The EMBO Journal vol.11 no.4 pp.1239-1249, 1992

Bracteomania, an inflorescence anomaly, is caused by the loss of function of the MADS-box gene *squamosa* in *Antirrhinum majus*

Peter Huijser, Joachim Klein, Wolf-Ekkehard Lönnig, Hans Meijer, Heinz Saedler and Hans Sommer

Max-Planck-Institut für Züchtungsforschung, 5000 Köln 30, FRG

Communicated by H.Saedler

Anomalous flowering of the *Antirrhinum majus* mutant *squamosa* (*squa*) is characterized by excessive formation of bracts and the production of relatively few and often malformed or incomplete flowers. To study the function of *squamosa* in the commitment of an inflorescence lateral meristem to floral development, the gene was cloned and its genomic structure, a well as that of four mutant alleles, was determined. SQUA is a member of a family of transcription factors which contain the MADS-box, a conserved DNA binding domain. In addition, we analysed the temporal and spatial expression pattern of the *squa* gene. Low transcriptional activity of *squa* is detectable in bracts and in the leaves immediately below the inflorescence. High *squa* transcript levels are seen in the inflorescence lateral meristems as soon as they are formed in the axils of bracts. *Squa* transcriptional activity persists through later stages of floral morphogenesis, with the exception of stamen differentiation. Although necessary for shaping a normal racemose inflorescence, the *squa* function is not absolutely essential for flower development. We discuss the function of the gene during flowering, its likely functional redundancy and its possible interaction with other genes participating in the genetic control of flower formation in *Antirrhinum*.

Key words: floral meristem development/flower reversion/homeosis/*in situ* hybridization/transcription factor

Introduction

The process of flowering in higher plants can be divided into two stages, initiation and differentiation, which have different sensitivity to changes in environmental and internal factors. Initiation of flowering strongly depends on external and internal factors such as light, temperature, nutrition, age and size (for review see Bernier, 1988). In contrast, the subsequent stage of floral differentiation in most plants seems to be an all-or-none process: once started, it goes to completion and it is difficult to influence it externally (Battey and Lyndon, 1990). Due to the different experimental accessibilities of the two stages, physiologists have focused mainly on the early stages of flowering whereas geneticists have felt more attracted by the later stages. What factors mediate between the presumptive flower promoting signal(s) and the morphological changes during flower development is still not known.

The process of flowering is under genetic control. Several distinct phases in this process are identified and defined by mutants with altered inflorescence and/or flower morphology. In *Antirrhinum*, floral organ identity and the determinate mode of growth of the floral meristem are established under the control of 'late' genes like *deficiens* (*def*), *globosa* (*glo*) and *plena* (*ple*) (Schwarz-Sommer *et al.*, 1990). Early stages in flower development, immediately after floral evocation, are characterized by the activity of genes like *floricaula* (*flo*), *squamosa* (*squa*), *squamata* (*squam*) and *centroradialis* (*cen*) (Carpenter and Coen, 1990; Coen *et al.*, 1990; Schwarz-Sommer *et al.*, 1990; Stubbe, 1966). These 'early' genes determine the identity of floral meristems and, thereby, exert temporal and spatial control over flower formation.

Recently, several floral morphogenetic genes have been isolated (Coen *et al.*, 1990; Sommer *et al.*, 1990; Yanofsky *et al.*, 1990) in an attempt to unravel the major molecular mechanisms governing floral development. One of these is the *deficiens* gene of *Antirrhinum* which, upon mutation, causes homeotic alterations of floral organs. Its putative protein product contains a conserved DNA binding domain (MADS-box; Sommer *et al.*, 1990) previously identified and characterized in the mammalian SRF (Norman *et al.*, 1988) and yeast MCM1 (Passmore *et al.*, 1989) transcription factors. A similar DNA binding domain was found in the putative protein product of the *Arabidopsis* gene *agamous* (*ag*), also known to affect the identity of floral organs (Yanofsky *et al.*, 1990). Most interestingly, the homeotic alterations in *ag* mutant flowers differ completely from those of *deficiens* mutants. This finding raised the question whether additional plant genes exist whose protein products also contain this conserved DNA binding domain (Schwarz-Sommer *et al.*, 1990). In fact, several other MADS-box genes could be identified in *A.majus*. Two of these were identical to two known morphogenetic genes, *glo* and *squa* (Schwarz-Sommer *et al.*, 1990).

The *squamosa* gene is particularly interesting because of its key role in the genetic control of flowering. Mutations in this gene result in the development of shoots at positions where the wild type inflorescence bears flowers, i.e. in the axils of the bracts formed by the shoot apical meristem. These secondary shoots also form bract-like leaves, and the whole process is repeated with the formation of new shoots in their axils. However, *squa* inflorescences regularly produce anomalous, proliferous flowers, a feature inherent to the *squa* phenotype. Excessive formation of bracteal leaves, often combined with the failure to form normal flowers, is called bracteomania and is known to occur in many flowering plant species (Penzig, 1922). The *squa* mutant of *Antirrhinum*, displaying this abnormality, has been known for more than half a century; Baur and coworkers obtained it several times in the course of chemical and physical mutagenesis experiments (Baur, 1930; Kuckuck and Schick, 1930; Stubbe, 1932, 1966).

The bracteomaniacal phenotype of *squamosa* suggests that molecular analysis of the corresponding gene, its regulation

and its function may help to identify the factors that merge initiation and differentiation in the integrated process of flowering.

Results

Morphological description of the squamosa phenotype

With the onset of reproductive growth in *Antirrhinum*, the vegetative shoot meristem is reorganized to form a racemose inflorescence (Figure 1A). The still indeterminately growing shoot apical meristem starts formation of scale-like leaves (bracts), the associated axillary meristems of which form single flowers in wild type plants (Awasthi *et al.*, 1984). In plants homozygous for the recessive *squa* mutation (Stubbe, 1966), regular transition of these axillary meristems to floral meristems fails. As a result of this defect the axillary meristems grow out as secondary shoots which resemble the main *squa* inflorescence in that they form bract-like leaves and reiterate the process of shoot formation in the axils of these bract-like leaves (Figure 1A and B).

Squa mutant plants have not completely lost their ability to flower. Axillary meristems of the *squa* mutant inflorescence occasionally undergo a (partial) transition to a floral meristem. Complete flowers, in which all organ types are present, may look virtually normal (Figure 1D) but are mostly malformed and are supported by elongated pedicels which sometimes bear bract-like leaves (bracteoles). Although flowers can be formed in the same position as in wild type inflorescences (i.e. in the axils of the bract-like leaves on the primary inflorescence), more regularly flowering is seen on secondary and higher order inflorescences (Figure 1C). Generally, flowering on a secondary shoot starts with formation of an incomplete flower before the appearance of more normal-looking flowers (Figure 1F). Petal-like leaves, often only two in an adaxial position and closely resembling the (adaxial) petals of the upper lip of the corolla of a wild type flower, may be found alternating with bract-like sepals clustered in a whorl-like organization. The development of axillary shoots associated with these bract-like sepals is generally suppressed. The central shoot

