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Ternary complex formation between the MADS-box proteins SQUAMOSA, DEFICIENS and GLOBOSA is involved in the control of floral architecture in *Antirrhinum majus*

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In *Antirrhinum*, floral meristems are established by meristem identity genes. Floral meristems give rise to floral organs in whorls, with their identity established by combinatorial activities of organ identity genes. Double mutants of the floral meristem identity gene *SQUAMOSA* and organ identity genes *DEFICIENS* or *GLOBOSA* produce flowers in which whorled patterning is partially lost. In yeast, SQUA, DEF and GLO proteins form ternary complexes via their C-termini, which in gel-shift assays show increased DNA binding to CArG motifs compared with DEF/GLO heterodimers or SQUA/SQUA homodimers. Formation of ternary complexes by plant MADS-box factors increases the complexity of their regulatory functions and might be the molecular basis for establishment of whorled phyllotaxis and combinatorial interactions of floral organ identity genes.

Keywords: ABC model/DNA binding/MADS-box/regulatory network/ternary complex

Introduction

The final architecture of multicellular organisms is the result of the coordinate activity of various developmental programs. In higher plants, after germination, development is divided into two major phases: vegetative growth and reproductive growth, characterized by distinct organ arrangement (phyllotaxy). Phyllotaxy is the result of the activity of the apical meristem, which produces new meristems in axial positions that give rise to new structures. During vegetative growth, *Antirrhinum* leaves show double decussate phyllotaxy, followed by a spiral phyllotaxy, which roughly correlates with the onset of the reproductive phase (Bradley *et al*., 1996). In flowers the phyllotaxy is whorled, thus five organs are initiated in the three outer whorls: sepals, petals, stamens and two fused carpels form the female organ (pistil) in the center of the flower.

Genetic analysis of mutants affecting flower morphology and development has identified several developmental programs that are required to create the complex floral structure of dicotyledonous plants. Initially, a genetic switch changes the fate of the axial meristems produced by the shoot apical meristem, which instead of maintaining a vegetative fate, produces floral organs. This process is controlled by meristem identity genes that comprise in *Antirrhinum FLORICAULA* (*FLO*) (Coen *et al*., 1990), *SQUAMOSA* (*SQUA*) (Huijser *et al*., 1992) and *CENTRORADIALIS* (*CEN*) (Bradley *et al*., 1996). *Squa* plants, for instance, flower rarely because most meristems that should adopt a floral fate remain as inflorescences (Huijser *et al*., 1992). Once the flower meristem is established, several parallel events occur: first, organ initiation changes from a spiral to a whorled fashion; secondly, the developing organs in the whorls adopt a specific identity; and thirdly, the floral meristem terminates.

Floral organ identity in angiosperms seems to be controlled by three conserved genetic functions that act in a combinatorial manner (Coen and Meyerowitz, 1991). The ABC model, which describes the role of these functions in floral development, proposes that sepal identity is controlled by the A-function, petals by the A- and B-functions, stamens by B- and C-functions, and carpels by the C-function (Haughn and Somerville, 1988). In *Antirrhinum*, floral organ identity genes include *DEFICIENS* (*DEF*) (Sommer *et al*., 1990) and *GLOBOSA* (*GLO*) (Tröbner *et al*., 1992), required for petal and stamen identity (B-function), and *PLENA* (*PLE*) (Bradley *et al*., 1993), required for stamen and carpel formation (C-function). Cadastral genes establish the expression boundaries of the organ identity genes (Weigel and Meyerowitz, 1994). Mutations in these genes, e.g. *FIMBRIATA* (*FIM*), have a dual effect by altering the whorl patterning as well as the organ identity boundaries (Simon *et al*., 1994).

The floral organ identity genes of *Antirrhinum DEF*, *GLO* and *PLE*, and the meristem identity gene, *SQUA*, are members of the MADS-box family, coding for transcription factors (Schwarz-Sommer *et al*., 1990). Plant MADS-box proteins share a similar secondary structure with the DNA binding domain, the MADS-box (M), located at the N-terminus of the protein. The MADS-box is followed by an intervening region (I), the K-box (K), involved in protein–protein interactions, and the C-terminus (C), in which the divergence among members is greater (Ma *et al*., 1991; Pnueli *et al*., 1991), and whose molecular function is unknown. The specificity of heterodimerization of the different MADS proteins, and the distinct phenotypes of loss-of-function alleles of several members of this protein family (see above), suggest that, although *in vitro* DNA binding specificity seems to be conserved, the target genes recognized *in vivo* by different combinations of MADS-box factors are very different. This might be due to distinct interactions that lead to formation of homo- or heterodimers and to additional interactions with other proteins known as ternary factors. Ternary factors are proteins that, upon formation of a

ternary complex with a transcription factor, modulate target gene recognition and/or activation and repression. One example of this kind of molecular mechanism is the recognition of **a** and α genes by the MADS-box protein MCM1 in yeast which is modulated by ternary complex formation with α-2 (Herskowitz, 1989).

