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Deficiens, a homeotic gene involved in the control of flower morphogenesis in *Antirrhinum majus*: the protein shows homology to transcription factors

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***Deficiens* (*defA*$^+$) is a homeotic gene involved in the genetic control of *Antirrhinum majus* flower development. Mutation of this gene (*defA*-1) causes homeotic transformation of petals into sepals and of stamina into carpels in flowers displaying the 'globifera' phenotype, as shown by cross sections and scanning electronmicroscopy of developing flowers. A cDNA derived from the wild type *defA*$^+$ gene has been cloned by differential screening of a subtracted 'flower specific' cDNA library. The identity of this cDNA with the *defA*$^+$ gene product has been confirmed by utilizing the somatic and germinal instability of *defA*-1 mutants. According to Northern blot analyses the *defA*$^+$ gene is expressed in flowers but not in leaves, and its expression is nearly constant during all stages of flower development. The 1.1 kb long mRNA has a 681 bp long open reading frame that can code for a putative protein of 227 amino acids (mol. wt 26.2 kd). At its N-terminus the DEF A protein reveals homology to a conserved domain of the regulatory proteins SRF (activating c-*fos*) in mammals and GRM/PRTF (regulating mating type) in yeast. We discuss the structure and the possible function of the DEF A protein in the control of floral organogenesis.**

Key words: GRM-*MCM1* yeast mating type/scanning electronmicroscopy/SRF-c-*fos* mammals/subtraction cDNA cloning/unstable mutation

Introduction

Floral organogenesis seems to be influenced and controlled by environmental as well as genetic factors, as indicated by physiological, cytological, morphological and genetic experiments (for monographs and reviews see Meyer, 1966; Wardlaw, 1967; Bernier, 1988; Davies, 1988; Steeves and Sussex, 1989). Virtually nothing is known about the molecular mechanisms by which environmental and genetic factors exert their influence on, or direct, flower development and differentiation. To untangle these fundamental regulatory processes is a fascinating challenge for plant molecular genetics.

We have chosen *Antirrhinum majus* (snapdragon) as a model system for studying flower development for the following reasons.

(i) Many developmental mutants have been isolated in the past (for compilation see Stubbe, 1966) and many of them are still available.
(ii) Transposable elements have been isolated and characterized (for reviews see Coen and Carpenter, 1986; Sommer *et al.*, 1988) that can be used as tools for the isolation of genes (transposon tagging) and generation of new mutants (transposon mutagenesis).
(iii) A good genetic map exists and construction of an RFLP map has been initiated (Zs.Schwarz-Sommer and E.Ritter, unpublished data).
(iv) The possibility of propagating plants vegetatively exists, thus allowing rescue of sterile mutants.
(v) The flower is large which makes it easy to collect a particular tissue or organ for molecular analysis.

A disadvantage of *A.majus* is that it has not been successfully transformed yet.

In animal systems (e.g. *Drosophila*) genetic and molecular analysis of morphogenetic mutants (homeotic, meristic and other morphological mutations) has led to deep insight into the underlying regulatory priciples of development (Gehring and Hiromi, 1986; Ingham, 1988). Although such mutants are also available in many plant species (reviewed by Meyer, 1966), they have not been used yet for the analysis of regulatory mechanisms controlling development. In a first attempt to gain insight into control mechanisms and genes governing flower development we have chosen a homeotic flower mutant of *A.majus*, *deficiens*^{globifera} (*defA*-1), for molecular analysis.

The male sterile and recessive *globifera* allele of the *defA* gene (Baur, 1924; Hertwig, 1926) conditions homeotic transformation of the male organ into an abnormal female organ and simultaneously causes transformation of petals into sepaloid leaves (Klemm, 1927). Similar phenotypes (carpellody), due to single gene mutations, occur in many other plants (for compilation see Meyer, 1966), e.g. pistillata and apetala-3 in *Arabidopsis* (Koornneef *et al.*, 1983; Bowman *et al.*, 1989) or stamenless-2 in tomato (Sawhney and Greyson, 1973).

What makes the *deficiens* locus attractive for molecular analysis is, firstly, the existence of an allelic series of mutants (Baur, 1924; Stubbe, 1966), displaying less pronounced phenotypes compared with the *globifera* allele. The charcterization of these morphoalleles should help in the structural and functional analysis of the *deficiens* gene. Secondly, mutant alleles of at least two other non-allelic loci, *globosa* and *viridiflora* (Stubbe, 1966), producing a phenotype very similar to *deficiens* alleles, have been identified. It is possible that all these loci are functionally linked together in a regulatory process directing flower development, although there is no genetic evidence indicating this at the moment.

