three CArG motifs upstream of the TATA-box are boxed. The insertion sites of the transposons Tam1, Tam7 and Tam9 are indicated by the three overlined nucleotides which are duplicated in the mutants and flank the inserts. Partial sequence analysis indicated that the Tam7 element present in the *glo*-1 allele and in the *def*A-gli allele are not completely identical. The Tam9 element does not have internal homology to other known transposons. The Tam1-like element in the *glo*-3D allele contains an ~6 kb internal deletion relative to Tam1 (Bonas *et al.*, 1984).

detectable within the flower meristem is when the sepal primordia emerge, in the area between sepal primordia and the centre of the meristem (Figure 3A). Before petal primordia are visible, *GLO* transcripts seem to accumulate in cells that will give rise to the petals and stamens, but not in the central part of the flower meristem (Figure 3B). In the course of development petals display an almost uniform pattern of elevated *GLO* transcription. The amount of *GLO* transcript in stamens also increases during development, except for the sporogenous tissue, where *GLO* expression is strongly reduced or absent (Figure 3D). The low level of *GLO* transcription in developing carpels (Figure 3C) was verified by Northern blot analysis with mRNA from dissected floral organs (not shown). Northern blot analysis

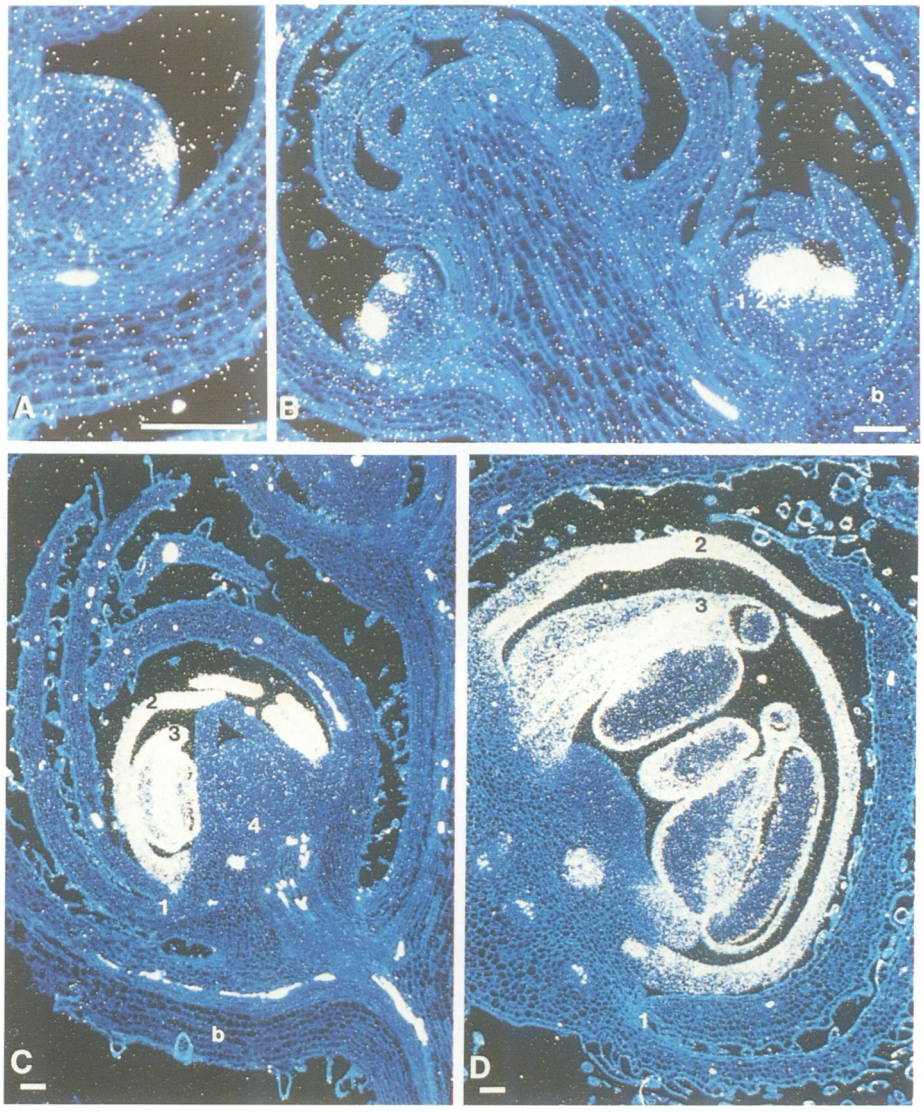

Fig. 3. Spatial and temporal pattern of *GLOBOSA* transcription during development of wild type flowers. Longitudinal sections of wild type flowers at different stages of development (increasing from A to D) were hybridized with ^{35}S-labelled antisense RNA derived from the 3′ end of the glo cDNA, not containing the MADS-box. The dark field exposure, detecting the silver grains, is superimposed by epifluorescense to visualize the underlying tissue. b = bract; 1, 2, 3 and 4 designate the whorls in which sepals, petals, stamens and carpels, respectively, develop in the wild type flower. Bar = 100 μm.

with mRNA from buds harvested at different stages of development and from different vegetative organs of the plant confirmed that *GLO* transcription is flower-specific and is maintained at a high level until flowers are fully developed (not shown). In summary, the temporal course of *GLO* expression and the spatial distribution of the *GLO* transcript are similar to those reported for *DEF*A (Schwarz-Sommer *et al.*, 1992).

Expression of GLO and DEF A in Glo mutants

To distinguish whether the *DEF*A and *GLO* genes function sequentially or combinatorially, their pattern of transcription in various mutants was studied *in situ*.