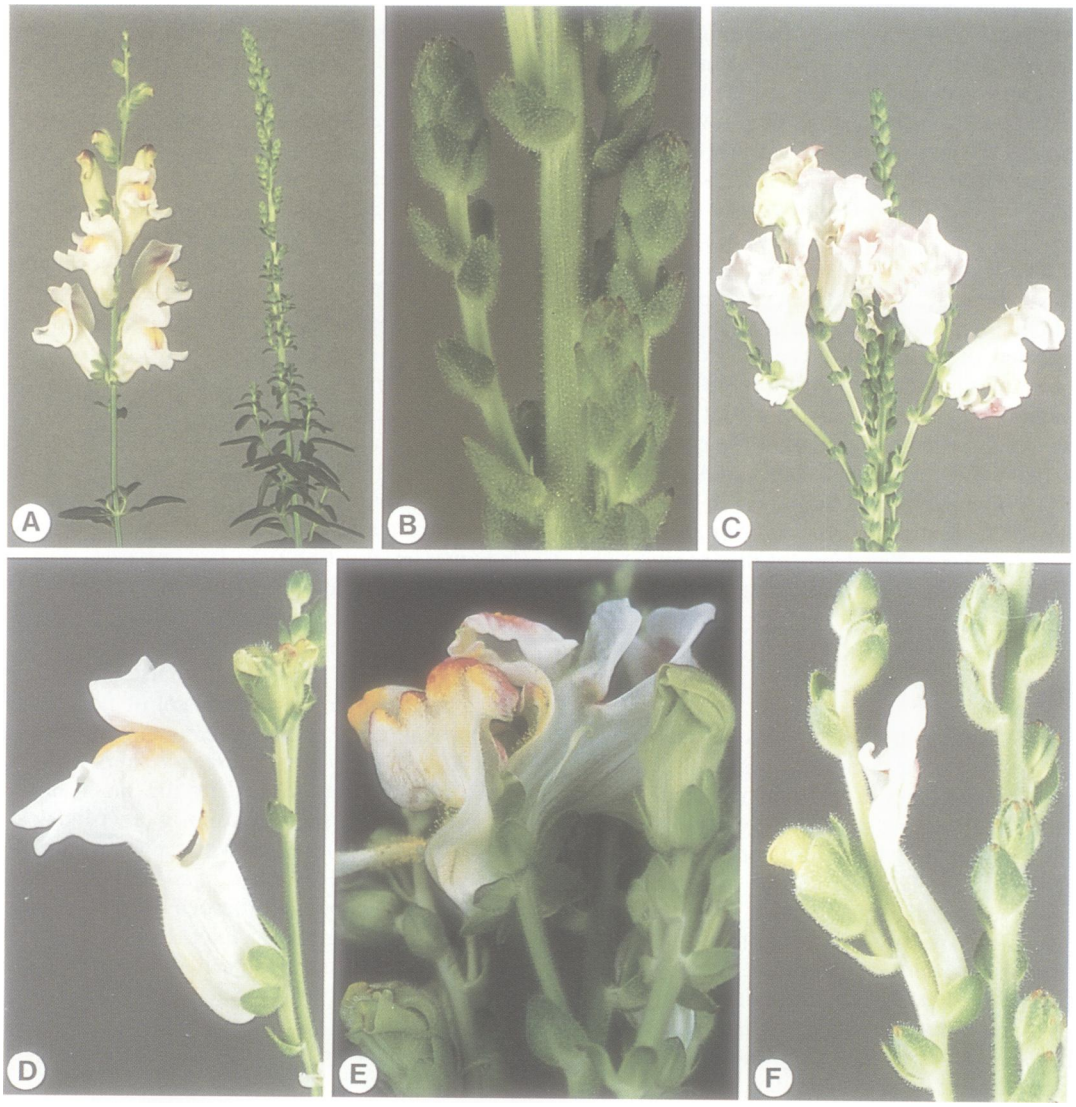

Fig. 1. Wild type and *squamosa* mutant inflorescences of *A.majus*. (**A**) Wild type inflorescence (line S50) next to *squamosa* mutant inflorescence (right). (**B**) Detail of *squa* mutant inflorescence showing the development of shoots in the axils of the bracts. (**C**) Flowering *squa* mutant. Some flowers show petaloid sepals, petals with greenish rims and/or bracteolated pedicels. (**D**–**F**) *Squa* mutant flowers. (**D**) Normal-shaped and fertile flower. (**E**) Complete but abnormal flower with twisted corolla. (**F**) Partial flower with only adaxial petals formed alternating with bract-like sepals.

proliferates, and again bracts are formed although even stamens and carpels may be initiated after petal formation. Then, in the axils of these bracts, complete but often abnormal flowers may develop. The number of sepals in the first whorl is sometimes higher than five and one or more of them may be filamentous or bract-like or may carry petaloid tissue (petaloid tissue has also been observed in the bracts subtending flowers in higher order inflorescences). Petals often display virescent sepaloid tissue at their margins which probably prevents normal cohesion and full closure of the corolla tube and, together with their possible aberrant initiation, may cause irregular growth resulting in a twisted corolla (Figure 1E). Although the number of stamens and carpels sometimes increase from a normal four to six and two to three, respectively, these organs seem less affected in the complete flowers, thus securing male and female fertility. However, stamens may show petalody.

The four different *squa* mutant lines we investigated (see Materials and methods) show very similar morphological characteristics. The high variability of the phenotype, probably caused by environmental factors, makes it difficult to recognize minor dissimilarities. Moreover, even significant differences do not necessarily point to different allelic effects but could equally well be explained by differences in the genetic backgrounds of the lines.

Instability of mutant alleles

Instability of mutations is often observed in *A.majus* (Stubbe, 1966) and is known to be due to the presence of transposable elements in the respective genes (Bonas *et al.*, 1984). Upon excision of the transposon from the mutated gene, the wild type phenotype may be partly or completely restored. Excision may happen in somatic and/or germinal cells. Only in the latter case is restoration of the wild type gene function heritable and, by this criterion, can be distinguished from leakiness of the mutation.

In order to determine if the flowers produced by the *squa* mutant *squa*293 (see Materials and methods) could be due to excision of a transposable element, we selfed 17 flowers of different plants of this line. Besides the expected squa phenotype, wild type plants were also observed in their progeny, indicating that instability of the squa phenotype is due to the presence of an active transposon.

It is important to notice that the relative numbers of wild type plants observed in the progeny of the different, selfed *squa*293 flowers varied between none and 67%. This could indicate that formation of fertile flowers on *squa* mutant plants does not require restoration of the wild type gene function in all the cells of the developing flower, at least not in the cells giving rise to the sporogenous tissues of stamens and carpels. In fact, molecular analysis of other *squa* alleles shows that the *squa* function is not required at all for the occasional formation of single flowers (see below).

Molecular cloning of the squamosa gene

In a search for transcription factors possibly involved in *Antirrhinum* flower morphogenesis, a cDNA library made from poly(A)$^+$ RNA isolated from young inflorescences was probed with a fragment of the Def1 cDNA clone comprising the conserved DNA binding domain, the MADS-box (see Materials and methods). Eight independent genes were identified. In Southern blot experiments, one of the cDNA clones (defH33) uncovered restriction fragment length polymorphisms (RFLPs) between genomic DNA isolated from wild type and *squa*293 plants (Figure 2A). As already mentioned, the genetic instability of line *squa*293 was suspected to be due to the insertion of a transposable element. To corroborate this further, we studied the detected RFLP in several wild type revertants as well as their mutant *squa* sisters in the progeny of selfed flowers of homozygous *squa*293 plants. Full restoration of the wild type phenotype was found to correlate with the reappearance of the wild type restriction fragment pattern (Figure 2B). This result strongly indicates that the *squa*293 mutation is caused by the insertion of a transposable element which, upon germinal excision, may restore the wild type gene function in a heritable way, and thus proves that the defH33 cDNA is derived from the *squa* gene.

By physical mapping (Zs.Schwarz-Sommer and I.Nindl, unpublished data) the *squa* locus was found to be linked to the *olive* (Luo *et al.*, 1991) and *plena* loci (*plena* probe kindly provided by R.Carpenter, D.Bradley and E.Coen, John Innes Institute, Norwich, UK).