In this paper we report on the molecular cloning of the

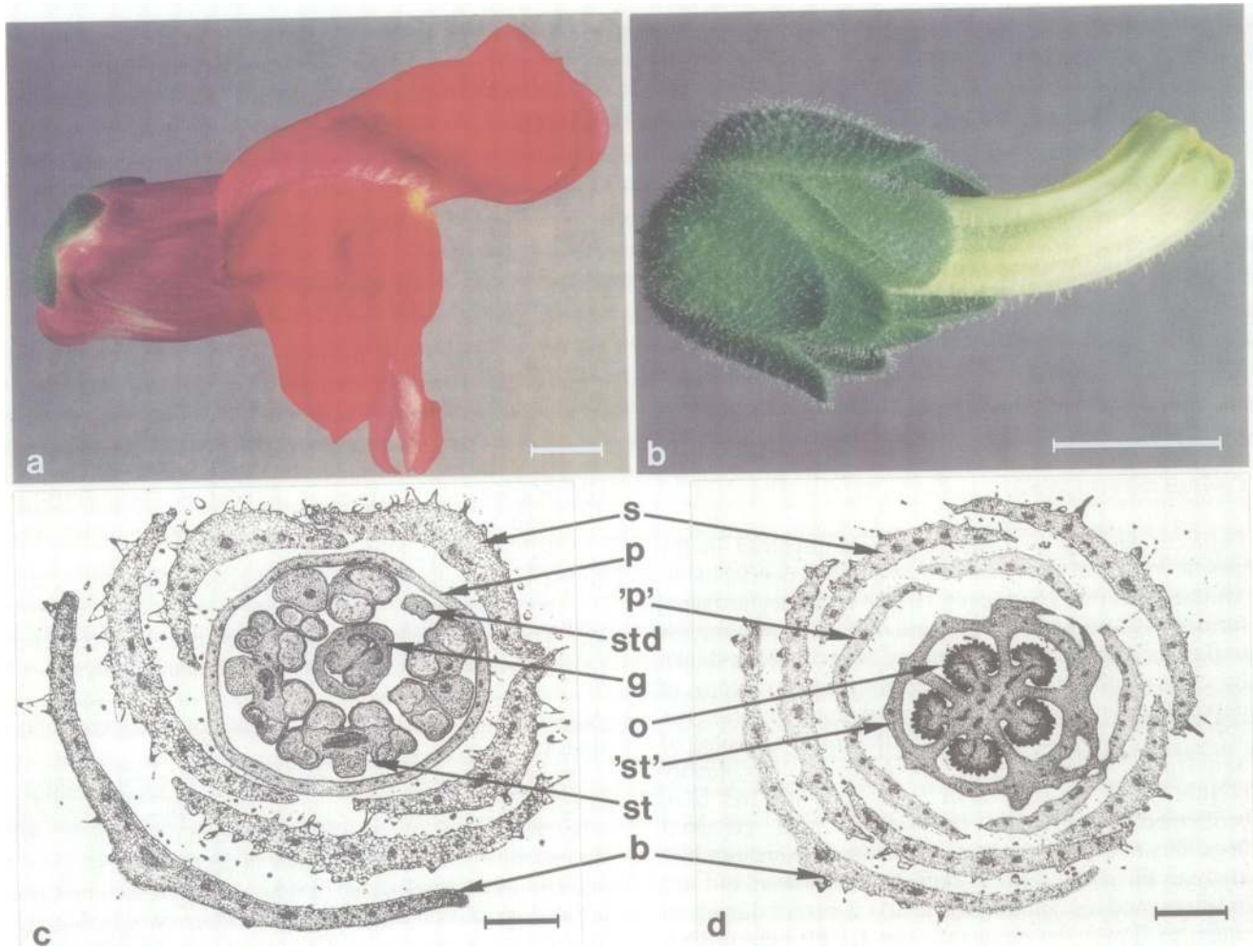

Fig. 1. Phenotypic expression of the *defA*-1 (*globifera*) mutation in *A.majus*. Photographs were taken from mature flowers of wild type (**a**) and globifera (**b**) plants. Cross sections of 5 mm long immature buds of wild type (**c**) and globifera (**d**) plants were prepared as specified in Materials and methods. Bar = 5 mm in (a) and (b) and 0.5 mm in (c) and (d). b = bract, s = sepal, p = petal, st = stamen, std = stamenoid, g = gynoecium, o = ovules. Quotation marks indicate transformed organs in globifera flowers.

deficiens gene by differential screening of a subtracted wild type cDNA library using subtracted (+) and (−) probes derived from wild type and globifera mutant inflorescences. A nearly full size cDNA was obtained and sequenced. The putative DEF A protein deduced from the cDNA sequence displays homology to transcription factors. Its possible role in the regulation of genes participating in flower development is discussed.

Results

Morphological changes due to the globifera (defA-1) mutation

Flower morphology of globifera (*defA*-1/*defA*-1) plants is strikingly different from that of wild type ($defA^+/defA^+$) plants (Figure 1a−d). The first and outermost whorl bearing five sepals is normal but the corolla, composed of five petals, is replaced by five large sepaloid leaves in the second whorl. The typical zygomorphic structure of the *Antirrhinum* flower is virtually lost. Cross sections reveal that the central part of the globifera flower is formed by a syncarpous and pentalocular gynoecium (Figure 1d) due to transformation and fusion of four stamens and one stamenoid constituting the androecium in the third whorl of the wild type flower (Figure 1c). The tubular structure which is characteristic for globifera flowers represents the upper parts of the transformed and fused stamens and resembles the style of wild type flowers. Its rim bears stigmatic tissue and also functions as a stigma in the mature (male sterile) globifera flower (Klemm, 1927). The genuine bilocular gynoecium formed in the fourth whorl of the wild type flower is missing. In addition, globifera flowers are reduced in size compared with the wild type.

The fundamental deviation in morphogenesis of wild type and globifera flowers can be visualized by scanning electron microscopy. Young inflorescences of 3−8 mm length carry flower primordia and buds at early developmental stages ranging from undifferentiated primordia to buds with developed but not yet mature organs. The photographs in Figure 2a and b show that the sequence of appearance of the organ primordia in the outer three whorls is not affected by the *defA*-1 mutation. Petals start to develop in the mutant similar to the wild type with regard to the disappearance of radial symmetry (Figure 2d). The fusion of the lower parts (forming the corolla tube in mature wild type flowers) is, however, suppressed in the mutant (Figure 2d). Subsequent petal development resembles that of sepals as judged by the appearance of hairs (Figure 2e). The first visible