In situ hybridization of longitudinal sections of Glo-75 flowers with the defA antisense RNA probe revealed fairly strong *DEF*A transcription at an early stage in emerging second whorl organ primordia and also a somewhat weaker signal in the third whorl decreasing toward the centre of the flower (Figure 4A). In older buds (Figure 4A lower panel) *DEF*A transcription was stronger in the upper part of the flower than in the lower part. During subsequent differentiation the intensity of the hybridization signal was almost identical in the second and third whorls and subsequently decreased more in the second whorl than in the third whorl (Figure 4A). Because no *GLO* transcription was detectable in Glo-75 flowers by Northern blot analysis, these observations indicate that induction and maintenance of a basal level of *DEF*A transcription is not under the control of *GLO*.

As mentioned before, in Glo-1 mutant plants excision of a transposon (Tam7) from the *glo*-1 gene results in sectorial restoration of the (cell autonomous) *GLO* gene function (see Figure 1B). *In situ* hybridization of cross-sections of phenotypically mutant buds with glo as a probe showed sectors of *GLO* expression in the second and the third whorls, indicating somatic restoration of *GLO* gene transcription (Figure 4B). The hybridization signal coincided spatially with partial restoration of morphological features, such as

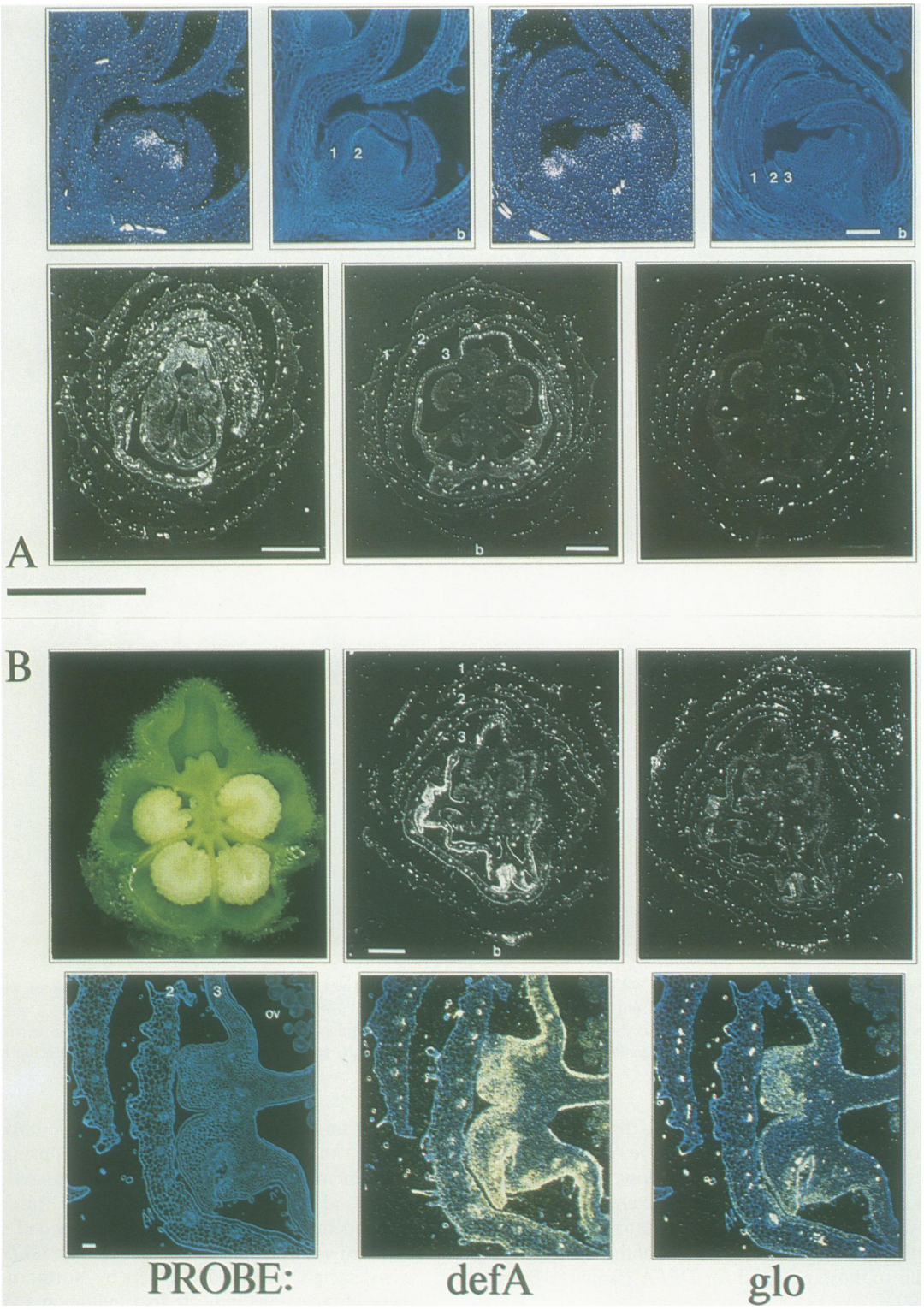

Fig. 4. *In situ* hybridization with *DEF A* and *GLO* probes of developing flowers carrying the stable *glo*-75 (**A**) and genetically unstable *glo*-1 alleles (**B**). In part A longitudinal sections (upper row) and cross-sections (lower row) of Glo-75 flowers at successive developmental stages were hybridized with the antisense defA RNA probe (upper row, panels 1 and 3; lower row, panels 1 and 2) as indicated in Figure 5. The picture on the right in the lower panel shows that no hybridization with the glo probe was detectable in the section consecutive to the section hybridized with the defA probe and shown in the middle. Epifluorescence (upper row, panels 2 and 4) was used to visualize the tissue, where silver grains were detected by superimposition of dark field exposure (upper row, panels 1 and 3) or by dark field exposure alone (lower row). Bars represent 100 μm in the upper row and 1 mm in the lower row. In part B phenotypically mutant Glo-1 flowers were selected from an inflorescence displaying reversion events (see Figure 1B). The photograph on the left in the upper row shows the third whorl of such a flower (bract oriented to the bottom of the picture). Consecutive cross-sections were used for *in situ* hybridization with the antisense RNA probes, as indicated below the panels. Dark field exposure at low magnification was used to detect the position of revertant sectors (upper row). The lower row shows morphological details (epifluorescence at the left) and spatial distribution of the hybridization signal (dark field exposure plus epifluorecence at the middle and at the right) of a revertant sector from the left side of the flower. b = bract; ov = ovules; 1, 2 and 3 designate the whorls in which sepals, sepaloid petals and carpelloid stamens, respectively, develop in the mutant. Bars represent 1 mm in the upper row and 100 μm in the lower row.