Structure of the putative SQUA protein

As deduced from the cDNA nucleotide sequence, the *squa* gene has the potential to code for a 248 amino acid protein of ~29 kDa M_r (Figure 3B). Like the DEF A protein and several other MADS-box proteins identified in *A.majus*, the SQUA protein carries the putative DNA binding domain at the N-terminus. A potential phosphorylation site (RQVT) for a calmodulin-dependent protein kinase (Cohen, 1988), present in the DNA binding domain of the DEF A protein (Schwarz-Sommer *et al.*, 1990), is also conserved in the SQUA protein at an identical position. Outside this well conserved domain, SQUA and the other MADS-box proteins have little homology, with the exception of the middle region which contains another conserved domain, the K-box (Ma *et al.*, 1991; Figure 4). Because of its similarity to a certain part of keratin these authors suggested that this domain is capable of forming two amphipathic helices. The functional

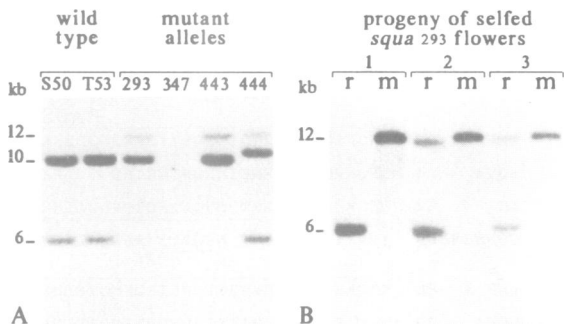

Fig. 2. Southern blot analysis of wild type and *squamosa* mutants. (**A**) Hybridization pattern of *Bam*HI digested genomic DNA of wild type (lines S50 and T53) and homozygous *squa*293, 347, 443 and 444 mutant plants with defH33 as probe. A 6 kb fragment observed in the wild type is altered to 12 kb in both the *squa*293 and 443 mutants. A 10 kb wild type fragment is replaced by two fragments of 10.5 and 12 kb in the *squa*444 allele. No hybridization is seen to genomic DNA of the *squa*347 mutant. (**B**) Hybridization of the genomic 6 kb *Bam*HI wild type fragment to *Bam*HI digested genomic DNA of phenotypically wild type revertants (r) and *squamosa* mutant (m) plants in the progeny of three selfed *squa*293 flowers (1, 2 and 3). The wild type phenotype of the revertants always correlates with the reappearance of the 6 kb fragment. Note that homozygous as well as heterozygous revertants can be observed.

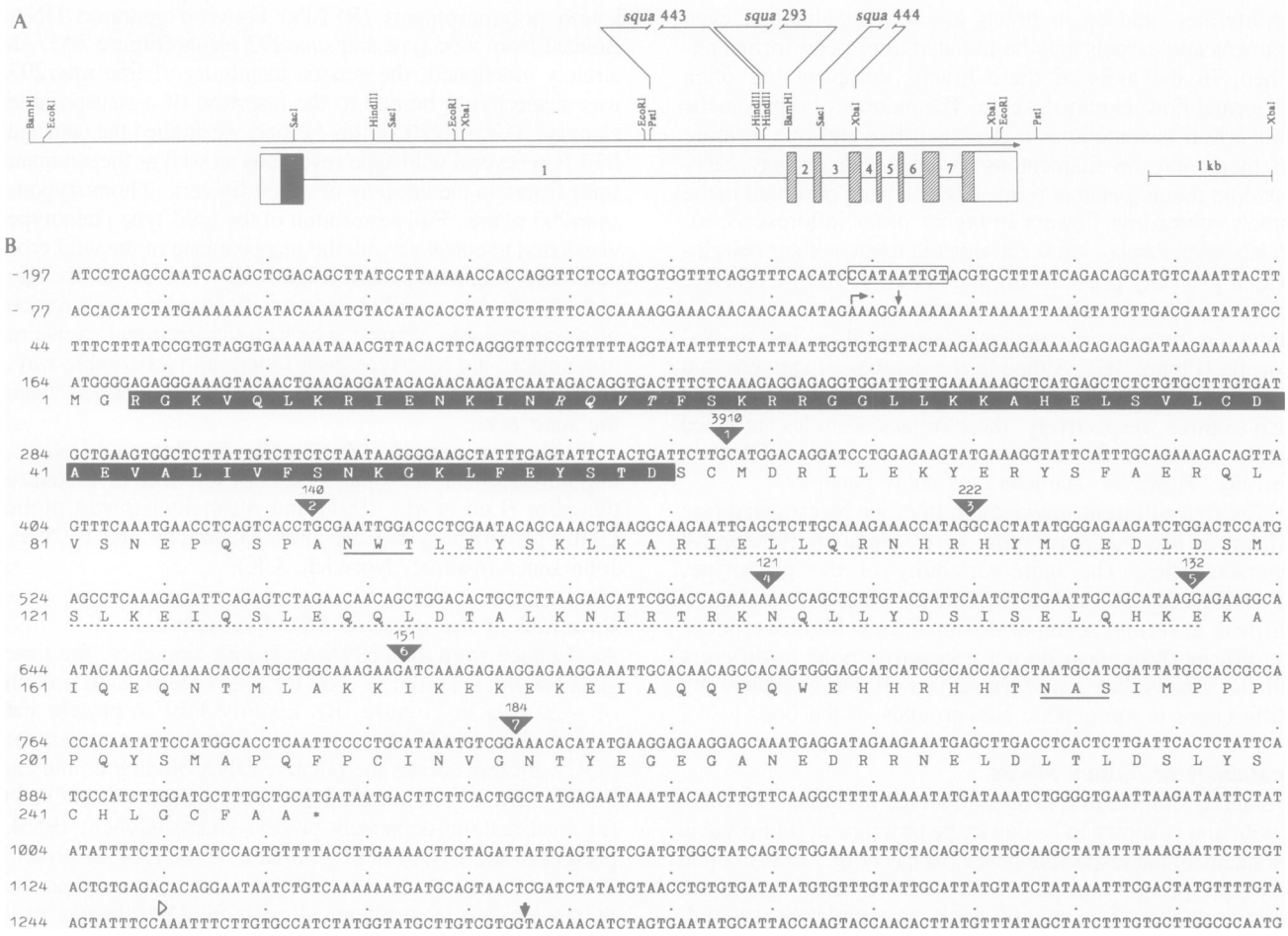

Fig. 3. Genomic organization and nucleotide sequence of the *squamosa* gene. (**A**) Exon−intron structure of the *squamosa* transcription unit is shown schematically below a restriction map of its genomic locus. The horizontal arrow indicates the direction of transcription. Boxes represent exons, horizontal lines introns. Filled boxes indicate protein coding domains within the exons; the MADS-box is in black and the rest of the protein coding domains are hatched. The approximate sites of the insertions found in the *squa* 293, 443 and 444 alleles are indicated above the restriction map. (**B**) Nucleotide sequence of the exons of the transcribed region including some upstream and downstream sequences. Numbered triangles mark the positions of introns with their respective size in nucleotides. The first nucleotide of the transcription start site and the first amino acid residue of the SQUA protein, shown below the nucleotide sequence, are numbered 1. The MADS-box is shown inverted; the K-box is underlined with a dashed line. A conserved potential phosphorylation site within the MADS-box is shown in italics and potential glycosylation sites are underlined with solid lines. A hooked arrow marks the transcription start site and a putative SRE-like binding site within the promoter region is boxed. A polyadenylation site is indicated by an open triangle. Small vertical arrows mark the start and end of the longest isolated cDNAs (see Materials and methods).