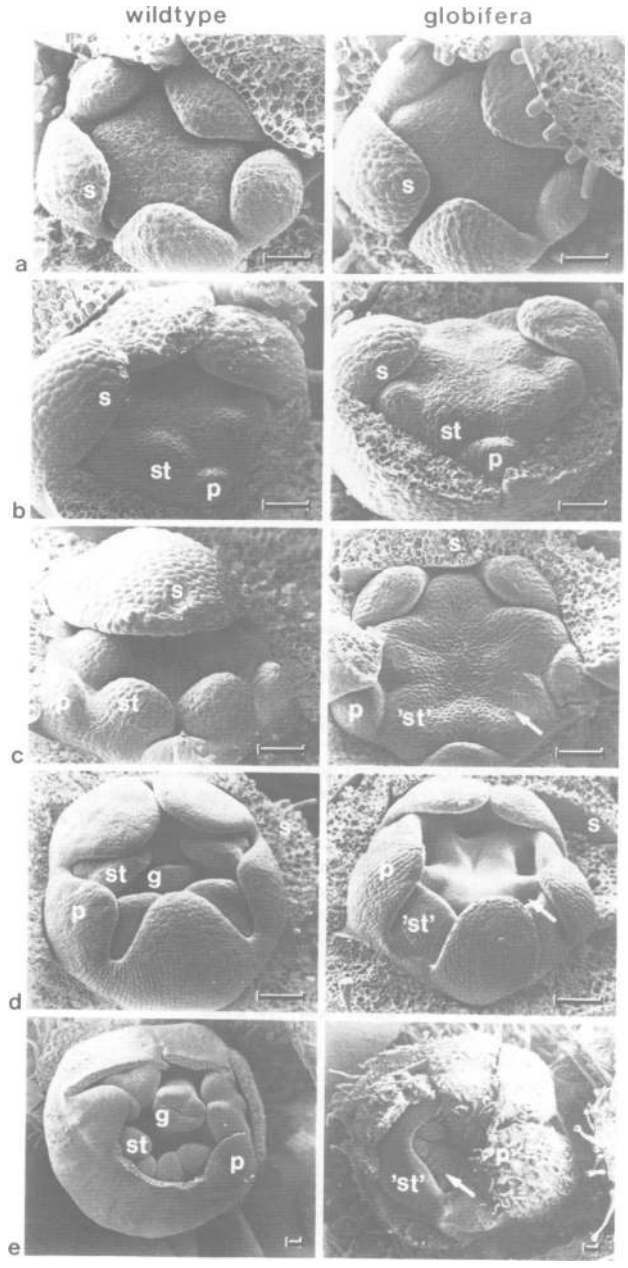

Fig. 2. SEM photographs of developing wild type and globifera mutant flower buds from young *A.majus* inflorescences. The photographs in each row (**a**–**e**) represent individual buds at a similar stage of development within young inflorescences of wild type (left) and globifera (right) plants. Maturity of buds increases from the top to the bottom. Organs in the first (**b**–**e**) and second (**e**) whorl were in part removed to detect inner whorl organs. Bars = 50 μm in the rows **a**–**c** and 100 μm in the rows **d** and **e**. For designation of organs see Figure 1. Arrows point to developing locules in row **d** which become filled with ovules in row **e**.

morphological difference between mutant and wild type is the sloping of the stamen primordia (Figure 2b) followed by indentations reminiscent of carpel development (Figure 2c). During further growth septa arise by fusion of the broadened filaments forming holes in the mutant where stamens develop in the wild type (Figure 2d). Still later these holes are filled with ovules readily visible because the ovaries remain open at the top (Figure 2e). At this stage the female organ of the wild type has not yet developed ovules but is already differentiated into ovary, style and what becomes later the stigma.

For clarity we have illustrated here the 'normal' globifera phenotype. However, variations can be detected when comparing globifera flowers on the raceme of a single plant as well as comparing flowers of plants differing in their genetic background. Variation includes the number of sepaloid petals and the number of locules and their placentation within the female organ. The cause of this variability is not clear yet.

Genetic instability of the globifera mutation

The *defA*-1 allele displays somatic and germinal instability (Baur, 1924). In our population somatic reversions most frequently resulted in restoration of one or more petals, or only restoration of parts of petals. In these latter cases petal-like structures emerged in the second whorl with a still sepal-like basis. Sometimes only half of the organ was reverted and the other half not, or the petaloid structure revealed green rims and stripes reminiscent of sepals. These observations indicate that the $defA^+$ gene acts cell autonomously. Restoration of the male organ was far less frequent and often resulted in petaloid structures. These flowers often contain the wild type female organ. We therefore assume that the disappearance of the genuine female organ in globifera flowers may be rather due to feminization of the third whorl than to non-function of the gene in the fourth whorl.

Twenty percent of the *defA*-1 plants displayed germinal reversions. These plants either carried on the inflorescence globifera and 'wild type' flowers or developed different inflorescences with either globifera or 'wild type' flowers. Some revertant flowers contained between one and four fertile anthers and hence could be selfed. Their selfed progeny segregated wild type revertant and globifera flowers in an almost 3:1 ratio indicating that the reversion event was heritable. These observations suggested that the phenotypic instability of *defA*-1/*defA*-1 plants is due to insertion of a transposable element which can excise, thereby restoring the wild type phenotype.

Differential screening of a subtracted cDNA library detects wild type genes with altered expression in the defA-1 mutant

The strategy for isolation of the *defA* gene was based on the assumption that its expression is abolished or significantly decreased in the *defA*-1 mutant. This should allow isolation of the locus by differential screening.

The principle of this approach is as follows. First strand cDNA synthesized from mRNA isolated from wild type inflorescences is subtracted with excess mRNA from young leaves. This step enriches the single stranded (ss) cDNA for flower specific sequences from which a cDNA library is prepared by conventional methods. This library is subsequently screened with a (+) probe (from wild type inflorescences) and a (−) probe (from mutant inflorescences). To increase the sensitivity of the procedure the (+) and (−) probes are also enriched for flower specific sequences. This is achieved by subtracting the sscDNA, synthesized from wild type and mutant inflorescence mRNA, with excess wild type leaf mRNA and labelling the remaining enriched (+) and (−) sscDNA by 'random priming' (for details see Materials and methods).