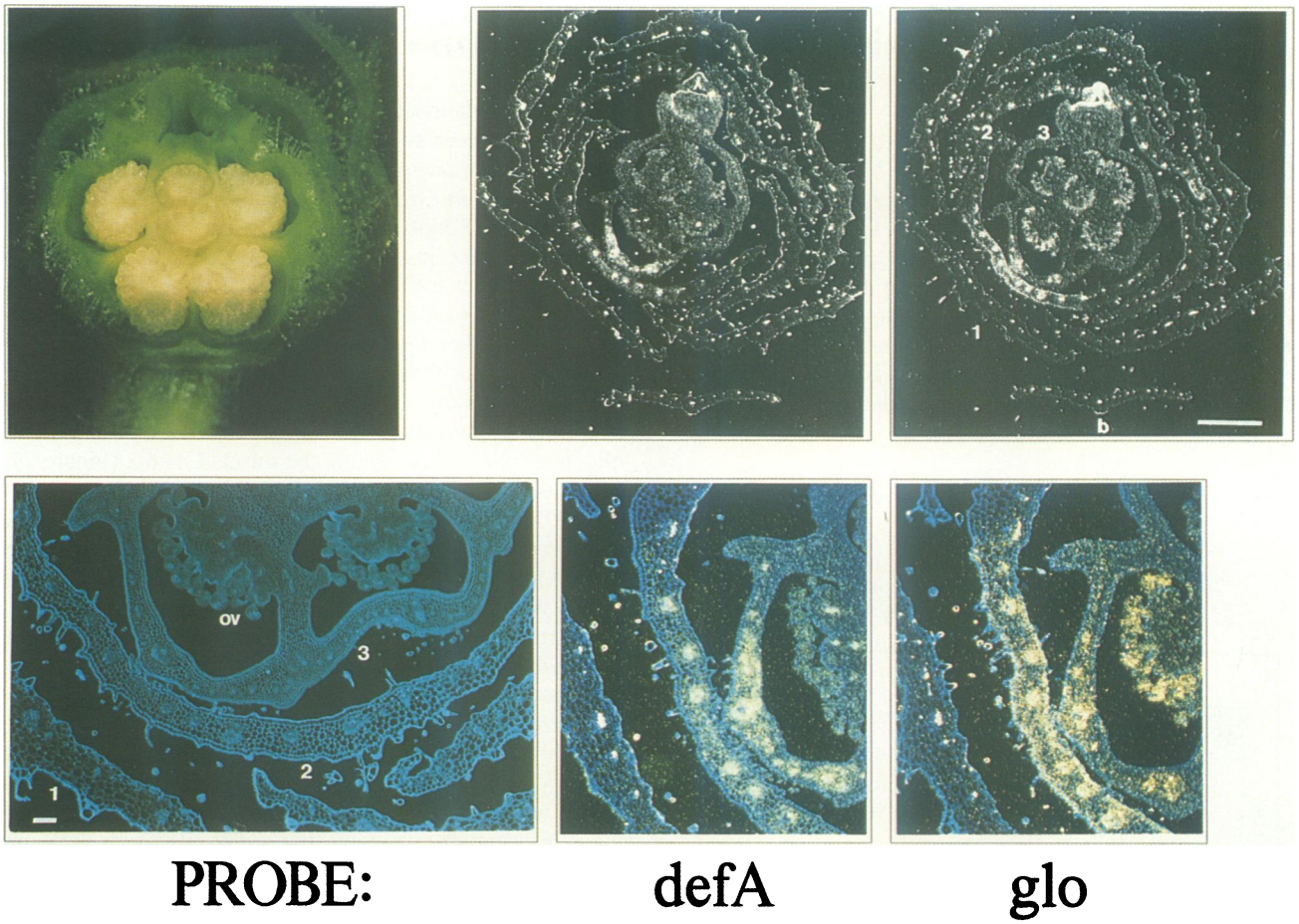

Fig. 5. *In situ* hybridization with *GLO* and *DEFA* probes of flowers carrying the genetically unstable *defA*-gli allele of the *DEFICIENS* gene. The photograph on the left shows the interior of a phenotypically mutant flower which was selected from a DefA-gli inflorescence displaying frequent somatic reversions. Except for the genotype of the plants, all details of the *in situ* hybridization experiment and the symbols used in the figure were the same as described in the legend to Figure 4B.

appearance of a distinct cell type, characteristic of petals but not of sepals, at the inner epidermal surface of second whorl organs. Similarly, epidermal cells in the third whorl showed a hybridization signal with glo. When a consecutive section was hybridized with the defA antisense probe, the same cells in the same regions of the *glo*-1 bud displayed strong hybridization signals, as observed with the glo probe (Figure 4B). Neither *GLO* hybridization nor such strong *DEF*A hybridization was detectable in other regions of the same section, thus indicating that restoration of *GLO* expression is a prerequisite for elevated *DEF*A transcription. It is interesting to notice that, due to late somatic reversion, the number of ovule-filled loculi was eventually reduced to four in the third whorl (Figure 4B).