In order to investigate possible interactions between meristem and organ identity genes, we analyzed double mutants between the meristem identity gene *SQUA* and the organ identity genes *DEF* and *GLO*. Although *SQUA* might be expected to control *DEF* and *GLO*, it is not completely epistatic to *DEF* and *GLO*. Rather, they show a complex genetic interaction that suggests that *SQUA*, *DEF* and *GLO* share a previously unknown function controlling genes required for the proper architecture of the *Antirrhinum* flower. *Def*, *glo* or *squa* single mutants can form flowers with a wild-type architecture, but the double mutants fail to form flowers with a normal architecture suggesting that they share a partially redundant function. Remarkably, this genetic interaction is also detectable at the protein level since DEF, GLO and SQUA proteins form ternary complexes in yeast. Furthermore, ternary complexes of DEF, GLO and SQUA show a dramatic increase in DNA binding affinity compared with DEF/GLO heterodimers or SQUA/SQUA homodimers.

Results

Genetic interactions between DEF, GLO and SQUA

In order to improve our understanding of the function of floral meristem and organ identity genes, we analyzed double mutants of *def-gli* (*def-globifera*) and *squa-347 or glo-75* and *squa-347*, resulting from selfing plants heterozygous for *def-gli* and *squa-347* or *glo-75* and *squa-347*. Single mutants from these F_2 populations did not differ in phenotype from the single-mutant parents (Figure 1A and B). The *squa-347* plants flowered rarely, but all plants analyzed showed at least one wild-type flower (Huijser *et al*., 1992). In *glo-75* plants, the first whorl consists of five sepals, six sepals were rarely observed. The second whorl consists of five sepals, but in some flowers from this particular F_2 a sixth sepal developed between the second whorl and the carpeloid tube in the third whorl that contained five locules. This additional organ was consistently positioned between the two dorsal second whorl sepals. This sixth organ was often composed of sepal and carpel tissue and was fused to the third whorl carpeloid tube. It had been noticed previously in flowers of the *def-gli* mutant but was not studied in detail (Sommer *et al*., 1990). This suggests that in *def* or *glo* single mutants, primordia initiation is sometimes disturbed, resulting in an additional organ that appears between the second and third whorl. This aspect of the phenotype is not fully penetrant.

The meristem identity defects observed in *def-gli squa-347 or glo-75 squa-347* double mutants included those observed in *squa-347* siblings or *squa-347* siblings heterozygous for *def-gli*, and included erratic flowering and bracteomanial development, suggesting that *def-gli* and *glo-75* mutations have no effect on the early events of the floral initiation program. However, the flower architectures of *def-gli squa-347* and *glo-75 squa-347* double mutants were dramatically affected compared with wild type or the single mutants described above, and could not be distinguished from each other. The rare flowers of these plants had long pedicels with two prophylls distinct from the first whorl organs (Figure 1B). The first whorl normally had five sepals as in wild type, but this number rarely varied, ranging between four and seven. In contrast to *glo-75* or *squa-347* single mutants, the number of organs inside the first whorl was dramatically increased. In some flowers, >20 sepaloid organs developed prior to the formation of carpels (Figure 1D), arranged in a whorled, spiral or alternate phyllotaxy. In 26% of the flowers, the number of sepals was a multiple of five, suggesting that whorl phyllotaxy was maintained but whorl number had been increased to three or four whorls of sepaloid organs. We tried to analyze changes in phyllotaxy by scanning electron microscopy (SEM), but due to the extremely rare flowering of these double mutants, meristems suitable for SEM could not be recognized as floral before their development was already too advanced to study the pattern of primordia initiation. In the center of the flower, a variable number of locules, between six and nine, were formed. The *glo-75 squa-347* double-mutant flowers could also be distinguished from the *glo-75* single mutants because they formed carpels in the center of the flower (Figure 1E). In some cases, the number and arrangement of locules resembled a *glo-75* flower, but in most cases the number of locules was increased compared with *glo-75*. Some flowers produced locules arranged in a spiral fashion (Figure 1F). Among the double-mutant flowers of either *glo-75 squa-347 or def-gli squa-347* analyzed (146 flowers from six *glo75 squa-347* and 39 *def-gli squa-347* double mutants), we could not find a flower with 10 sepaloid organs and five locules that corresponded to the architecture of the 'normal' *glo-75* or *def-gli* single-mutant flowers. A typical organ arrangement of the double mutants analyzed is shown in the schematic representation in Figure 1G.