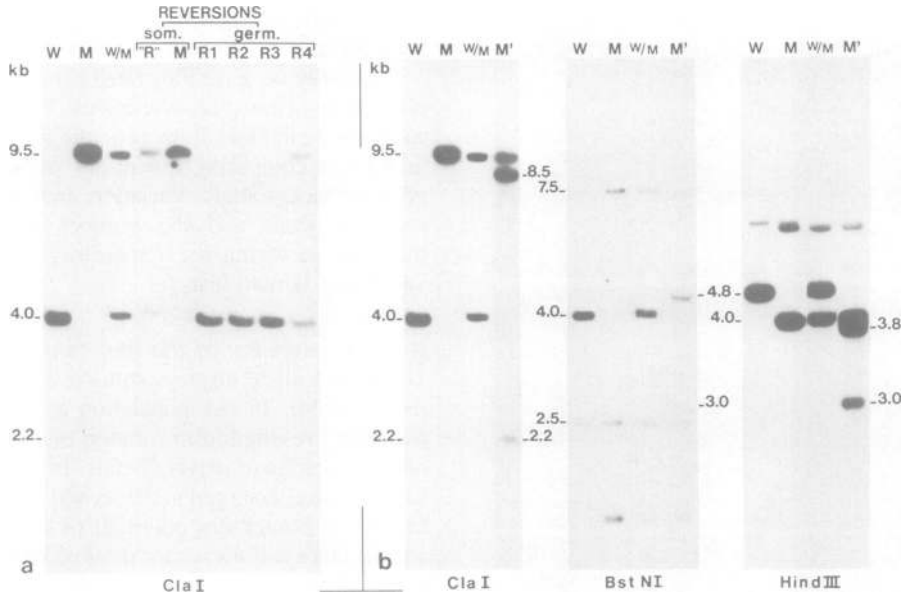

Fig. 3. Hybridization pattern of cDef1 with genomic DNA confirms its homology with the *deficiens* gene of *A.majus*. Southern blots were prepared from genomic DNAs digested with restriction enzymes as indicated at the bottom of each panel. For hybridization a 463 bp long *Hin*dIII−*Bam*HI (between positions 239 and 604 in Figure 5) fragment of cDef1 was used as radioactive probe. The probe detects a 4 kb long *Cla*I fragment in wild type (W = *defA*$^+$/*defA*$^+$) plants and two bands of 9.5 and 2.2 kb size in globifera (M = *defA*-1/*defA*-1) plants. The weak signal of the 2.2 kb band is due to the asymmetrical position of the transposable element insertion within the *defA* gene in *defA*-1 plants. Since the element contains a *Cla*I restriction site the portion of the probe hybridizing to this band is smaller than the portion hybridizing to the 9.5 kb band. In heterozygotes (W/M = *defA*$^+$/*defA*-1) the 2.5 kb band is hardly detectable. (**a**) Demonstration that somatic (som) and germinal (germ) reversions are due to excision of the transposable element insert. Phenotypic restoration of wild type gene activity can be correlated with restoration of the wild type size band in revertant ('R') branches of a globifera plant and also in germinal revertants. The germinal revertants used in the analysis were either homozygotes (R1 and R3) or heterozygotes (R2 and R4). Due to subsequent somatic excisions in a heterozygous (*defAR*/*defA*-1) revertant plant (exemplified by R2 in **a**) the *globifera* band is present but its intensity is very low. Such additional excisions in the mutant allele of the heterozygote lead to distorted segregation ratios between the wild type and *globifera* progeny, as was found after selfing of such revertants. The cDef1 also detects novel rearrangements (fragment sizes at the right of the panels in (**b**) in a newly obtained allele of the *defA* gene (M' with the genotype *defA*-4/*defA*-1). The fourth lane in the respective panels shows that the fragments corresponding to the wild type (*defA*$^+$) allele disappear in the new mutant which is still heterozygous with the *defA*-1 allele.

For preparing the enriched library we isolated mRNA from young wild type inflorescences (3−8 mm in length), since analysis of the temporal pattern of morphological changes in globifera plants indicated that the *defA*$^+$ gene must be expressed before the third whorl primordia appear. The sscDNA synthesized from the mRNA was subjected to two cycles of subtraction with an excess of leaf mRNA (40- to 50-fold enrichment) and the remaining sscDNA was converted into double stranded (ds) cDNA. dscDNA was cloned into the lambda vector NM1149. About 7 × 10^5 recombinants were obtained. Differential screening of 7 × 10^4 phages yielded 68 candidates showing clear differences in hybridizations with the (+) and (−) probes. Subsequently, experiments were carried out (i) to test independence or identity of the recombinants and (ii) to identify those candidates that were derived from the *defA* gene.

Genomic Southern blots using cDNA inserts as probes revealed that the 68 recombinants belong to 12 independent groups consisting of one to 10 group members (data not shown). Northern blot experiments confirmed the distinctness of these groups and also their differential behaviour with respect to their expression in mutant and wild type (data not shown).

Subsequently we tested by cosegregation experiments which of the independent recombinants may be the product of the *defA* gene. For this analysis genomic DNA was prepared from 46 individual F_2 plants displaying globifera phenotype (*defA*-1/*defA*-1) obtained by selfing the wild type (*defA*/*defA*-1) heterozygote. Only group VII, consisting of a single member (c23), revealed a restriction polymorphism that cosegregated with the chromosome marked by the *defA*-1 mutation (data not shown). Subsequent experiments with this group were carried out with the nearly full size (1042 bp long) cDef1 cDNA clone obtained by using c23 (250 bp long)

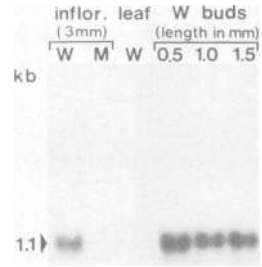

Fig. 4. Differential expression of the *defA* gene. Northern blot analysis was carried out with 2 μg mRNAs isolated from wild type (W) leaves and from young (3 mm long) influorescences (inflor.) of wild type and globifera mutant (M) plants. The absence of a signal in *defA*-1 inflorescences was proved to be true in several other genetic backgrounds (not shown). For analysis of expression during flower development mRNA was prepared from wild type buds of different sizes (the length is indicated above the lanes). cDef1 was used as radioactive probe.

to screen a non-subtracted cDNA library prepared from the mRNA of young wild type inflorescences. Two recombinants, hybridizing to c23, were detected in a library of 2×10^5 recombinant phages indicating that the abundance of the *defA* transcript in the mRNA population is $\sim 10^{-5}$.

cDef1 is homologous to the defA gene

RFLP cosegregation of a probe with a particular chromosome marker is a strong indication for linkage of the probe with this marker but it does not prove the identity of the probe with the gene. Therefore, we sought for independent ways to confirm the identity of cDef1 with the *defA* gene. Correlation of excision of a transposable element with restoration of wild type morphology provides an unequivocal criterion for identity of a molecular probe with the unstable gene (Wienand *et al.*, 1982).