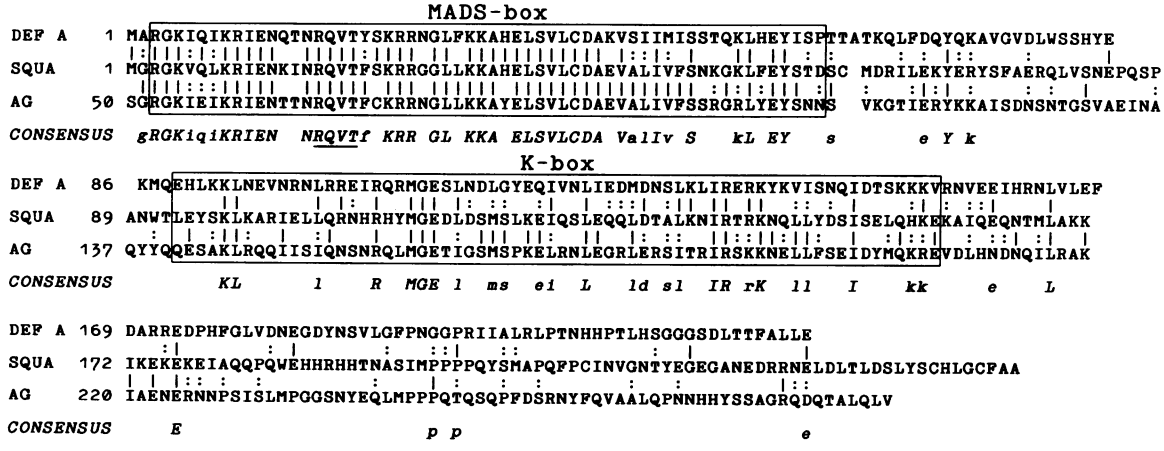

Fig. 4. Comparison of the SQUA protein with two other plant MADS-box proteins. Homology between the deduced amino acid sequences of the SQUA protein with the MADS-box proteins DEF A (Sommer *et al.*, 1990) and AG (partly shown; Yanofsky *et al.*, 1990) is shown. Lines and colons represent amino acid identity and conservative amino acid changes, respectively. The MADS-box and K-box domains are indicated. Note that the gaps necessary to improve the alignment are outside the conserved domains and coincide with exon boundaries. A consensus sequence is shown in italics. A conserved putative phosphorylation site is underlined.

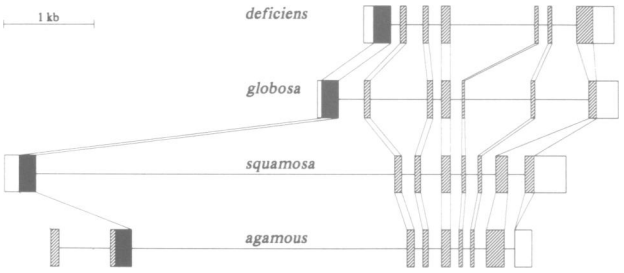

Fig. 5. Comparison of exon−intron structure of the *squamosa* gene with other floral MADS-box genes. The exon−intron structures of the MADS-box genes *squa*, *def* (Schwarz-Sommer *et al.*, 1992), *glo* (Tröbner *et al.*, in preparation) and *ag* (Yanofsky *et al.*, 1990) are represented schematically as described in Figure 3A. Exons of the different genes encoding comparable protein domains are connected with thin lines.

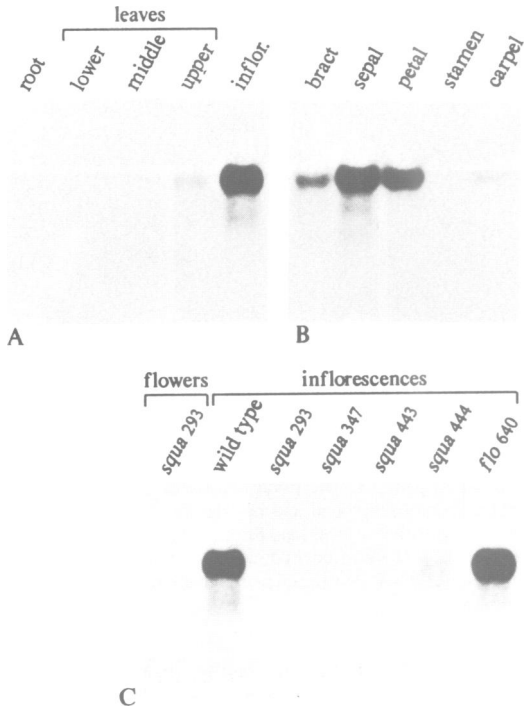

Fig. 6. Northern blot analysis of the *squamosa* expression pattern. (**A** and **B**) Poly(A)$^+$ RNA was isolated from different organs of wild type plants as indicated above the lanes and probed with the 3′ part of the *squamosa* cDNA (without the MADS-box). Leaf RNA was isolated from the first two true leaves (lower), leaves of nodes 5 and 6 (middle) and from the two leaves just below the inflorescence (upper) of flowering plants with 1 cm long inflorescences. Floral organs were dissected from 1−2 cm flower buds (i.e. before anthesis).
(**C**) Poly(A)$^+$ RNA from wild type, and from different *squamosa* and *floricaula* mutant inflorescences, as well as of young *squa*293 flowers (genotypes indicated above the lanes) was hybridized with *squamosa* cDNA as in panels A and B. Size of the hybridizing RNA species in panels A−C is 1.25 kb.

importance of this domain in the DEF A protein has recently been demonstrated by us (Schwarz-Sommer *et al.*, 1992). Two non-conserved glycosylation sites, NWT and NAS (Fishleigh *et al.*, 1987), are found C-terminal to the MADS-box in the SQUA protein (Figure 3B).

Structure of the squamosa gene

The cDNA clone defH33 was used as a probe to isolate the corresponding genomic clone. The genomic organization (Figure 3A) and the nucleotide sequence of the *squa* gene, as well as part of its flanking DNA (Figure 3B), were determined from one of the identified genomic clones. Its exon−intron structure was derived from comparison of the sequence of the genomic clones with the defH33 cDNA sequence. The donor and acceptor sites for splicing, GT and AG respectively, are conserved in all of the seven introns which separate the eight exons encoding the SQUA protein. Primer extension and S1 nuclease mapping (data not shown) revealed a transcriptional start site 163 nucleotides upstream of the first nucleotide of the translation initiation codon (Figure 3B). No putative consensus plant gene TATA box (Joshi, 1987) is detected upstream of this transcriptional startpoint. A sequence motif, CCATAATTGT, is found in the *squa* gene promoter which resembles the consensus DNA binding sequence (CArG box) of the mammalian SRF and yeast MCM1 transcription factors (Hayes *et al.*, 1988).

The structure of the *squa* transcription unit is similar to that of other known MADS-box genes (Figure 5). For example, the first six exons of the *squa* gene are of almost the same size as the first six exons of the *def* gene (Schwarz-Sommer *et al.*, 1992) and those of the *glo* gene (Tröbner *et al.*, in preparation). In both genes, the putative DNA binding domain (MADS-box) is encoded by their first exon. The middle region of the protein, which contains the conserved K-box, is encoded by the third, fourth and fifth exons in the case of *squa* and *def*. Except for an additional exon upstream of the MADS-box encoding exon in the case of *agamous*, a similar organization was found for the *agamous* and *agamous*-like genes in *Arabidopsis* (Yanofsky *et al.*, 1990; Ma *et al.*, 1991).

Analysis of the squamosa mutant alleles 293, 443 and 444

Fragments of the wild type gene were used as probes to analyse the genomic organization of the four different *squa* mutations (Figures 2A and 3A). The RFLP observed with genomic DNA of *squa*293 was found to be due to an ~6 kb insertion within the 3′ end of the large first intron and the first nucleotides of the second exon. In the mutant *squa*443, another insertion of ~6 kb was found at the end of the first large intron. The *squa*444 mutation is caused by an insertion in a region spanning the third and fourth exons and the intron separating them. The exact nature of these inserts has not been analysed. However, results described previously (see above) suggest that at least the *squa*293 insertion represents a mobile transposable element.

Analysis of the squamosa mutant allele 347

When the mutant allele *squa*347 was analysed using the transcribed region of the wild type *squa* gene or its immediately flanking sequences (covering a total of 16 kb of genomic DNA; not shown) as a probe, no signal was detected on Southern blots made from genomic DNA of plants homozygous for this mutation (Figure 2A). Thus, in this mutant line the *squa* gene is completely deleted. Since this allele occasionally produces flowers (selfing of which never resulted in wild type progeny), flowering does not seem to be absolutely dependent on the *squa* function.