Our results show that, in a *squa* background, *DEF* and *GLO* are absolutely required to establish the wild-type number of whorls, and are partly required to maintain the whorled phyllotaxy. Remarkably, the flower architecture of the double mutants described (Figure 1G) is similar to that observed for null alleles of *FIM* (Ingram *et al*., 1997).

DEF and GLO form ternary complexes with SQUA in yeast

The genetic interactions described above suggest that *SQUA*, *DEF* and *GLO* might interact at the molecular level, possibly by binding of DEF/GLO heterodimers and SQUA/SQUA homodimers to promoter sequences of common target genes. We have shown previously that neither DEF nor GLO can interact with SQUA in yeast two-hybrid assays (Davies *et al*., 1996). However, it was still possible that they could interact by forming a multimeric protein complex.

In order to test this hypothesis, the three proteins DEF, GLO and SQUA were co-expressed in yeast. A third vector was constructed to express the GLO cDNA in yeast (pTFT; see Materials and methods for details). The yeast strain HF7c was sequentially transformed with three constructs expressing DEF fused to the GAL4 DNA binding domain (DEF–BD), pTFT–GLO and SQUA fused to the GAL4 activation domain (SQUA–AD). Surprisingly,

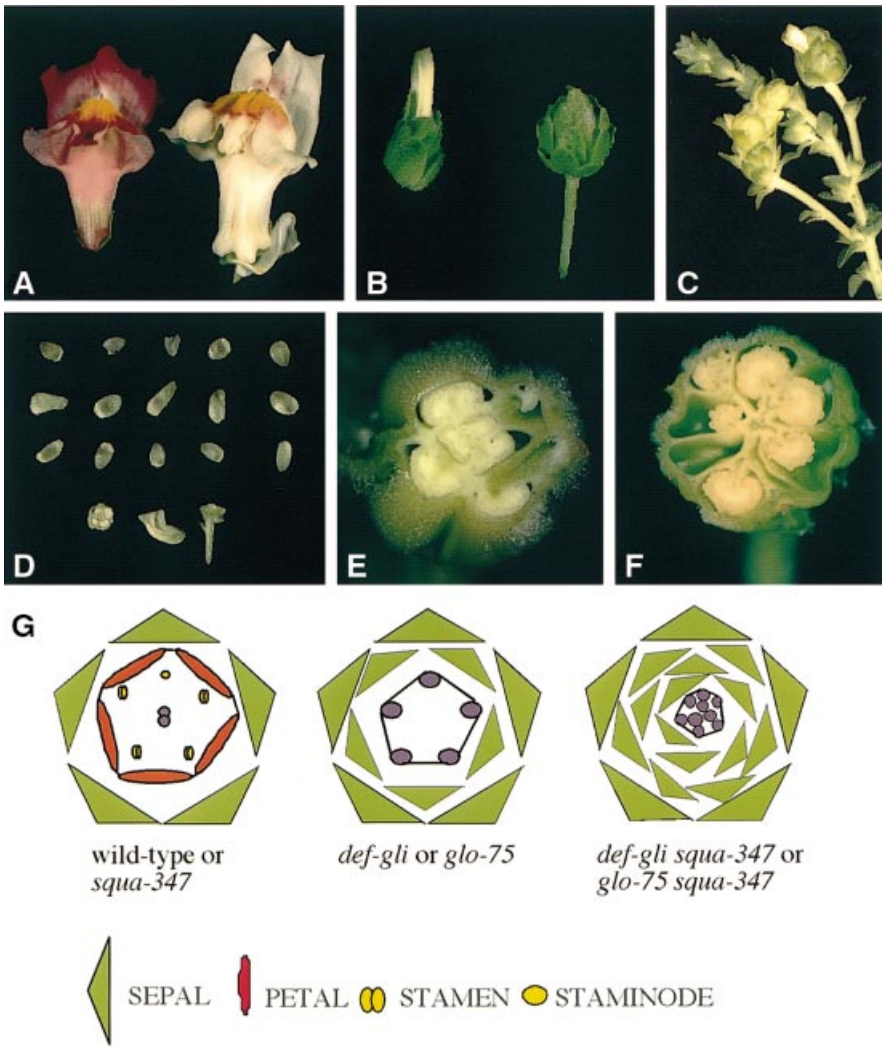

Fig. 1. Phenotypes of wild type, single and double mutants. (**A**) Wild-type (left) and *squa-347* flowers (right). The *squa* flower has wild-type architecture and shows a petaloid sepal in the lower right part. (**B**) Flowers of *def-gli* single and *def-gli squa-347* double mutant. The *def-gli squa-347* double-mutant flowers can easily be distinguished from *def* single-mutant flowers because of the longer pedicels. This long pedicel is characteristic of *squa-347*. (**C**) *Glo-75 squa-347* double-mutant inflorescence showing a single flower with long pedicel. (**D**) Organ composition of a *def-gli squa-347* double-mutant flower. This one has 15 sepals and four locules. (**E**) Cross-section through the center of a *glo-75 squa-347* double-mutant flower. A total of nine locules can be seen, five in outside positions and four in the center of the flower. (**F**) Cross-section through the center of a *glo-75 squa-347* double-mutant flower showing a spiral phyllotaxy in the center of the flower. (**G**) Floral diagrams depicting the phenotypes of the mutants. Diagrammatic representation of wild-type or *squa-347* (left), homozygous *def-gli* or *glo-75* (middle) and *def-gli squa-347* or *glo-75 squa-347* double-mutant flowers (right).