As shown by Southern hybridization (Figure 3a), somatic reversion events generating a 'revertant' branch on a globifera (*defA*-1/*defA*-1) plant correlate with the appearance of a band with the size of the wild type DNA fragment. Somatic reversion, therefore, may be due to excision of a transposable element. The weak wild type band in a 'globifera' branch of the same plant indicates that in this branch the number of cells derived from the somatic revertant sector is low and hence does not confer an altered phenotype. Furthermore, after selfing revertant flowers from four independent plants, individuals with wild type flowers appearing in their progeny display the wild type DNA band. Heritable reversion events hence correlate with germinal excisions. These observations prove that instability of *defA*-1 plants is due to excision of a transposable element, and that cDef1 is a molecular probe identifying the *defA* gene. In fact, the genomic clone isolated from *defA*-1 plants with cDef1 as probe contains a 7kb long insert (named Tam7) in contrast to the corresponding wild type clone.

Additional proof for identity of cDef1 with the *defA* gene came from a transposon mutagenesis experiment during the summer of 1989. Several new *defA* alleles were obtained by screening a large population of heterozygous *defA*$^+$/*defA*-1 plants. Plants displaying phenotypic alteration, compared with the wild type phenotype of these heterozygotes, represent either a dominant mutation not related to the *defA* gene or recessive alleles of it. Investigation of the F_1 plants in Southern blots revealed rearrangements within the wild type *defA* locus as documented by one example (M′) in Figure 3b. Hence this plant represents a new recessive allele of the *defA* locus.

Expression of the defA gene in vivo

Northern blot analysis revealed that the 1.1 kb long mRNA hybridizing with cDef1 is only expressed in wild type inflorescences and is not detectable in either the mutant inflorescence or in wild type leaves (Figure 4). The lack of expression in vegetative tissues was true for all stages we tested including young shoots of seedlings (not shown). Furthermore, the level of expression of the *defA* gene seems to be nearly constant during the whole period of flower development, since *defA* is also expressed in earlier and later stages than those depicted in Figure 4 (not shown).

Northern hybridization experiments indicate that the *defA* mRNA is present in all floral organs, albeit its abundance in the mRNA populations varies in different organs (data not shown). Accordingly, *in situ* hybridization experiments did not detect a specific time or a specific tissue of expression (P.Huijser, unpublished data).

The structure of the putative DEF A protein as deduced from the DNA sequence of cDef1

The full size cDef1 clone has been sequenced (Figure 5) and its colinearity with the corresponding wild type genomic clone was confirmed (data not shown).

Translation of the cDNA sequence into a putative protein sequence revealed a 227 amino acids long open reading frame (ORF). Searching of the EMBL and Genbank databases indicated the presence of a conserved domain with homology to the proteins of the *FUN80* and *ARGRI* genes of yeast (Dubois *et al.*, 1987a,b), and to the SRF protein of mammals (Norman *et al.*, 1988). *FUN80* (or *ARG80*) corresponds to the *MCM1* gene of yeast (Passmore *et al.*, 1988) encoding the GRM/PRTF protein (Bender and Sprague, 1987; Keleher *et al.*, 1988; Jarvis *et al.*, 1989; Passmore *et al.*, 1989). Comparison of the DEF A protein with these proteins reveals a strongly conserved region of 58 amino acids containing 28 identical amino acids and 12 representing conservative exchanges (Figure 6).

As shown by Norman *et al.* (1988) SRF contains DNA binding and dimerization domains within the conserved region. The degree of homology between the DEF A protein and the other two proteins is higher within the DNA binding domain although the dimerization domain still shows conservation (Figure 6). The protein domain required for DNA binding and dimerization extends in SRF beyond the sequences presented in Figure 6 where in DEF A no homology is found anymore. In this extended region the homology between SRF and GRM is still maintained, albeit at a low degree (Norman *et al.*, 1988).

These observations strongly suggest that DEF A is a DNA binding protein like SRF and GRM/PRTF and is probably involved in the regulation of expression of a set of genes determining normal petal and stamen development in the flower of *A.majus*.

Discussion

Strategies to isolate genes involved in flower development

Molecular cloning and analysis of genes affected in homeotic mutants promises insight into the regulatory mechanisms directing flower morphogenesis. Two strategies to isolate such genes with unknown products and functions are available: transposon tagging and chromosome walking (discussed by Meyerowitz *et al.*, 1989). While transposon tagging has been successfully used to isolate plant genes (for compilation see Gierl and Saedler, 1989) the feasibility of chromosome walking still has to be demonstrated in plants.

In this report we have described a third alternative to the two strategies mentioned above: a combination of differential cDNA screening and transposon mutagenesis was used to isolate the *defA* gene. The strategy is very sensitive as indicated by the successful cloning of a rare mRNA (the *defA* mRNA has an abundance of $\sim 10^{-5}$ in the mRNA fraction used). The high sensitivity is achieved by screening a subtracted (enriched) cDNA library with subtracted (+) and (−) probes derived from mutant and wild type respectively. In addition to a probe for the *defA* gene, probes for 11 other genes affected by the mutation were obtained. In many cases