Expression of GLO in defA alleles

Like *glo*-1, the *def*A-gli mutant also displays somatic instability due to excision of the Tam7 transposon (Sommer *et al.*, 1990). Cross-sections of buds were used to analyse *in situ* the consequences of restoration of the *DEF*A function on transcription of *GLO*. Figure 5 demonstrates that restored *DEF*A transcription is accompanied by elevated *GLO* transcription in the same revertant sector, in a manner similar to that described above for *glo*-1 somatic excisions. Transcription of *DEF*A and *GLO* extends to the ovules formed within the sector. Since ovules in wild type flowers do not usually display strong *in situ* hybridization with defA or glo probes, we conclude that transcription of *DEF*A or *GLO* does not *per se* interfere with ovule formation. Interestingly, in addition to four still feminized third whorl organs, the flowers contained a central bilocular gynoecium, like in the wild type flowers, although no morphologically visible reversion events were observed, except for the cell type of revertant sectors in the second whorl (Figure 5). This may indicate that *DEF*A expression in a sector of the third whorl suffices to initiate fourth whorl development.

The expression of *GLOBOSA* in phenotypically distinct DefA mutants was analyzed by Northern-blot experiments with mRNA isolated from whole flower buds (Figure 6). The phenotype of the mutants and the molecular features of the *def*A morphoalleles are described elsewhere (Schwarz-Somer *et al.*, 1992). In defA-gli flowers which carry a null allele of *DEF*A (Figure 1A, also see Sommer *et al.*, 1990), a weak hybridization signal was obtained in Northern blots with the *GLO* cDNA probe when 10 μg of mRNA was loaded onto the gel (Figure 6). Thus, expression of *DEF*A is not a prerequisite of basal *GLO* transcription. In the *def*A-chl (*chlorantha*) allele the mutation affects the promoter of the gene and strongly decreases *DEF*A transcription, whereas *GLO* transcription is only slightly decreased (Figure 6). In contrast, *GLO* transcription is affected in all other *def*A morphoalleles where structural alterations within the DEF

A protein are responsible for the altered phenotype, albeit the effects on *DEF*A and *GLO* transcription are different in different morphoalleles. A point mutation in the MADS-box of *def*A-nic (*nicotianoides*) still results in ~70% of the *def*A-nic message compared with wild type and also only slightly affects *GLO* transcription. Mutation in the putative K-box region of DEF A, which confers temperature sensitivity on the *def*A-101 allele, concomitantly affects *GLO* and *def*A-101 transcription in flowers developing at the permissive (15°C) and non-permissive (25°C) temperature. In contrast, alteration in the carboxy-terminal region of the DEF A-23 protein has less severe effects on *GLO* transcription than on that of the *defA*-23 allele. These observations indicate that the DEF A protein has an important role in the control of *GLO* transcription during organogenesis, following early induction of *GLO*.

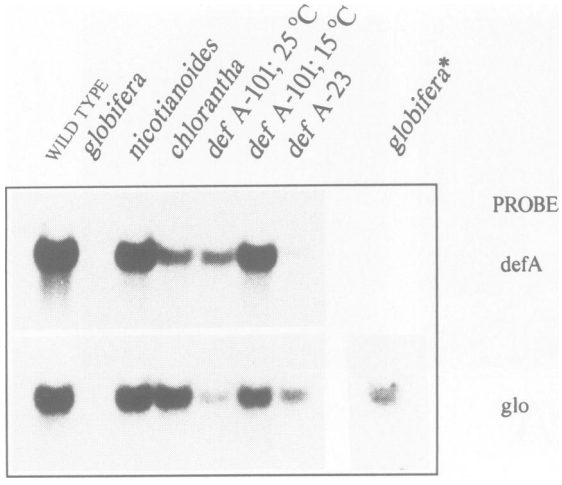

Fig. 6. Northern blot analysis of *DEFA* and *GLO* transcription in flowers carrying mutant *deficiens* alleles. Poly(A)$^+$ RNA (1.5 μg per lane; except for the lane designated *globifera**, which contained 10 μg) was isolated from 0.5–1 cm long flower buds of plants carrying different *deficiens* alleles (genotype indicated above each lane; *globifera* = *def*A-gli, *nicotianoides* = *def*A-nic, *chlorantha* = *def*A-chl). The temperature at which the temperature sensitive DefA-101 mutant was grown is also indicated. After hybridization with the first probe and exposure, the filter was washed and reused for hybridization with the second probe (probes indicated on the right). Neither the defA nor the glo cDNA probe contain the MADS-box region.

In vitro DNA-binding studies with the DEF A–GLO protein heterodimer

We have shown previously that a DEF A–GLO heterodimer obtained by *in vitro* co-translation can bind to an oligonucleotide containing the consensus binding motif for MADS-box proteins, which is present in the yeast *STE6* promoter (Schwarz-Sommer *et al.*, 1992). These studies were extended to such CArG motifs (Pollock and Treisman, 1991; for further references see Schwarz-Sommer *et al.*, 1992) present in the *DEF*A and *GLO* promoters (Table 1).

The glo and defA cDNAs were translated *in vitro* and the expressed proteins were used in gel retardation assays with oligonucleotides representing the selected motifs (Figure 7). When translated alone, neither GLO nor DEF A proteins show DNA binding (not shown). In contrast, when the defA and glo cDNAs were co-translated, the proteins were able to bind to one of the *DEF*A promoter motifs (CArG-1) and (more weakly) also to the three *GLO* motifs (Figure 7). Thus, it seems that protein–protein interaction, occurring during *in vitro* co-translation between the DEF A and GLO proteins, is a prerequisite for DNA binding, as has been previously described (Schwarz-Sommer *et al.*, 1992). Experimental evidence that the DEF A–GLO complex is a heterodimer will be provided in a forthcoming report (I.Hue and W.Tröbner, manuscript in preparation), together with information on how the *def*A-101 and *def*A-nic mutations in the DEF A protein interfere with DNA binding.