yeast cells containing DEF–BD, pTFT–GLO and SQUA–AD grow very robustly in medium lacking histidine (Figure 2A, row 2), whereas yeast cells containing DEF–BD together with SQUA–AD and an empty pTFT vector show only residual growth.

These results suggest that DEF, GLO and SQUA form ternary complexes in yeast. The formation of homo- and heterodimers among plant MADS-box proteins occurs through the I-region and K-box (Davies *et al*., 1996; Riechmann *et al*., 1996; Fan *et al*., 1997), but ternary complex formation is a novel property of MADS proteins. The protein domains involved in this interaction could not be predicted from the current knowledge about MADS-box protein heterodimerization (Shore and Sharrocks, 1995).

In order to determine the domains involved in ternary complex formation, we expressed DEF and GLO together with truncated SQUA proteins in yeast. Figure 2A shows that the N-terminal MADS domain, part of the I-region and part of the C-terminus of SQUA, is dispensable for ternary complex formation (rows 3–5). However, the region of the C-terminus immediately adjacent to the K-box is required (Figure 2, row 6). Growth of cells expressing GLO-MIK is still ~10-fold stronger than that of those without GLO suggesting that weak protein–protein interactions still occur via the K-box or the two C-termini of DEF and SQUA (see below).

Since some MADS-box proteins require a portion of the I-region for dimerization (Davies *et al*., 1996; Riechmann *et al*., 1996; West *et al*., 1998), it was not clear if the loss of ternary complex formation was due to loss of SQUA homodimerization or loss of interaction with DEF/GLO. Thus, we tested interaction of the different SQUA truncations fused to the GAL4 activation domain with full-size SQUA fused to the GAL4 DNA binding