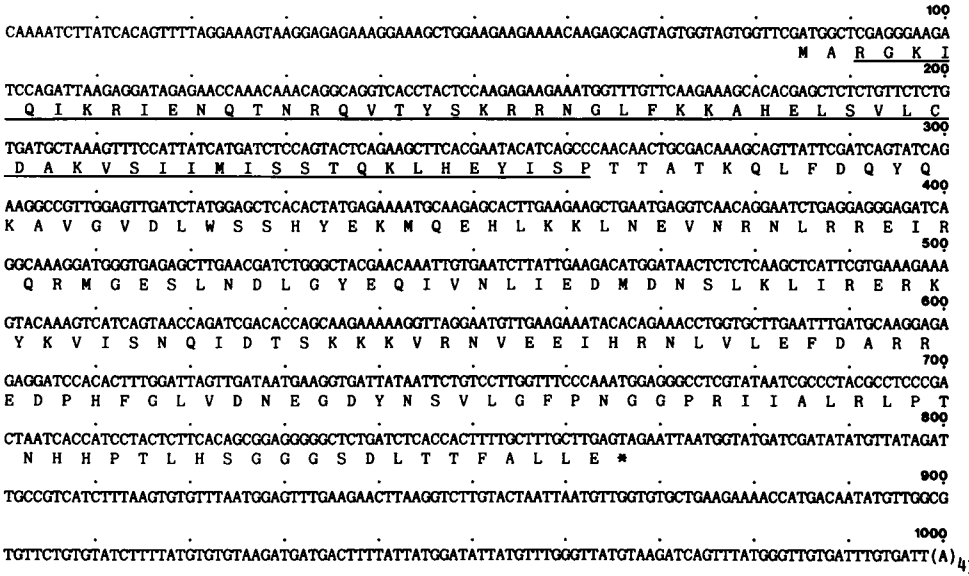

Fig. 5. Nucleotide and deduced amino acid sequence of the cDef1 cDNA corresponding to the gene product of the *deficiens* gene of *A.majus*. The region of conserved amino acids (see Figure 6) is underlined.

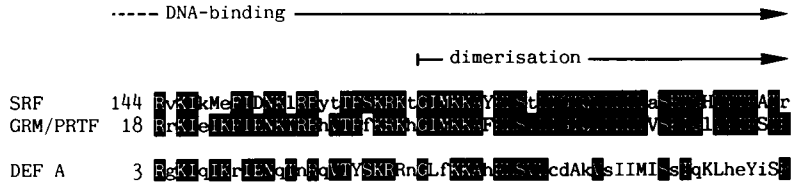

Fig. 6. Amino acid sequence homology between the DEF A protein and a conserved domain detected in transcription factors in mammals (SRF) and yeast (GRM/PRTF). The conserved domain between SRF and GRM/PRTF (Norman *et al.*, 1988) is aligned with the deduced amino acid sequence of the DEF A protein. Amino acids conserved in two out of the three proteins are shown in dark boxes and conservative exchanges by capital letters. Non-conserved amino acids are depicted with small letters. For comparing conservation the following groups of residues have been used: P,A,G,S,T (neutral or weakly hydrophobic); Q,N,E,D (hydrophylic, acid amine); H,K,R (hydrophylic, basic); L,I,V,M (hydrophobic); F,Y,W (hydrophobic, aromatic); C (cross-link forming). The position of the conserved domain within each protein is indicated by numbers at the left. The positions of particular domains within the conserved region (see Norman *et al.*, 1988) are shown at the top. The dashed line towards the N-terminal end and the arrowhead at the C-terminal region indicate that the domains determined for SRF extend upstream and downstream of the sequence depicted in the Figure.

these might represent organ specific genes, but a few could be direct target genes under the control of *defA*. These would allow the function of the DEF A protein to be studied at the molecular level. The identity of clone c23 with the *defA* gene was shown unequivocally in Southern experiments by utilizing the instability of a mutant allele, its revertants and rearrangements in *de novo* induced independent alleles.

This strategy has obvious advantages. Firstly, no complementation of the mutation in transgenic plants is required as for chromosome walking. Secondly, in contrast to transposon tagging, where a known transposable element has to be 'moved' into the gene to be isolated, our approach allows the use also of mutants due to insertion of unknown transposable elements. Obviously the strategy requires 'transcriptional' mutations and would fail for mutations affecting, for example, mRNA processing or translation.

The functional significance of homology of the DEF A protein with SRF and GRM/PRTF

The determination of cell fate during differentiation is fundamentally different comparing plants and animals (Walbot, 1985; Sussex, 1989), but the basic processes at the molecular level of gene regulation may be very similar. The structural conservation of the putative DNA binding domain in the DEF A protein might therefore be of significance in that it reflects functional similarities to the SRF and GRM/PRTF proteins. Since DEF A is the first protein, participating in flower organogenesis, accessible for molecular analysis it is tempting to discuss these aspects.

Concerning similarities in function, SRF and GRM/PRTF are regulatory proteins responding to general cellular signals (Bender and Sprague, 1987; Norman *et al.*, 1988; Keleher *et al.*, 1988). SRF is essential for the serum inducible transcriptional activation of the c-*fos* nuclear protooncogene of mammals. c-*fos* itself is involved in the transcriptional regulation of genes controlling cell growth in response to growth factors, but the precise function of the protein is not known. The yeast proteins GRM/PRTF are suggested to be similar, if not identical, products interacting in a cooperative manner with the $\alpha 2$ repressor or with the $\alpha 1$ activator thereby regulating the expression of cell type specific genes. The DEF A protein, in turn, has to be involved in the determination of floral meristem cells to differentiate into the appropriate organ in the right place, e.g. petals and not sepals in the second whorl and stamens instead of female organs in the third whorl. DEF A, therefore, could also be a regulatory protein responding to general cellular signals. Interestingly, SRE the *cis*-acting binding site of SRF and

the operators of α1 and α2 have closely related sequences (see Norman *et al.*, 1988; Passmore *et al.*, 1989). It will be interesting to learn whether or not the *cis*-acting binding site of DEF A will be similar to SRE and the yeast operators.

Concerning similarities in their expression pattern, SRF and GRM/PRTF are constitutively produced (Norman *et al.*, 1988; Keleher *et al.*, 1988; Jarvis *et al.*, 1989), SRF expression being in addition inducible. Remarkably, the *defA* mRNA is also constantly expressed during flower development (but not in leaves) and its expression does not seem to be organ specific. In this sense, *defA* belongs to the 'common denominator' type of genes suggested to exist in flower development (Lifschytz, 1988). Permanent expression, however, apparently contradicts specific action in space and time as required for developmental regulation, although evidence is emerging for the participation of pleiotropic regulatory genes in developmental processes (Cline, 1989). The models suggested for SRF and GRM/PRTF to describe their role as specific transcription activators/repressors assume that efficient interaction of the regulatory proteins with their *cis*-acting target is accomplished by their interaction with accessory factors.