The three CArG motifs in the *GLO* promoter can compete with the *DEF*A CArG-1 oligonucleotide for binding to the heterodimer while oligonucleotides that show no binding in gel retardation assays with the DEF A–GLO complex, such as the binding site of an unrelated DNA-binding protein (CREB, not shown) or *DEF*A CArG-2 (Table I; Figure 7), cannot compete with the CArG related promoter motifs. This suggests that the band shifts in the gel retardation assay reflect specific binding of the DEF A–GLO protein complex to the motifs present in the *DEF*A and *GLO* promoters. It should also be noted that all of the sequence motifs used for the binding assay contained the CArG motif (Table I). Thus, the differences in the strengths of binding of these sequences

Table I. Oligonucleotides for *in vitro* binding of the DEF A–Glo complex

Gene		Oligonucleotide	Position[a]	Binding
DEF A[b]	CArG-1	GGCAACTCTTT CC TTTTTA GG* TCGCATATGG	1207 bp	strong
	CArG-2	GCAATTCTGTTCTTA CC TTTGTA GA* TTTGTAAGTT	1051 bp	none
	CArG-3	GAACACTAAATCCA CC ACAATT GA* AAGAAAAC	785 bp	none
GLO	CArG-1	GTCTTCTTGT CC TAAATAT GG* CTAAGGACCC	527 bp	weak
	CArG-2	GACCATAAATT CC ATTTTC GA* ACTATCTTTTG	107 bp	weak
	CArG-3	GTTGTCGCACAATC CC ACAATA GA* AAAATGC	53 bp	weak
STE6[c]		CCATGTAATTA CC TAATAG GG* AAATTTACACGCT	166 bp	strong

[a]Position of the CArG motif (beginning at the internal G residue of the core, indicated by an asterisk) with respect to the transcription initiation site within the promoter of the respective gene.
[b]From Schwarz-Sommer *et al.* (1992).
[c]From Keleher *et al.* (1988).

suggest that not only the CArG motif but also the flanking sequences are important for DNA binding affinity and specificity.

Discussion

The molecular mechanism establishing interdependent control of DEFICIENS and GLOBOSA expression in petals and stamens

The similarity of homeotic alterations displayed by DefA and Glo mutants points to their regulatory interactions in the control of the identity of petals and stamens during floral organogenesis. Previously we speculated that the GLO protein, as a potential partner for heterodimerization with DEF A, might be a (combinatorial) component in the autoregulatory mechanism which governs up-regulation and maintenance of expression of the *DEFICIENS* gene during petal and stamen organogenesis (Schwarz-Sommer *et al.*, 1992). The data obtained from analysis of the *GLOBOSA* gene are consistent with the proposed model and allow it to be expanded to include the regulation of the *GLOBOSA* gene, as summarized in Figure 8. In this model, high level transcription of the *DEF*A and *GLO* genes depends on pre-existing low basal expression of the GLO and DEF A proteins, respectively. The positive autoregulatory control is then established by a heterodimer formed between the DEF A and GLO proteins, which recognizes cognate binding sites (the CArG motif) present in the promoters of both genes. The primary induction of both genes is independent. In the following section, evidence for the validity of this complex regulatory circuit will be discussed.

Low level expression of DEFICIENS and GLOBOSA is the prerequisite for transcriptional up-regulation of the DEFA and GLO genes. Subsequent to their induction early during floral morphogenesis, the *DEF*A and *GLO* genes are intensively transcribed in developing petals and stamens of *Antirrhinum* flowers. The observation that mutations in the *DEF*A gene that affect the structure of the DEF A protein also affect the amount of *DEF*A transcript in the mutant flowers indicates that DEF A is involved in transcriptional up-regulation of the *DEF*A gene (Schwarz-Sommer *et al.*, 1992). It was found that *GLO* transcription is also down-regulated in the flowers of these same DefA mutants. Furthermore, up-regulation and maintenance of *GLO* transcription must be intimately related to expression of DEF A, because in a genetically unstable *def*A background, generating somatic revertant sectors, *GLO* gene transcription strictly follows the pattern of restoration of *DEF*A transcription. These observations are indicative of control of *GLO* transcription by the DEF A protein.

There is also evidence for the opposite situation, i.e. the regulation of the *DEF*A gene by the GLO protein. Support

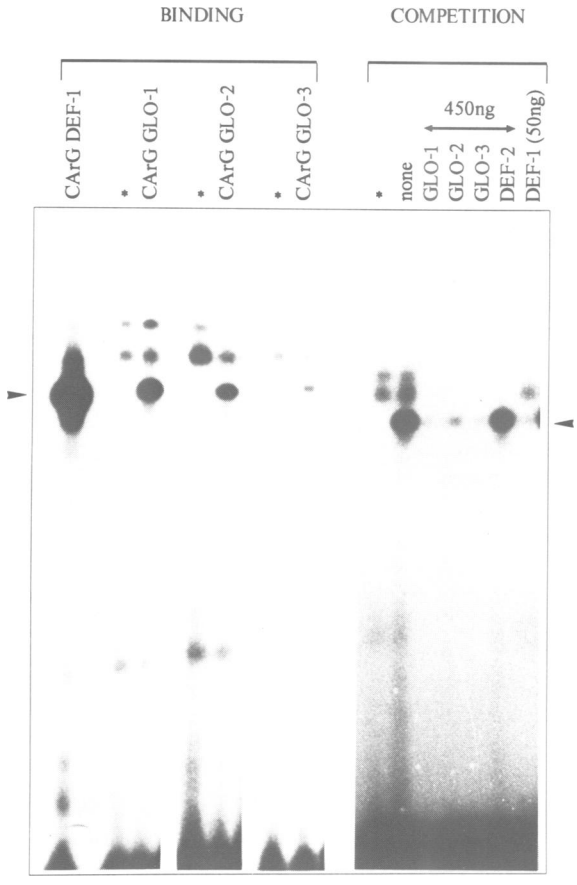

Fig. 7. DNA binding of the *in vitro* translated DEF A and GLO proteins in gel retardation assays. In the DNA-binding assays (left panel) the ^{32}P-end-labelled CArG motifs from the DEF A and GLO promoters (compiled in Table I) were incubated with the DEF A and GLO proteins obtained by *in vitro* co-translation. As a control for binding specificity (specific complex indicated by arrowheads) the same promoter probes were incubated with reticulocyte lysates without mRNA (indicated by an asterisk above the lanes). For the competition assay shown in the right panel the labelled CArG DEF-1 motif and an excess of unlabelled promoter probes (amount and origin indicated above the lanes) was incubated with the DEF A/GLO proteins.