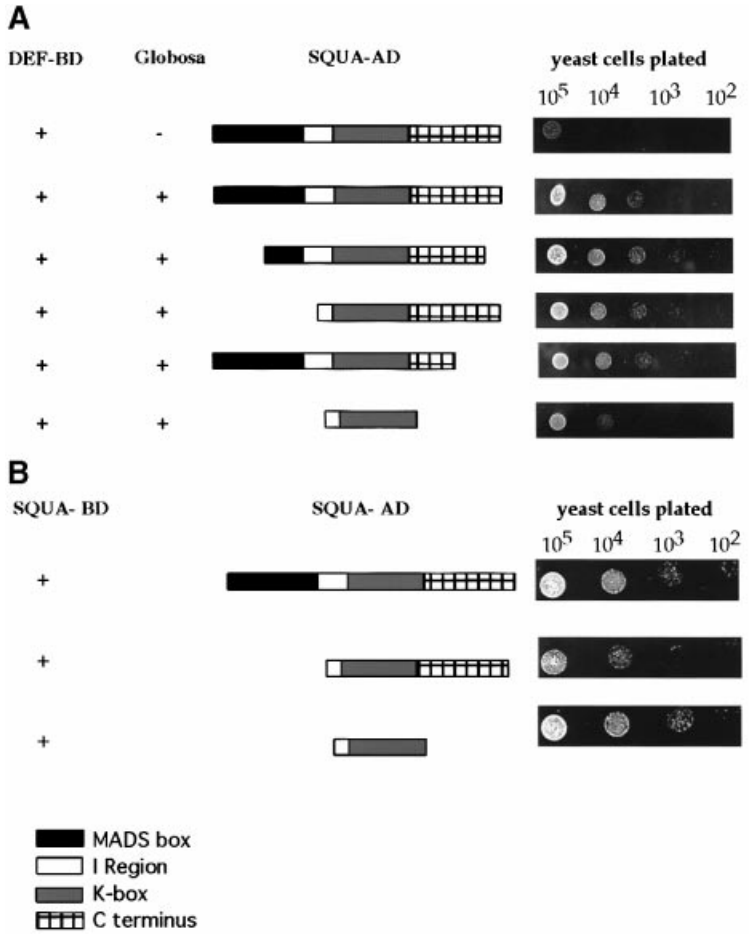

Fig. 2. Ternary complex formation of DEF and GLO with SQUA in yeast. The yeast strains Hf7c containing the plasmids listed were grown on liquid culture lacking adenine, tryptophan and leucine. Serial dilutions of 10^5–10^2 cells were plated on selective media lacking tryptophan, adenine, leucine and histidine, and the effect of the different constructs on growth was monitored. The presence (+) or absence (–) of full-size constructs expressing the protein listed above is shown. Several truncations of the SQUA cDNA were expressed, and the corresponding drawings of the constructs show the different domains expressed. The patterning of the constructs represents the protein domains expressed as proteins fused to the GAL4 activation domain. (**A**) Effect on growth of different truncations of SQUA. The negative control, showing lack of growth of DEF and SQUA in the absence of GLO, contains the pTFT1 vector without insert. It allows cells to grow on plates without adenine. (**B**) Effect of truncations on the homodimerization of SQUA.

domain. Figure 2B shows that growth of yeast cells containing the SQUA constructs expressing proteins lacking most of the MADS domain (row 3), the MADS-box and the N-terminal half of the I-region (row 4) or the I-region and the C-terminus, is similar to that of full-size constructs, indicating that SQUA homodimerization still occurs. Since SQUA forms homodimers in yeast (Figure 2B), it is possible that the ternary complex is a tetramer composed of one DEF/GLO heterodimer and one SQUA/SQUA homodimer.

These results show that DEF, GLO and SQUA can form ternary complexes via the C-terminus of SQUA, which suggests that the C-terminus of plant MADS-box proteins mediates ternary complex formation, and thus expands the regulatory possibilities of MADS-box transcription factors.

We investigated whether ternary complex formation with other MADS-box genes was a general feature of DEF and GLO. We tested directly for interaction with the C-function genes PLE and FAR (Davies *et al*., 1999), but we could not find any interaction in yeast. However, interaction with other MADS-box proteins, or with PLE via additional MADS-box proteins, can not be ruled out.

Ternary complex formation between DEF, GLO and SQUA requires the C-termini of the proteins

In order to determine whether the C-terminus of GLO was also required for ternary complex formation, three different GLO truncations were co-expressed with DEF and SQUA in yeast and tested for interaction.

As already shown for SQUA, ternary complex formation also requires the C-terminus of GLO. Figure 3A shows that both full-size GLO (GLO-MIKC) and a GLO construct lacking a small part of the C-terminus (GLO-MIK1/2C) can promote yeast cell growth under selection. However, a truncated GLO protein lacking the C-terminus is unable to maintain growth (Figure 3A, row 3) indicating that the ternary complex is no longer formed. This is not due to a disruption of DEF/GLO heterodimer formation since GLO-MIK and GLO-MIKC can still interact with DEF (data not shown). To test these observations further, we investigated the effect of DEF truncations. SQUA was

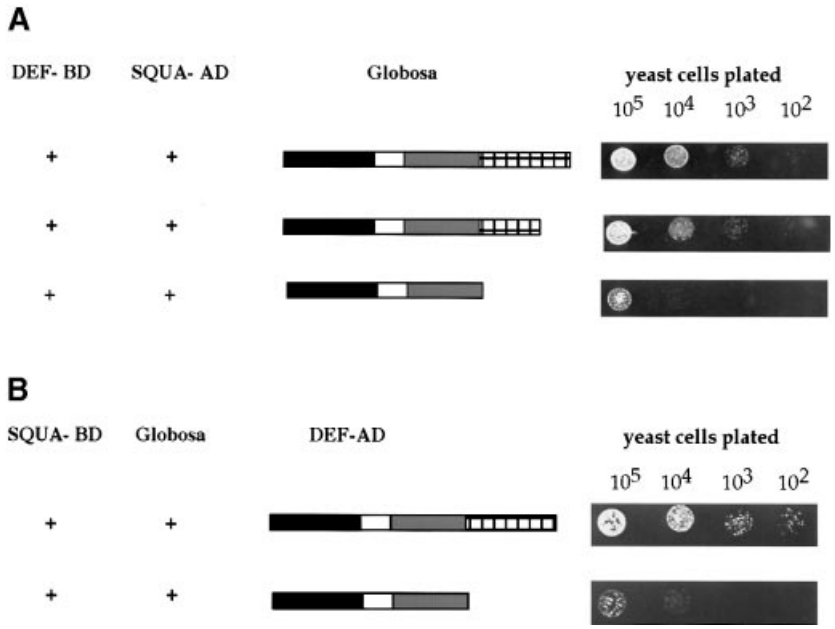

Fig. 3. Identification of domains in DEF and GLO required for ternary complex formation. (**A**) Effect on growth of truncations at the C-terminus of GLO. (**B**) Effect on growth of truncations at the C-terminus of DEF.