Post-translational phosphorylation may modify DEF A specificity

Post-translational modification of regulatory proteins is one of the mechanisms to explain their spatial and temporal specificity. As indicated by Norman *et al.* (1988) SRF is a potential phosphoprotein with several recognition sites for phosphorylation. One of these is located within the DNA binding domain and hence is conserved in all proteins compiled in Figure 6 (RHVT for yeast, RYTT in SRF and RQVT in DEF A). The consensus RxxS/T represents a phosphorylation site for the calmodulin dependent multiprotein kinase of mammalian proteins (Cohen, 1988). Calmodulin governed phosphorylation of proteins is Ca^{++} dependent, and changes in calmodulin activity and Ca^{++} concentration are thought to be linked to environmental and hormonal influences in plants (for reviews see Kelly, 1984; Gilroy *et al.*, 1987). If true, this aspect is interesting because it would explain, in part, how carpellody, the phenotypic expression of *defA*-gene malfunction, can also be induced in many species by environmental and hormonal factors (Meyer, 1966; Wardlaw, 1967).

Accessory proteins may confer organ specificity on the DEF A protein action during flower development

Alternatively, the interaction of a constantly (and not tissue specifically) expressed regulatory protein with accessory proteins, binding cooperatively to the same regulatory region, could explain their specific function in space and time. This type of control has been suggested not only for GRM/PRTF and SRF but also for homeotic genes in *Drosophila* (Scott and Carroll, 1987; Cline, 1989). The partial conservation of the dimerization domain, especially that of the hydrophobic core sequence (VsIIMI in Figure 6), may be an indication for the involvement of DEF A in protein−protein interactions (homo- or hetero-oligomerization). In addition, DEF A does not contain any of the sequences characteristic for transcription activation (compiled by Mitchell and Tjian, 1989). It could well be that this function is provided by an independent protein(s) interacting with DEF A, whose expression is controlled spatially and temporally. Such interactions may in fact exist as suggested by the following observations.

Firstly, *defA* gene malfunction has differential effects on petal and stamen development. These are revealed (i) by the different phenotypic reversion response of petals and stamens to late somatic excision events in the unstable *defA*-1 mutation and (ii) by the observation that *defA* morphoalleles exist with simultaneously increasing effects on both petals and stamens which show a clear dominance relationship with respect to petal development but which do not show the same dominance order with respect to feminization of the male organ (Klemm, 1927; unpublished results). These observations provide suggestive evidence for the idea that for the regulation of organ specific expression of various genes DEF A interacts with different sets of proteins expressed in petals or stamens. Alternatively, organ specific *cis*-acting regulatory regions of 'organ-determinant' target genes may exist differing in DEF A binding affinity.

Secondly, the existence of non-allelic loci, like *globosa* and *viridiflora* (and several others obtained recently by us), whose non-function conditions globifera-like phenotypes indicates that the products of these genes either control or interact with each other.

Taking these arguments together it seems reasonable to hypothesize that *defA* and several other genes involved in determination of flower morphogenesis are organized in a regulatory network. The role of the DEF A protein within this network can now be elucidated. On the other hand, analysis of its expression pattern in non-allelic *deficiens* mutants should allow the determination of a possible relationship between the products of these regulatory genes. On the other hand, analysis of the structure and function of 'target' genes, whose expression is altered in the *defA*-1 mutant, should help to uncover the mechanism by which the DEF A protein exerts its control at the molecular level. These genes may be represented in the cDNA collection obtained by differential screening.

Materials and methods

Plant material

Plants were grown in the glasshouse at 20−25°C under additional light during the winter. Tagging experiments with larger populations were carried out in the field and plants of interest were transferred into the glasshouse for further analysis.

Genetic stocks

Seeds of genetic stocks were obtained from the Gatersleben (GDR) seed collection with exception of the lines *niv*::98 and T53 which were provided by Rosemary Carpenter (Norwich, UK) and have the genotype *niv*-53::Tam1 and *niv*-98::Tam3 respectively. In order to address phenotypes and genotypes correctly we wish to introduce a nomenclature which is based only in part on traditional designations as follows:

deficiens: wild-type phenotype of mutations conferring similar morphological alterations

def^{+}: genotype of wild type *deficiens* loci

defA: allelic series of mutations not complementing the classical *deficiens*^{globifera} mutation. Mutations conferring upon similar phenotypes but complementing each other are designated *defB* (e.g. *globosa*), *defC*, etc.

defA-1: genotype of the *deficiens*^{globifera} allele. Other mutant alleles are designated by successive numbers (e.g. *deficiens*^{nicotianoides} = *defA*-2, *deficiens*^{chloranta} = *defA*-3, etc.)

globifera: phenotype of *defA*-1.

The wild type plant used in our studies was a selfed progeny of line 50. For propagation *defA*-1 plants were pollinated with pollen obtained from plants with wild type flower morphology (line 50, T53, *niv*-98). The resulting wild type heterozygotes (*defA*/*defA*-1) were selfed and globifera (*defA*-1/*defA*-1) plants of their F_2 progeny were maintained vegetatively.

Transposon mutagenesis

To obtain new alleles of *defA* 45000 *defA*/*defA*-1 F_1 heterozygote plants were grown during the summer season of 1989. Heterozygotes were obtained by crossing globifera (*defA*-1) plants with the line T53 (*defA*$^+$). Seventeen candidates with altered flower morphology were obtained. The putative genotype of these plants is *defA*-(4-21)/*defA*-1. Their genetic analysis is in progress. In this report we used an F_1 plant directly obtained from the field.

Microscopy

Cross sections for microscopy (10 μm thick) were prepared after fixation of young buds and embedding in paraffin according to standard procedures. Subsequently the thin sections were stained with toluidin blue.