Temporal and spatial expression of the squamosa gene

To study the expression of the *squa* gene, poly(A)$^+$ RNA was isolated from different plant organs and analysed in

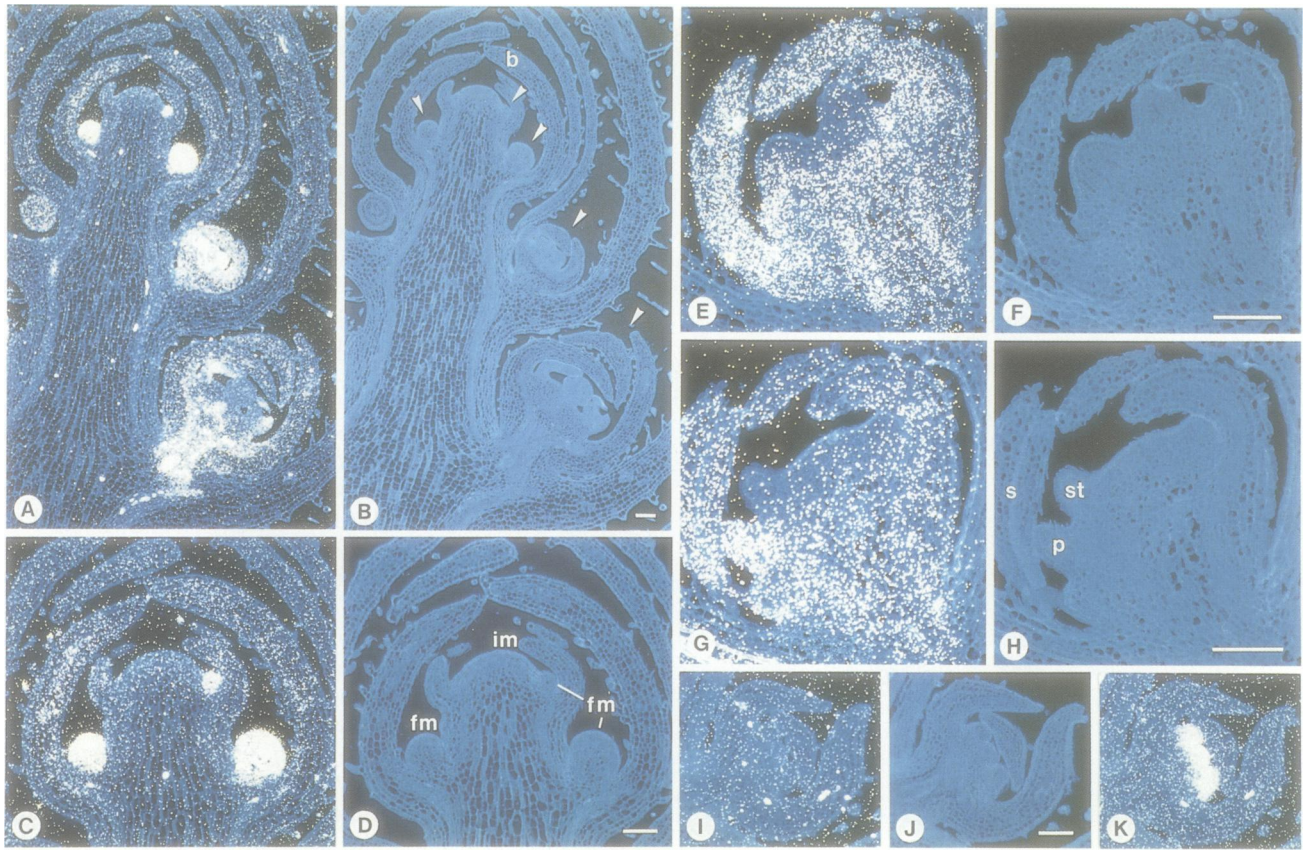

Fig. 7. Analysis of *squamosa* mRNA distribution by *in situ* hybridization. *In situ* hybridization of ^{35}S-labelled *squamosa* (A–I) or *deficiens* (J and K) antisense RNA (not containing the MADS-box) to tissue sections. Autoradiographic signal was studied in darkfield illumination (A, C, E, G, I and K). Epifluorescence was used to visualize the underlying tissue (A–K). Bar = 100 μm. (**A** and **B**) Longitudinal section through a young wild type inflorescence. Hybridization signal is clearly seen in the floral primordia and the floral buds (arrowheads) developing in the axils of the bracts. Weak signal can be detected in young bracts (b). (**C** and **D**) Detail of the inflorescence apex shown in panel A. The floral meristems (fm) are strongly and uniformly labelled in contrast to the infloral meristem (im) where the signal is only slightly above background and not significantly different from that obtained with a sense probe (not shown). (**E** and **F**; **G** and **H**) Two longitudinal sections at different positions through the same young flower bud showing expression in the sepals (s) and petal primordia (p) but not in stamen primordia (st). (**I–K**) Longitudinal and serial sections through a young *squa*443 mutant flower bud hybridized with *squamosa* (I) or *deficiens* (J and K) antisense RNA. No significant signal can be detected with the *squamosa* probe, whereas the *deficiens* probe strongly hybridizes to petal and stamen primordia.

Northern blot experiments. When probed with the defH33 cDNA clone (to avoid cross-hybridization to other MADS-box genes we deleted the MADS-box coding region), a signal corresponding to a 1.25 kb mRNA could be detected in poly(A)$^+$ RNA from wild type inflorescences. This RNA species seems to be absent from vegetative parts of the plant such as roots and leaves, except for the leaves immediately below the inflorescence where a weak signal is detected (Figure 6A). When expression was investigated in bracts and floral organs at later stages of development, no *squa* transcript was found in developing stamens. Expression is relatively strong in sepals and petals but weak in bracts and carpels (Figure 6B). *Squa* gene transcripts are not detectable in plants homozygous for the *squa*293, 347 and 443 mutations. However, a weak hybridization signal is seen in Northern blots with *squa*444 RNA, at and below the position where wild type *squa* transcripts are detected (Figure 6C).

Mutant inflorescences of the *Antirrhinum floricaula* gene (Carpenter and Coen, 1990) display some similarities to the *squa* phenotype. However, expression of *squa* is detectable in *flo* mutant inflorescences at a level comparable to that of the wild type (Figure 6C).

To determine the spatial and temporal expression pattern of the *squa* gene more precisely, we performed *in situ* hybridization experiments on young inflorescences using ^{35}S-labelled *squa* antisense RNA as a probe (see Materials and methods). *Squa* gene transcripts are detectable in the earliest stage of the developing flower, from the moment the flower primordium becomes visible microscopically (Figure 7A–D). No significant hybridization signal is detected in the apical meristem of the inflorescence, but weak hybridization is seen in young bracts. The *squa* gene remains transcriptionally active in the differentiating cells giving rise to the floral organs, with the exception of the third whorl organs where the signal disappears as soon as stamen primordia become visible microscopically (Figure 7E–H). Since the floral meristem seems to be uniformly labelled before the floral organs arise, indicating that *squa* is expressed in all meristematic cells, *squa* has to become actively repressed in those cells of the floral meristem that give rise to the stamens. In early carpel development, *squa* gene expression can be detected in the basal parts of the carpels forming the ovary wall, but seems to be absent from those cells which differentiate into the style and stigma. During further development, *squa* transcription remains detectable in the elongating pedicel, receptacle and floral organs, with the exception of the stamens. The overall hybridization signal becomes weaker and is hardly detectable