fused to the GAL4 DNA binding domain (SQUA–BD), GLO was expressed in pTFT1 and two different DEF fragments, DEF-MIKC (full size) and DEF-MIK (lacking the C-terminus), were fused to the GAL4 activation domain. The results indicate that, as with SQUA and GLO, ternary interaction also requires the C-terminus of DEF (Figure 3B). These chimeric DEF proteins had previously been shown to interact with GLO (Davies *et al*., 1996). We conclude that DEF, GLO and SQUA can form ternary complexes in yeast and that protein–protein interaction between DEF, GLO and SQUA occurs through the C-termini, a region that is not required for DEF/GLO heterodimer and SQUA/SQUA homodimer formation or for DNA binding as hetero- or homodimers (see below).

DEF, GLO and SQUA can form DNA binding complexes

Since ternary complex formation in yeast occurs when the proteins are bound to a heterologous promoter (GAL1 or GAL4 binding sites), we assume that the interaction is heterodimer-specific, and that the DEF/GLO/SQUA complex might show biochemical properties that are different from those of the DEF/GLO heterodimer or the SQUA/SQUA homodimer. DEF/GLO heterodimers and SQUA/SQUA homodimers bind DNA motives known as CArG boxes (Pollock and Treisman, 1991). Since the DEF/GLO/SQUA complex has the potential to form a tetramer with two different DNA binding domains, one supplied by DEF/GLO and the other by SQUA/SQUA, we performed DNA binding experiments using a region of the *GLO* promoter, a putative target gene of *DEF* and *GLO* (Tröbner *et al*., 1992; Zachgo *et al*., 1995), which had two adjacent CArG boxes, and compared the DNA binding capacity of the separate DEF/GLO and SQUA/SQUA dimers with that of mixtures which contained all three proteins.

Figure 4A shows that the SQUA protein forms two different complexes with the GLO promoter fragment, one strong lower complex (S–L) and a weak higher complex (S–H) (Figure 4A, lane 5), whereas the DEF/GLO heterodimer forms two complexes of approximately equal strength (DG–L and DG–H; Figure 4A, lane 6). When the DEF/GLO and SQUA proteins were co-incubated, a strong major complex was formed, migrating with slower mobility than the corresponding slower DEF/GLO or the faint upper SQUA complex (DGS; Figure 4A, lane 8). The large complex observed suggests that, as observed in yeast, DEF, GLO and SQUA can also form ternary complexes *in vitro*. Furthermore, based on the difference in intensity of the bands on the autoradiogram, DNA binding affinity seems to be strongly increased. To investigate whether this was due to protein–protein interactions between DEF/GLO and SQUA, we assayed truncations of DEF and GLO (DEF-MIK and GLO-MIK) unable to interact with SQUA in yeast (Figures 2 and 3). Indeed, incubation of DEF-MIK/GLO-MIK (DG-MIK) with full-length SQUA results in almost complete loss of the slower complex (Figure 4A, lane 9). This result suggests that the slowest DNA binding complex is a ternary complex containing DEF, GLO and SQUA and that its formation requires the C-termini of DEF and GLO, as observed in yeast.

Since the slowest DNA binding complex (DGS) was much more intense than all the other complexes together (DG–H or S–H) (Figure 4A, compare lanes 5 and 6 with 8), it is likely that DNA binding of the ternary complex is much stronger than that of the separate homo- or heterodimers. To test if any of the separate dimers exhibit preferential binding for one of the two CArG boxes present in the Glo promoter fragment, two separate DNA fragments carrying CArG2 or CArG3 (Tröbner *et al*., 1992) were used in DNA binding assays. DEF and GLO (Figure 4B, lanes 3 and 6), or DEF-MIK/GLO (lanes 4