For scanning electron microscopy (SEM) young inflorescences were fixed for 48 h with 2% formaldehyde, dehydrated with dimethoxymethane overnight and critical point dried in liquid CO_2 as described by Gerstberger and Leins (1978). After mounting on SEM stubs, all bracts and, if necessary, several outer whorl organs of individual flowers, were removed manually. The prepared inflorescences were then gold-coated in a sputtercoater (SCD 004, Balzers/Liechtenstein). SEM was performed with a Zeiss DSM 940 apparatus. Photographs were taken on a Kodak TX-400 film.

Preparation of plant DNA and RNA

Plant DNA minipreps were prepared as suggested by Dellaporta *et al.* (1983) with some modifications as follows. Cellular extracts were prepared from 0.5 g of fresh or frozen leaves using a sap-extractor (Pollähne-press, MEKU/Wennigsen) allowing constant flow of extraction buffer. The total volume was adjusted to 10 ml and processed as cited above. 1/20 (0.5−1 μg) of the total DNA yielded was digested with restriction enzymes and subjected to Southern blot analysis.

Plant mRNA was prepared from frozen tissues as described by Logemann *et al.* (1987).

Subtraction of sscDNA

sscDNA was synthesized by priming with oligo $d(T)_{15}$ from 2−3 μg of poly(A)$^+$ RNA isolated from 3−8 mm wild type inflorescences in the presence of [α-^{32}P]dCTP (Maniatis *et al.*, 1982). Specific activity was ~10^6 c.p.m./μg sscDNA. sscDNA was hybridized with 30 μg (10- to 15-fold excess) of leaf mRNA in 20 μl HP buffer containing 0.12 M NaCl, 1 mM EDTA and 0.1% SDS for 20−24 h at 68°C (Sargent, 1987). Separation of sscDNA and double stranded hybrid was carried out by chromatography on hydroxylapatite (Bio-Gel HTP, DNA-grade from BioRad) in a jacketed column (0.3 ml bed volume) at 60°C according to Sargent (1987) with modifications. The hybridization mixture was diluted with 40 mM Na-phosphate (pH 6.8) and applied to the column. sscDNA was eluted with 0.12 M Na-phosphate (pH 6.8) in 400 μl fractions and purified on Qiagen-tip5 (Diagen, Düsseldorf) according to the manufacturer's protocol. The sscDNA was eluted with 2 × 300 μl buffer G (50 mM MOPS, pH 7.5, 1.2 M NaCl, 30% EtOH, 5 M urea) and precipitated with 0.8 vol of 2-propanol. The enrichment was ~10- to 12-fold. The sscDNA was then subjected to a second cycle of subtraction with 15 μg of leaf mRNA (50-fold excess), etc. About 25−30 ng of ssCDNA, 40- to 50-fold enriched for flower specific sequences, were obtained that were used to construct a cDNA library.

Construction of an enriched cDNA library

The enriched sscDNA was converted into double strand cDNA (dscDNA) using the classical 'hairpin' method with the four dNTPs and Klenow polymerase at 15°C overnight (Maniatis *et al.*, 1982). The hairpin loops were digested with mung bean nuclease (2 units in 50 μl 30 mM Na-acetate, pH 4.6, 50 mM NaCl, 1 mM $ZnCl_2$) at 27°C for 15 min and the ends of the dscDNA repaired with T_4 DNA polymerase (2−3 units in 50 μl Tris−HCl, pH 8.3, 6 mM $MgCl_2$, 50 mM NaCl, 10 mM DTT, 0.1 mM dNTPs) for 30 min at 37°C. *Eco*RI linkers were added according to Maniatis *et al.*, (1982) in 20 μl volume at 16°C overnight. After digesting with *Eco*RI (100 units, 30 min at 37°C) the excess linkers and small dscDNA (<150−200 bp) were removed on a Qiagen tip5 (see above). About 5−6 ng *Eco*RI linkered dscDNA were obtained. 2 ng were ligated into the *Eco*RI restriction site of 200 ng lambda NM119 vector (Murray, 1983); incubation was 40 min at room temperature, then at 16°C overnight in 5 μl total volume. The ligation mixture was packaged *in vitro* and for screening plated with the Hfl strain POP13b (Murray, 1983). 7 × 10^5 recombinants were obtained.

Preparation of (+) and (−) probes

For preparation of probes sscDNA synthesized from poly(A)$^+$ RNA isolated from wild type (+) and mutant inflorescences (−) were subtracted with 20-fold excess of leaf mRNA as described above (10- to 15-fold enrichment). Of the remaining sscDNA 5−10 ng were then 'oligo-labelled' by random priming in the presence of 150−200 μCi [α-^{32}P]dCTP and the other three dNTPs, following the Amersham protocol. Excess radioactive dCTP was removed on a Sephadex G-50 column.

Library screening

For differential screening 10^3 recombinants were plated per plate (90 mm φ). Two replica nitrocellulose filters were prepared from each plate, one hybridized with the 'plus', the other with the 'minus' probe. Hybridization was at 68°C for 12−15 h in 5 × SSPE. Filters were washed twice in 2 × SSPE at 68°C for 25 min (wash buffer not prewarmed). The 'plus' and 'minus' filters were mounted side by side on blotting paper, covered with Saran wrap and exposed at −70°C with intensifying screen for 1−3 days.

Blotting techniques and hybridizations

After agarose gel electrophoresis RNA and DNA were transferred to nitrocellulose filters (Hybond N or Hybond N$^+$, Amersham) by standard techniques (Maniatis *et al.*, 1982). Hybridization with labelled probes was overnight at 42°C in 50% formamide and 5 × SSPE for Northern blots and at 68°C in 5 × SSPE for Southern blots.

Radioactive labelling

Probes used for Nothern and Southern blots were labelled by 'random priming' according to the protocol supplied by Amersham. 5′ and 3′ end-labelling of DNA fragments was performed according to standard protocols (Maniatis *et al.*, 1982).

DNA sequencing

The 1.1 kb long cDef1 insert was subcloned into a Bluescript plasmid vector. Both DNA strands have been sequenced by plasmid sequencing (according to the protocol devised by Pharmacia) and by the chemical degradation method (Maxam and Gilbert, 1980).

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