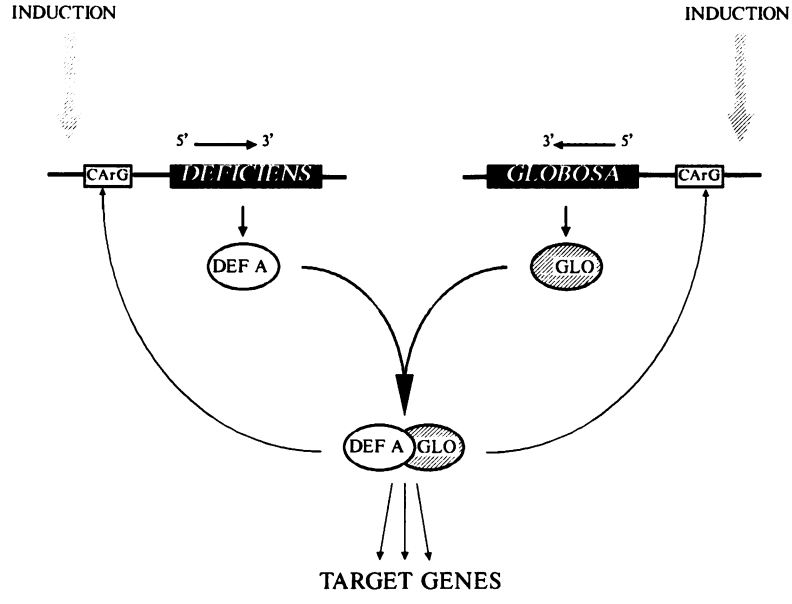

Fig. 8. Possible mechanism of regulatory interactions between *GLOBOSA* and *DEFICIENS*. Open and shaded circles represent the DEF A and GLO proteins, respectively. CArG is a potential binding site of the DEF A–GLO heterodimer present in the *DEF*A and *GLO* promoters. Independence of induction of the two genes is shown by shaded vertical arrows. The scheme also indicates a regulatory function of the heterodimer in the control of several downstream target genes (Schwarz-Sommer *et al.*, 1992). Not shown in the scheme is the possibility of a regulatory function of DEF A or GLO as homodimers, or of their heterodimerization with other proteins which cannot be ruled out. For further explanations see Discussion.

for this comes from the observation that in revertant sectors of the genetically unstable *glo*-1 mutant, elevated *DEF*A transcription occurs which is always correlated with restoration of the GLO function. In other words, the *DEF*A and *GLO* gene products are required to regulate each other's expression positively at the level of transcription in developing petals and stamens.

GLOBOSA and DEFICIENS are DNA-binding proteins: heterodimerization and autoregulation. The presence of the MADS-box at the amino-terminal end of the deduced 215 aa GLO protein indicates that *GLO* codes for a DNA-binding protein and thus for a putative transcription factor. Gel retardation assays presented here and in a previous report (Schwarz-Sommer *et al.*, 1992) have shown that the *in vitro* produced GLO protein, in combination with DEF A, can bind DNA, whereas the GLO or DEF A proteins alone do not bind under the same conditions. The *in vitro* binding assay thus reflects the capability of the GLO polypeptide to interact with the DEF A protein. Recent results (I.Hue and W.Tröbner, manuscript in preparation) with truncated GLO proteins show that the DEF A and GLO proteins bind to CArG motifs as a heterodimer.

The CArG binding motifs used in the gel retardation assay are found in the promoters of the *DEF*A or *GLO* genes. At least one CArG motif from each promoter produced a band shift, indicating affinity of the DEF A−GLO heterodimer for these potential binding sites. These observations provide support, although not direct proof, for an autoregulatory control mechanism of *DEF*A and *GLO* transcription. That autoregulation is the most likely mechanism for up-regulation of *DEF*A transcription in petals and stamens is also suggested by the temperature dependence of *DEF*A transcript levels in plants homozygous for the temperature sensitive *def*A-101 allele (Schwarz-Sommer *et al.*, 1992).

Induction of DEFICIENS and GLOBOSA occurs independently. The cross-regulatory mechanism of transcriptional control discussed above can only operate when both DEF A and GLO are already expressed at a low level. Thus, prior to their up-regulation, transcription of the two genes has to be independently induced. Two lines of evidence suggest that floral induction and organ-specific up-regulation of the two genes are independently regulated. Firstly, *DEF*A is transcribed in flowers homozygous for the null allele *glo*-75 and *GLO* is transcribed in flowers homozygous for the null allele *def*A-gli. Second, a mutation in the promoter of the *def*A-chl allele specifically interferes with transcriptional up-regulation of the gene in petals and stamens whereas its induction and basal level of expression in all floral organs of the *chlorantha* mutant are unaffected (Schwarz-Sommer *et al.*, 1992).

A cross-regulatory relationship of DEFA and GLO gene transcription for the control of organogenesis: implications and questions. The mechanism that positively regulates the *DEF*A and *GLO* genes is unexpected, because a combinatorial interaction or a hierarchical regulatory relationship would be sufficient to establish interdependence between the two genes and thus could account for the phenotypic similarity of their respective mutants. Thus the cross-regulatory transcriptional control by a heterodimer formed by the two gene products may have a role in proper function in morphogenesis. For example, it could secure a co-ordinate and balanced synthesis of the two proteins. This would imply that an excess of DEF A or GLO protein interferes with the control of organogenesis, perhaps by favouring homodimerization and/or heterodimerization with other (MADS-box) transcription factors. If this were true then mutational disturbance of the balance should confer an aberrant phenotype on the flowers. An example supporting this could be the altered floral morphology of plants homozygous for the *def*A-chl allele, where *GLO* gene transcription is only slightly affected whereas *DEF*A transcription is severely reduced. However, higher sensitivity of downstream target genes towards changes in the amount of DEF A protein (or of the DEF A−GLO complex) could also account for such a mutant phenotype.