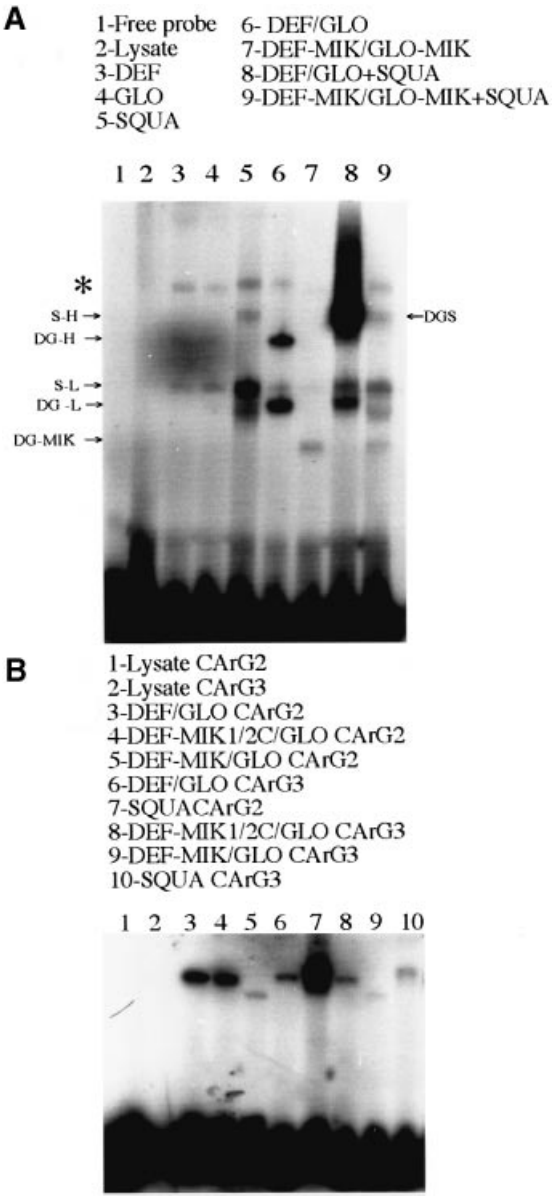

Fig. 4. Electrophoretic mobility shift assay of DEF, GLO and SQUA using Glo promoter sequences as target. When DEF and GLO were translated together it is referred to as DEF/GLO. Each lane has 12 µl of binding cocktail. When DEF/GLO were co-incubated with SQUA, 6 µl of each reaction were mixed. Thus, the amount of DEF/GLO and SQUA is half of that loaded in the slots in which only DEF/GLO or SQUA reactions were loaded. (**A**) Binding to *GLO* promoter. The Glo2/3 probe corresponds to the wild-type GLO promoter between bp –150 and –25 (see Figure 5A). The SQUA protein was always translated separately from DEF and GLO. The different complexes formed are indicated with arrows. DG-MIK, DEF-MIK/GLO-MIK; DG, DEF/GLO; S, SQUA; DGS, DEF/GLO + SQUA. Note that all lanes with programmed lysate show an upper band (*) of an unknown nature (under study). Transcription–translation of SQUA consistently gives two proteins of slightly differing size (one full size and one truncated), which might account for the two lower bands (S–L and a lower one) seen in slots 5 and 9. (**B**) Differential binding of DEF/GLO and SQUA to CArG2 and CArG3 from the Glo promoter. Two probes of equal specific activity were generated with CArG2 and CArG3.

and 9) heterodimers, bound CArG2 more strongly than CArG3, but binding strength was within the same order of magnitude. SQUA, however, showed a marked preference for CArG2 (Figure 4B, compare lane 7 with 10). Thus, since DEF and GLO bind both CArG2 and CArG3 with comparable affinities, whereas SQUA binds CArG3 with very low affinity, it is likely that DEF and GLO increase binding of SQUA to CArG3, possibly in a cooperative manner. We also performed DNA binding experiments using smaller DNA fragments with a single CArG motif, but under these conditions we could not observe complexes other than the corresponding hetero- or homodimers (data not shown), suggesting that with a single CArG motif the ternary complex is not stable *in vitro*.

DEF/GLO/SQUA ternary complexes display strongly increased DNA binding affinity

In order to test the previous assumption further, the *GLO* promoter was mutagenized to generate two artificial DNA fragments carrying either two copies of CArG2 (Glo2/2) or two copies of CArG3 (Glo3/3), but leaving the flanking regions unchanged (Figure 5A). These DNA fragments were used in gel-shift assays. Figure 5B shows that the SQUA homodimer binds Glo2/2 and forms two different complexes (S–L and S–H; lane 3). DEF and GLO also formed two complexes (DG–L and DG–H; Figure 5B, lane 4). Co-incubation of DEF/GLO with SQUA resulted in a strong upper complex (DGS; Figure 5B, lane 6), but coincubation of DEF-MIK/GLO-MIK with SQUA resulted in an upper complex of similar intensity to that formed by SQUA alone (Figure 5B, lane 7). Glo3/3, however, showed a different pattern of complex formation. Both SQUA/SQUA and DEF/GLO hardly bound Glo3/3 compared with Glo2/2 (compare lanes 3 and 4 in Figure 5B with lanes 3 and 4 in Figure 5C), but the combination of DEF/GLO and SQUA resulted in strong binding (DGS; Figure 5C, lane 6), suggesting that the affinity for CArG3 has been dramatically increased. This effect was lost when the truncated DEF-MIK/GLO-MIK proteins were used in combination with SQUA (Figure 5C, lane 7), indicating that the C-termini of DEF and GLO are indispensable for this interaction. Since the region between the CArG2 and CArG3 motifs had not been changed, it does not seem to play a role in this interaction. Indeed, we could not compete out any of the specific complexes with a 300-fold excess of a cold fragment containing only the intervening region between CArG2 and CArG3.