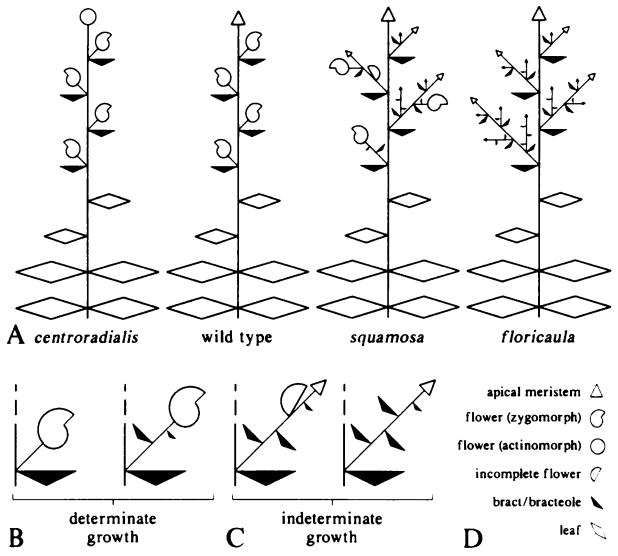

Fig. 8. Organization of wild type, *squamosa* and other mutant inflorescences of *Antirrhinum*. (**A**) Schematic representation of the wild type and mutant phenotypes (genotypes indicated below the graphs). Vegetative parts of mutants are unaffected. In wild type the apical meristem of the main shoot grows indeterminately, whereas that of *centroradialis* mutant differentiates into a terminal (actinomorphic) flower. The axillary meristems in the axils of bracts formed by the wild type inflorescence apical meristem become determinate and form (zygomorphic) flowers. In the *floricaula* mutant these axillary meristems do not differentiate into flowers and show indeterminate growth like the main infloral shoot. Inflorescence axillary meristems of the *squamosa* mutant may differentiate into flowers as in wild type or remain indeterminate as in the *floricaula* mutant. (**B** and **C**) Schematic representation of possible fates of axillary meristems formed on the *squamosa* mutant inflorescence. The two alternative modes of growth indicated below the graphs result either in formation of flowers (B) or in bract-forming shoots (C). Temporal variation in the switch between the two alternative modes of growth could explain occasional occurrence of bracteolated and elongated pedicels (B, right), as well as the formation of incomplete flowers (C, left). (**D**) Explanation of the symbols used in A, B and C.

in older bracts. This may be due to elongation of the cells and the enlargement of their vacuoles. The presence or absence of *squa* transcripts at later stages of flower development is therefore more convincingly demonstrated by Northern blots (see Figure 6B). Absence of detectable *squa* transcripts in *in situ* experiments in flowers of the *squa*443 mutant (Figure 7I) is in agreement with Northern blots. It confirms the conclusion drawn from the analysis of the *squa*347 allele that flowers can be formed in the complete absence of the *squa* gene function. Although flowers formed by *squa* mutants are usually malformed, organ identity might be correctly established. This is reflected by a strong transcriptional activity of the *def* gene in second and third whorl organs formed in the occasionally developing complete flowers in *squa* mutant inflorescences (Figure 7J and K). Elevated expression of *def* in the second and third whorl organs of wild type flowers is a prerequisite for differentiation of petals and stamens, respectively (Schwarz-Sommer *et al.*, 1992).

Discussion

We have taken advantage of the genetic instability of the recessive *squa*293 mutation to isolate and identify the *squamosa* locus. We have shown that the *squa* gene belongs to the recently discovered MADS-box gene family in flowering plants. All members of this gene family encode a protein with a conserved DNA binding domain (Schwarz-Sommer *et al.*, 1990; Ma *et al.*, 1991; Pnueli *et al.*, 1991). The fact that two of the newly isolated MADS-box genes can be correlated with the mutant phenotypes of two morphogenic genes, *squa* and *glo* (Schwarz-Sommer *et al.*, 1990; this report; Tröbner,W., Hue,I., Ramirez,L., Huijser,P., Saedler,H., Sommer,H. and Schwarz-Sommer,Zs., in preparation), points to the important role of this gene family in the control of floral morphogenesis.

The SQUA protein is a putative transcription factor

As a member of the MADS-box gene family, which includes the mammalian transcription factor SRF and the yeast transcription factor MCM1, the *squa* gene protein product probably represents a nuclear transcription factor. The DNA binding properties of these proteins as homo- or heterodimers have been demonstrated for SRF and MCM1 (Norman *et al.*, 1988; Passmore *et al.*, 1989), and for DEF A and GLO (Schwarz-Sommer *et al.*, 1992; Tröbner *et al.*, in preparation), respectively. Although the *in vivo* target sequences of the floral MADS-box proteins are unknown, they will probably resemble the consensus sequence $CC(A/T)_6GG$ (SRE) for SRF and MCM1 (Hayes *et al.*, 1988). A similar sequence, CCATAATTGT, is found upstream of the *squa* transcriptional start site, suggesting autoregulation of *squamosa* expression. An autoregulatory control, by binding of DEF A/GLO heterodimers to *cis*-acting sequences in the promoter, has been suggested for the *deficiens* gene, for upregulation of its expression as well as for its persistent expression during organogenesis (Schwarz-Sommer *et al.*, 1992).

The possible role of squa in determination of shoot meristem identity

Lateral shoot primordia develop in the axils of newly initiated leaf primordia recruited from the proliferating cells at the tip of growing shoots called the apical meristem (see Lyndon, 1990; Steeves and Sussex, 1989). The defined order as well as the defined positions at which primordia arise on the flank of an apical meristem may change during development, as is seen most dramatically upon the onset of flowering.

In *Antirrhinum*, the transition from vegetative to reproductive growth is manifested when initiation of leaf and vegetative shoot primordia by the shoot apical meristem is replaced by initiation of bract and reproductive shoot (i.e. flower) primordia. The vegetative apical meristem is thus converted to an inflorescence apical meristem (infloral meristem) and the identity of the axillary meristems is changed from vegetative to reproductive, i.e. to a floral meristem. This transition seems to be controlled genetically.

The phenotypes of two recessive mutations in *Antirrhinum*, *cen* and *flo* (Figure 8A), suggest that the genetic control of infloral and floral meristem differentiation is closely linked. In the *cen* mutant, a central flower terminates the inflorescence after formation of a limited number of lateral flowers, whereas the wild type infloral meristem grows indeterminately and is capable of forming hundreds of flowers. A floral meristem can thus arise from the conversion of an apical meristem. Conversely, axillary meristems initiated in *flo* mutant inflorescences are converted to indeterminately growing infloral meristems (Carpenter and

Coen, 1990). One explanation of these homeotic alterations in meristem identity is that in the *flo* mutant the floral transition is blocked and that the primordia in the axils of the bracts now show their underlying identity, which is that of an infloral meristem. Hence, an infloral meristem is considered as an intermediate in the transition from a vegetative to a floral meristem (Coen *et al.*, 1990; Coen, 1991). However, it is possible that the transition resulting in infloral and floral meristems are not sequential but represent simultaneous and alternative conversions of a common meristem type (for a comparable alternative see also Ferguson *et al.*, 1991). This assumption is substantiated by the *cen* mutant phenotype, where the first of the—usually only eight and in normal acropetal order—initiated lateral flowers develops and opens at the same time as the central terminal flower [Stubbe, 1966; P.Huijser and W.-E.Lönnig, unpublished data; compare with the very early commitment to terminal flower formation in tobacco (Gebhart and McDaniel, 1987)]. Moreover, the organization of the *cen* mutant inflorescence suggests a functional partition of the shoot apical meristem; the terminal flower develops from the central part of the apical meristem, while, at the moment of its initiation, the remaining peripheral meristem cells become partitioned to form a limited number of bracts and lateral flowers (see also Shannon and Meeks-Wagner, 1991).