Autoregulation of a homeotic gene has been suggested to be the mechanism for maintenance of transient primary positional information during subsequent differentiation in the case of patterning genes in *Drosophila* (e.g. Serfling, 1989), and was also proposed previously for the function of *DEF*A (Schwarz-Sommer *et al.*, 1992). Our observations on the maintenance of a low level of transcription of *DEF*A (or *GLO*) in absence of GLO (or DEF A) expression, respectively, argues more in favour of a stable 'primary' signal, present throughout flower and floral organ development. The basal level of concomitant *DEF*A and *GLO* gene transcription in the fourth whorl, however, does not result in their up-regulation. Thus, either an additional factor is positively involved in autoregulation of both the *DEF*A or *GLO* genes in the second and third whorls, or their autoregulation is suppressed by a negative regulator in the fourth whorl. That a positive regulatory factor may be involved in autoregulation of the *DEF*A gene in the second and third whorls is suggested by the decreased transcription of the *def*A-chl mutant allele, where the mutation affects a site close to the DEF-1 CArG motif (Schwarz-Sommer *et al.*, 1992). Therefore, this 'chlorantha motif' could represent the binding site of a *trans*-acting regulatory protein which cooperates with DEF A and GLO in the autoregulatory control of the *DEF*A gene. At present the molecular nature of this function is not known, but genes like *FIMBRIATA* (*FIM*; Harte, 1951) or *VIRIDIFLORA* (*VIR*; Stubbe, 1966), whose mutants display homeotic alterations of petals and stamens similar to Glo and DefA mutants, are good candidates for participating in the regulation of the *DEF*A and *GLO* genes. The question as to whether this positive regulatory influence is direct or indirect can be approached experimentally by isolating the protein whose binding to the *DEF*A promoter is abolished in the *def*A-chl allele.

Role of DEFICIENS and GLOBOSA in the control of meristematic functions in the centre of the flower

The number and position of organs formed in the second and third whorls of *def*A and *glo* mutants and their early developmental pattern are indistinguishable from those of the wild type flower. This suggests that the basic developmental control underlying the primary determination of whorl identity does not include *DEF*A or *GLO* functions. Instead, DEF A and GLO are required, as homeotic organ identity genes, for the manifestation of this 'positional information' during subsequent organogenesis. The mechanism depicted in Figure 8 thus reflects regulatory interactions between DEF A and GLO which are important

only for their role as regulators of floral organogenesis in the second and third whorls of the flower.

Carpel development in the fourth whorl, however, is absent when *DEF*A or *GLO* are non-functional, although formation of carpels in the third whorl of their mutants indicates that neither of these functions is essential for carpel organogenesis. Absence of fourth whorl development in the mutants could be an indirect consequence of carpel formation in the third whorl (Carpenter and Coen, 1990; Sommer *et al.*, 1990). Alternatively, activity of DEF A and GLO in the third whorl of the wild type flowers could be necessary for growth and cell proliferation of the central meristem, which is required for formation of organs in the fourth whorl. In the somatically unstable DefA-gli mutant, somatic restoration of *DEF*A expression in the third whorl is sufficient for carpel formation in the fourth whorl, although still carpels and not stamens develop in the third whorl. This observation suggests a direct relationship between the DEF A function in the third whorl and maintenance of meristematic activities in the centre of the flower. Thus, in the third whorl of the wild type flower the DEF A protein apparently has an antiterminator function in that it prevents the premature termination of meristem growth.

Somatic reversion events which restore the (cell autonomous) DEF A function in the third whorl are sectorial, yet this is sufficent to prevent termination of cell proliferation in the centre of the flower. To resolve this apparent contradiction, we suppose that the DEF A control over meristematic functions may be established via control of synthesis of a diffusible factor or by a different signalling mechanism. An alternative explanation could be that the excision event results in low level of *DEF*A expression in certain cells of the third whorl, which is not detectable by *in situ* hybridization, but is sufficient to maintain meristematic functions for initiation of organogenesis in the fourth whorl. Whether the GLO protein is also involved in this function can neither be stated nor excluded. Somatically unstable Glo-1 flowers often contain four instead of five female organs in their third whorl, indicating suppression of carpel formation at the position of the stamenoid. Fourth whorl formation, however, has not been observed in Glo-1 flowers so far.

It is interesting to note that mutations in other floral homeotic genes such as *PLENA* (*PLE*; Stubbe, 1966; Carpenter and Coen, 1990) or *FIMBRIATA* (see above) dispense with the need for DEF A and GLO functions to counteract termination of flower development. Thus double mutant flowers of either Ple or Fim plants, carrying in addition mutant *def*A or *glo* alleles, maintain the tendency of Ple and Fim single mutant flowers for indeterminate growth (L.Ramirez, W.-E.Lönnig and Zs.Schwarz-Sommer, unpublished). With respect to the maintenance of meristematic activities, the *PLE* and *FIM* functions are therefore possible targets for negative regulation by DEF A and GLO in the third whorl of the wild type flower.

In summary, *DEF*A and *GLO* control organ type in the second and third whorls of the flower and control meristematic functions in the centre of the flower. In this sense these genes do not differ from other homeotic genes such as *PLENA* in *Antirrhinum* or *APETALA2* and *AGAMOUS* in *Arabidopsis*, which also control both organ identity and meristematic functions (Coen and Meyerowitz, 1991; Bowman *et al.*, 1992).