Analysis of the *squa* mutation may shed light on the initial identity of the inflorescence axillary meristems and the way in which their fate is determined. Different types of anomalous flowering modes are observed which suggests that establishment of floral commitment of infloral axillary meristems is temporally limited. In the first type a terminal zygomorphic flower is formed on an often elongated and bracteole-bearing shoot (pedicel; Figure 8B) indicative of complete conversion of the axillary meristem to a floral meristem with its characteristic determinate mode of growth. In the second type, apical growth of a lateral shoot remains indeterminate. Occasionally, the meristem continues to form bract-like leaves after formation of partial or complete whorls of floral organs, indicating that floral conversion is not, or not fully, accomplished. Thus, the *squa* mutant phenotype suggests that the floral transition, instead of being blocked completely as in the *flo* mutant, may be delayed or may occasionally become reversed. As a consequence, only some of the axillary meristems on the inflorescence become floral and hence the *squa* mutant shows partial flowering.

The flowering abnormalities seen in *squa* inflorescences are reminiscent of the reversion phenomena observed after floral induction in a few plant species such as *Impatiens balsamina* (Krishnamoorthy and Nanda, 1968). Under experimentally controlled environmental changes, reversion can proceed quickly and during virtually all stages of flower development. Moreover, an axillary meristem seems to pass rapidly through a phase of maximum sensitivity for the presumptive floral stimulus (Fontaine, 1972). Meristems that are either too young or too old do not respond fully, thus resulting in reverted flowers (for review on flower reversion see Battey and Lyndon, 1990 and references therein). Therefore, we propose that *squa* may have an important function in enhancing the response to and/or increasing the sensitivity of the meristem for flower promoting signals of a so far unknown nature. The temporally limited ability of the axillary shoot to form a flower could be due to a gradual establishment of the *centroradialis* function (see above). If this interpretation is correct, then the axillary meristems of an inflorescence acquire their identity gradually after their initiation.

The differentiation of an axillary meristem, and that of an organ primordium initiated on its flank, starts from a multicellular structure. We assume that conversion into an infloral or a floral meristem is the result of two alternative developmental pathways which are induced simultaneously in all cells of the meristem. Early factors of both pathways must affect their mutual expression such that the whole meristem becomes converted in one or the other direction. If this is not accomplished before the first organs arise, it results in mosaic organs, as often displayed by *squa* mutant flowers, especially in the perianth. Therefore, the *squa* activity observed in all flower primordial cells could serve another important function: to achieve and maintain a coordinate response of the individual primordial cells to the presumptive flower promoting signals.

Low level expression of squamosa

Low levels of *squa* transcripts can be found in the leaves just below the inflorescence and the bracts subtending the flowers. Although *squa* could affect processes in these organs other than morphogenesis, the functional role of this low expression remains obscure. It could be a prerequisite for the induction of high level transcription via heterodimer-mediated autoregulation of the gene at the moment when its function is actually required. The overlap in expression of *squa* with other regulatory genes would then define the precise borders of its function. Such mechanisms seem to govern pattern formation in *Drosophila* (for reviews see Akam, 1987; Ingham, 1988).

Possible interactions of squamosa with floricaula and centroradialis

In *Antirrhinum*, mutants of the genes *squa*, *flo* and *cen* display phenotypes which indicate involvement of the corresponding functions in early events of wild type flower development. In the following section we discuss how these genes may interact in determination of identity of floral meristems.

Transcription of squamosa and floricaula is not interdependent. The temporal and spatial expression patterns of *squa* and *flo* show interesting similarities. Transcriptional activity of both genes is detectable in bracts, flower primordia and floral organ primordia, with the exception of the stamens. This, together with the similarities of their mutant phenotypes, strongly suggests that there is some form of interaction between these two genes. Mutual transcriptional regulation seems unlikely for the following reasons. Firstly, *flo* activity is found in early stages and declines later, whereas *squa* activity persists throughout floral organ differentiation. Secondly, the *squa* transcript is present in *flo* mutant inflorescences and, in turn, the *flo* gene is expressed in the *squa* mutant (P.Huijser and H.Meijer, unpublished data). Since the FLO protein, which has no MADS-box, has been suggested to represent a different type of transcription factor (Coen *et al.*, 1990), it seems more likely that the two proteins interact during the early stages of flower development in the transcriptional regulation of common target genes.

Squamosa and centroradialis may have antagonistic functions. As described in a previous section, mutations in the *cen* gene apparently do not affect the identity of wild type inflorescence axillary meristems. Nevertheless, several observations suggest a mutual negative regulatory dependence between *squa* and *cen*. Because strong transcription of *squa* is detectable in floral meristems but not in the inflorescence apical meristem, the *cen* function could repress *squa* activity in this meristem, as has been suggested also for *flo* (Coen *et al.*, 1990) and for the apparently equivalent genes *terminator* and *leafy* in *Arabidopsis* (Schultz and Haughn, 1991). Conversely, the induction of *squa* in the floral primordia may repress the *cen* function or prevent its establishment in these meristems. Thus, in the two different types of meristems (floral and infloral) the *cen* and *squa*/*flo* functions may be mutually exclusive.

The squamosa function is not strictly required for flowering

Sporadic development of normal flowers remains possible in *squa* null mutants. Assuming that *squa* is activated in response to flower promoting signals, its target genes must be able to respond directly or indirectly to the same signals. In the absence of the *squa* function, a temporal and/or local increase in the level of these signals, perhaps reflecting fluctuations of external factors, may provide a by-pass in *squa* mutants by directly activating (or repressing) its target genes and thus occasionally trigger flower development. Alternatively, other MADS-box genes could partly complement the missing *squa* function in mutants. Some recently identified MADS-box genes in *Antirrhinum* show expression patterns similar to that of *squa* and the respective protein products show remarkable overall homology to SQUA, including regions outside of the MADS-box (data not shown). If these MADS-box proteins can only in part mimic the *squa* function, due to their lower affinity for the target genes and/or accessory factors of SQUA, this could explain the abnormal flowering of the *squa* mutant.

The possible role of squamosa in the origin of flowers and inflorescences

From an evolutionary point of view, the remarkable structural similarities between different MADS-box genes in *Antirrhinum* and in *Arabidopsis* (Ma *et al.*, 1991) strongly suggest that these genes evolved from a common ancestor (see also Pnueli *et al.*, 1991; for a critical discussion on the origin of gene families see Lönnig, 1988). MADS-box genes like *def* and *glo* determine the identity of the lateral organs formed by the floral meristem. In contrast, the *squa* gene is involved in determining the floral identity of the axillary meristems produced by the inflorescence apical meristem. Hence the *squa* gene is the earliest acting MADS-box gene known to control flower development. Taking into consideration its influence on the identity of axillary meristems and the development of inflorescences and flowers in the wrong places in mutants, the *squa* gene may be called homeotic. However, flower development in *squa* mutants can also be looked at as occurring at the wrong time: before flowers are formed, the programme of inflorescence formation is reiterated. Thus, *squa* may be regarded as a heterochronic gene. In the origin of racemose inflorescences, establishment of its function may have played a role in the reduction of the number of flowers per primary branch, provided that the hypothesis of an originally compound cyme is correct (Stebbins, 1977; see also Coen, 1991). In this case the mutant phenotype of *squa* could reinforce the role of heterochrony in the evolution of form (Lord and Hill, 1987). One may speculate that in *Antirrhinum*, the origin of the flower and the inflorescence could thus be linked to the establishment of the MADS-box gene family.

The heritable floral homeotic mutations observed in distantly related species like *Antirrhinum majus* and *Arabidopsis thaliana* (Schwarz-Sommer *et al.*, 1990; Coen and Meyerowitz, 1991) show striking similarities. They suggest that the basic mechanisms governing flower development in these and other species (compare Meyer, 1966; Meyerowitz *et al.*, 1989) are conserved, which is also reflected in the conservation of the MADS-box gene family. Therefore, a *squa* homologue might be expected to act in *Arabidopsis*, too. *Apetala*-1 (*ap*1; Koornneef *et al.*, 1983) is a good candidate for such a homologue. Certain features of *squa* mutant effects in *Antirrhinum* can also be recognized in *Arabidopsis ap*1 mutants. In the *ap*1 mutant, vegetative growth is not affected but sepals are transformed into bract-like structures with flowers developing in their axils (Irish and Sussex, 1990). This process is reiterated with respect to the first whorl organs of these higher order flowers. The similarity to the reiterated development of inflorescences in the *squa* mutant is obvious if one realizes that wild type *Arabidopsis* flowers are not subtended by bracts and that, therefore, bracts and sepals in *Antirrhinum*, which both express *squa* although at different levels, may be related to sepals in *Arabidopsis* (for discussion of the supposed evolutionary relationships of floral organs see Stebbins, 1977). Additional similarities can be recognized in the incomplete and irregular development of mosaic organs of higher order *ap*1 mutant flowers and *squa* mutant flowers. The *ap*1 gene could thus represent the *squa* counterpart in *Arabidopsis*.

Materials and methods

Plant material

The inbred line S50 used in our studies as morphological wild type and the *squamosa* mutant lines S443 and S444 were obtained from the Gatersleben (FRG) seed collection. Although many of the different *Antirrhinum* mutants isolated by Baur and coworkers are maintained in this seed collection, it is difficult if not impossible to establish the precise origin of these *squa* mutants. Two additional lines showing the *squa* phenotype, *squa*293 and *squa*347, arose from line T53 in the course of a transposon mutagenesis programme performed in the summer of 1988 at the Max-Planck-Institut für Züchtungsforschung (Köln, FRG). The allelic identity of these lines was genetically tested. The line T53 (*niv*-53::Tam1), carrying an active Tam1 transposon, and the *floricaula* mutant line 640 were kindly provided by R.Carpenter (John Innes Institute, Norwich, UK). Plants were grown as described by Sommer *et al.* (1990).

Extraction of nucleic acids

DNA was isolated from 0.5–1.0 g of frozen leaves according to Dellaporta *et al.* (1983), with the modifications described by Sommer *et al.* (1990). Poly(A)$^+$ RNA was prepared from frozen tissues as described by Logemann *et al.* (1987).

Construction and screening of libraries

A cDNA library in a λNM1149 synthesized from poly(A)$^+$ RNA of young wild type inflorescences was screened with a 240 bp *Eco*RI–*Hin*dIII fragment of the cDef1 clone containing the conserved MADS-box sequence (Sommer *et al.*, 1990). From this first screen two independent and almost full-size cDNAs, defH33 and defH76, were identified. Both cDNAs differed

by only 14 bp at their 5′ ends and lacked a poly(A) tail. A second screen, using defH33 as a probe, yielded four additional cDNAs. Two of these carried poly(A) tails starting at the same position (see Figure 3B). The other two showed, compared with the other isolated cDNAs, an extended non-translated 5′ leader and a 3′ trailer sequence, respectively, but both again lacked a poly(A) tail.

For the construction of a genomic library, total cellular DNA was partially digested with *Mbo*I and isolated fragments of 15–25 kb were cloned in the *Bam*HI sites of EMBL4 phages. From this library the *squa* genomic locus was isolated by screening with defH33 as a probe.

Southern and Northern blot hybridizations

Blotting techniques, probe labelling and hybridizations of Southern and Northern blots were performed as described by Sommer *et al.* (1990).

DNA sequencing

The DNA sequence of both strands of the *squa* genomic locus and its corresponding cDNAs were determined by both the dideoxy chain termination method (protocol supplied by Pharmacia), after subcloning overlapping restriction fragments into Bluescript (Stratagene), and the chemical degradation method (Maxam and Gilbert, 1980).

Primer extension and S1 nuclease mapping

Primer extension and S1 nuclease mapping to determine the squamosa transcriptional start site was performed according to Sambrook *et al.* (1989) and Sommer and Saedler (1986) respectively.

In situ hybridization and microscopy

We applied different fixation methods for *in situ* RNA hybridization to young inflorescences and small flower buds. Although we obtained positive results with all fixatives tested, in general the strongest hybridization signals were obtained with FAA (formalin–acetic acid–alcohol; 24–48 h at 4°C) and 2–4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 (16 h at 4°C) After fixation the tissues were carefully dehydrated in a series of increasing ethanol concentrations. Subsequently the ethanol was replaced by xylol and the tissues were embedded in paraffin according to standard procedures. Sections (6–8 μm thick) were fixed on gelatin coated slides, deparaffinized in xylol and rehydrated in a series of decreasing ethanol concentrations. Before hybridization the slides were immersed in 0.2 M HCl for 20 min at room temperature, rinsed with H_2O and incubated in 2 × SSPE for 20 min at 70°C. Subsequently the slides were treated with 1 μg/ml proteinase K in 2 mM $CaCl_2$, 20 mM Tris–HCl, pH 7.4, for 20 min at 37°C. After brief washing in 0.1 M phosphate buffer, pH 7.0, the slides were rinsed in 0.1 M triethanolamine, pH 8.0, for 15 s and acetylated in 0.5% (v/v) acetic anhydride in 0.1 mM triethanolamine, pH 8.0, for 20 min at room temperature. After washing in 2 × SSPE for 5 min and rinsing in H_2O, the slides were then dehydrated in 70 and 95% ethanol for 5 min each and air dried.

For hybridization the sections on the pretreated slides were incubated with 1 × 10^4 c.p.m./μl ^{35}S-labelled RNA in 30–40 μl of the following mix: 500 μg/ml yeast tRNA, 100 μg/ml poly(A), 300 mM NaCl, 100 mM DTT, 1 × Denhardt's, 10% dextran sulphate, 50% formamide, 2 mM EDTA, 20 mM Tris–HCl, pH 8.0. The slides were covered with siliconized coverslips, placed in a humid chamber and incubated for 16 h at 45°C. After hybridization, coverslips were removed by rinsing in 3 × SSPE and the slides washed three times for 10 min in 3 × SSPE at room temperature. After 30 min incubation with gentle agitation in 20 μg/ml RNase A, 500 mM NaCl, 1 × TE, pH 8.0, at 37°C the slides were washed for 30 min in 500 mM NaCl, 1 × TE, pH 8.0, at 37°C. Two further 30 min washes were done at 45°C in 1.5 × SSPE followed by two 30 min washes in 0.75 × SSPE. The slides were dehydrated at room temperature by 5 min treatments in 70 and 95% ethanol, both with 0.3 M NH_4OAc, and then air dried.

For autoradiography, slides were dipped in Kodak NTB-2 nuclear track emulsion melted at 45°C and diluted with an equal volume of distilled water. Dry slides were stored for up to 3 weeks in a lightproof box (with some desiccant) at 4°C. They were developed for 2 min in Kodak D-19b at 16°C, rinsed for 10 s in water and fixed for 5 min in Agfafix diluted 1:5. After washing for 15 min in running tap water, the sections were stained for 10 min in 0.01% Calcofluor, washed again (2 × 5 min) in distilled water, air dried and mounted in Entellan. The distribution of grains was best seen using dark-field illumination. The underlying tissue was examined using UV fluorescence.

^{35}S-labelled RNA was synthesized from restriction fragments subcloned into Bluescript plasmid vectors according to the protocol supplied by Stratagene. For *in situ* hybridization the labelled RNA probe was hydrolysed to give fragments with an average length of 100 nucleotides in 100 mM carbonate buffer, pH 10.2, at 60°C.

Acknowledgements

We thank Rita Berndtgen and Isa Will for excellent technical assistance, and Johannes Müller and Anita Satzinger for excellent plant care. Also, we wish to thank Zsuzsanna Schwarz-Sommer for discussion and her critical reading of the manuscript, and Brendan Davies for correcting the English.

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Received on December 16, 1991; revised on January 15, 1992

Note added in proof

The *squamosa* sequence reported here has been deposited in the EMBL Data Library under the accession number X63701.