Homology between homeotic control genes in floral organogenesis of different species

Mutants of *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) in *Arabidopsis* (Bowman *et al.*, 1989, 1991; Hill and Lord, 1989; Jack *et al.*, 1992) display sepaloid petals and carpelloid third whorl organs, thus resembling mutants of *DEFICIENS* and *GLOBOSA* in *Antirrhinum*. In addition, both genes are involved in the control of determinate growth of the flower, like *DEF*A and *GLO* (Schultz *et al.*, 1991; Bowman *et al.*, 1992). Since *AP3* and *DEF*A seem to code for homologous proteins, and since it has been suggested that *PI* is the cognate homologue of *GLO* (Jack *et al.*, 1992), one might expect that the mechanism of regulation of the *Arabidopsis* genes *AP3* and *PI* would be similar to that of *DEF*A and *GLO*. In fact, induction of both *AP3* and *DEFA* transcription in the second and third whorls is independent of PI or GLO function, respectively, and in the third whorl of the flower transcriptional up-regulation of *AP3* and *DEFA* is positively controlled by the respective partners (see Jack *et al.*, 1992 and this report). It will be interesting to learn whether the autoregulatory mechanism controlling up-regulation of *DEF*A and *GLO* transcription also operates in the control of *AP3* and *PI* expression in *Arabidopsis*.

Not all aspects of function and regulation of *DEFA/GLO* and *AP3/PI* are similar, though. Firstly, *AP3* and *PI* seem to be involved in the control of organ number in the third whorl of *Arabidopsis* flowers (Hill and Lord, 1989; Schultz *et al.*, 1991; Bowman *et al.*, 1992; Jack *et al.*, 1992), whereas in *Antirrhinum* the only whorl-specific function we can possibly assign to the *DEF*A and *GLO* genes is the retardation of development of the fifth stamen. Secondly, *PI* does not seem to be involved in up-regulation of *AP3* transcription in the second whorl, in contrast to the strong dependence of *DEF*A transcription on GLO function. It is thus possible that these differences reflect different mechanisms of control of floral morphogenesis, which may be responsible for species-specific differences of floral organization.

Materials and methods

Plant material

Plants were grown in the glasshouse at 18–25°C with additional light during winter. Vegetative cuttings of plants with a given phenotype were used to exclude the influence of different genetic backgrounds. Growth conditions for the temperature sensitive *def*A-101 mutant were as described previously (Schwarz-Sommer *et al.*, 1992).

Genetic stocks

Line T53 (*niv*-53::Tam1) with wild type flower morphology was obtained from Rosemary Carpenter (John Innes Institute, Norwich, UK). Genetic stocks of *deficiens* morphoalleles have been described elsewhere (Schwarz-Sommer *et al.*, 1992). Seeds of the genetic stocks of the *glo*-1 mutation (Baur, 1918, 1924) and *fimbriata* (Kuckuck and Schick, 1930) were obtained from the Gatersleben seed collection.

Transposon mutagenesis

The *glo*-75 allele was uncovered in a large scale transposon mutagenesis experiment in the selfed progeny of one of the *NIV* colour revertant T53 plants. The F2 progeny of this plant (88-5/75) segregated plants with wild type or Globosa flowers in an almost 3:1 ratio.

Glo-3D was an unexpected isolate from a transposon tagging experiment aiming at isolation of new alleles of the *FIMBRIATA* (*FIM*) gene. For this purpose T53 (niv::Tam1) plants were crossed to the *fim*-1 mutant (Stubbe, 1966). Plant 87-3D displayed the Fimbriata phenotype and was analysed further as a candidate of a newly tagged *fim* allele. To separate the *fim*-1 allele from the new *fim* alleles, plant 87-3D was crossed to the wild type (in this case c.v. 'Snowman') and the resulting heterozygotes were self-

pollinated. In half of the cases wild type and Fimbriata plants appeared in the progeny, and in the other half, plants with Globosa flowers appeared among plants with Fimbriata and wild type flowers. The segregation ratio in these was 9:3:3:1 (wild type:Fim:Glo:double mutant Fim/Glo). Thus, the 87-3D plant contained a new *fim* allele (named *fim*-3D) and was heterozygous for a new *glo* allele (named *glo*-3D).

Glo-75 and Glo-3D mutants were fertilized with pollen of a heterozygote between the wild type and the *glo*-1 allele. The occurrence of Globosa flowers in the resulting progeny confirmed that the newly isolated mutants were alleles of the *GLOBOSA* gene. Both mutations are caused by insertion of a transposable element (see Figure 2), whose mobility is low. Somatic excision of Tam9 from the *glo*-75 allele was obtained only in one instance. Mobility of the Tam1-like element in *glo*-3D is indicated by analysis of two stable germinal derivatives, where imprecise excision of the element generates a frameshift in the encoded protein (insertion of ATAT between the nucleotides at position 518 and 519 in the first case and deletion of a GT at position 519/520 plus addition of an A at position 518 in the second case).

Nomenclature

We use a combination of original gene and allele designations (Stubbe, 1966), combined with those previously used (Sommer *et al.*, 1990) and written according to the nomenclature of *Arabidopsis* mutants (e.g. *deficiens*^{globifera} = *def*A-gli). The classical mutant allele, identified first when only one allele was described, has been given the allele number 1 (e.g. *glo*-1) and newly isolated alleles are numbered according to the order in which they were isolated (e.g. *glo*-75). Wild type alleles are written in upper case italics (e.g. *DEFICIENS*) and mutant phenotypes in lower case, with the first letter capitalized (e.g DefA-gli).

Methods

All methods, including scanning electron microscopy, isolation of plant genomic DNA and mRNA, molecular cloning of genomic and cDNA, subcloning into plasmid vectors, DNA sequence analysis, blotting techniques, hybridization procedures, *in vitro* translation and DNA-binding assays were performed as described previously (Sommer *et al.*, 1990; Schwarz-Sommer *et al.*, 1992). Techniques applied for *in situ* hybridization are also described elsewhere (Huijser *et al.*, 1992). Sequence analysis of the wild type *globosa* gene and of all mutant alleles was performed with genomic *Eco*RI fragments cloned into lambda EMBL4 phages and subcloned into plasmids pBR322 or pUC18. Computer analysis was conducted with the Genetics Computer Group Sequence Analysis Software Package, version 7.0 (Devereux *et al.*, 1984).

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