Our results demonstrate that DEF, GLO and SQUA are able to form ternary complexes *in vitro*, which results in a dramatic increase in DNA binding affinity. This ternary complex formation, as previously shown *in vivo* in yeast, occurs via the C-termini of DEF and GLO.

Discussion

Floral architecture in Antirrhinum

The genetic analysis of mutants affecting floral architecture has allowed a number of developmental programs that are responsible for their structure to be uncovered. Mutations in genes required to establish the floral identity program produce plants in which floral primordia are substituted by inflorescence primordia. *Squa* plants occasionally produce wild-type flowers (Huijser *et al*., 1992), suggesting that in *Antirrhinum* there is genetic redundancy for all the functions exerted by *SQUA*. Our genetic data show that, in a *squa* background, absence of function of the organ identity genes *DEF* or *GLO* results in flowers with an

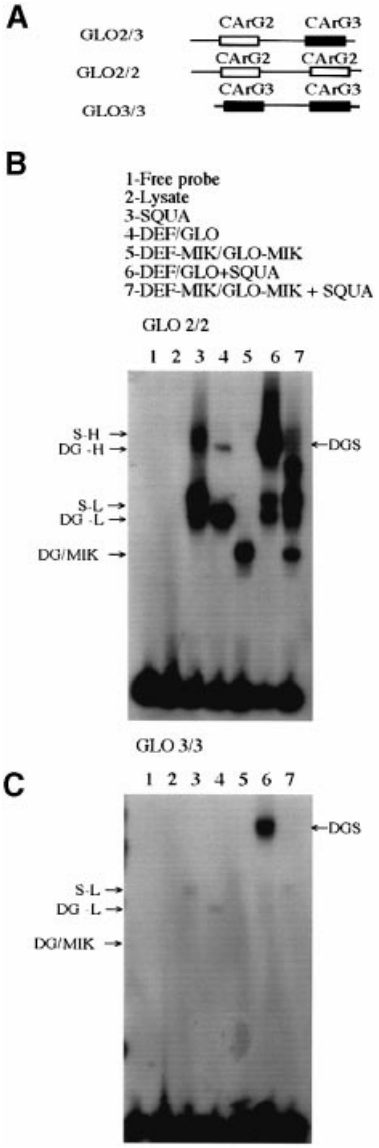

Fig. 5. Effect of ternary complex formation on DNA binding affinity. (**A**) Schematic representation of wild-type and mutated Glo promoter sequences. Glo2/2 and Glo3/3 represent strong binding sites (GLO2/2) or two weak binding sites (GLO3/3). The mutated sequences correspond to CArG2 and CArG3 as described in Tröbner *et al*. (1992) (see Materials amd methods for details). Binding of different protein combinations to GLO2/2 (**B**) or GLO3/3 (**C**). One hundred nanograms of Glo2/2 and Glo3/3 were labeled and similar incorporation of radioactivity and efficiency of recovery from polyacrylamide gels was obtained for both probes. The same number of radioactive counts was loaded on every lane of this autoradiogram allowing comparison of the binding affinities of the proteins between GLO2/2 and GLO3/3. The gel was exposed overnight. Exposure for more than a week allowed the complexes of SQUA, DEF/GLO and DEF-MIK/GLO-MIK bound to GLO3/3 to be seen (data not shown). The complexes formed in the gel are indicated as in Figure 4.

altered architecture. In all cases whorl number is increased, and in most cases (74%) additional organs are formed. Whether these additional organs are due to whorls with an increased number of organs or result from a change in phyllotaxy is currently not clear. Thus, the genetic redundancy observed in *squa* might be the result of a multigenic function, in which expression of several genes, when properly coordinated, can overcome the lack of SQUA protein.

The formation of a whorl requires the coordinated allocation of a group of cells forming a ring around the center of the flower meristem from which the organ primordia arise. The control mechanism which establishes this whorled pattern is currently not understood. In *fim* null alleles (Ingram *et al*., 1997) and *def-gli squa-347* or *glo-75 squa-347* double mutants, organs sometimes arise in opposite orientation, similar to the double decussate phyllotaxy of leaf primordia. This suggests that the two non-floral phyllotaxy programs, spiral and decussate, are repressed in the flower but can be revealed in certain mutant backgrounds. Genetic evidence supporting this can be found both in *Antirrhinum*, where loss-of-function of the meristem identity gene *flo* results in a spiral phyllotaxy (Carpenter *et al*., 1995), and in *Arabidopsis*, where mutations in two genes affecting organ identity, *APETALA2* and *PISTILLATA* (Bowman *et al*., 1989; Kunst *et al*., 1989), show pairwise loss of opposite organs. In the *Antirrhinum* double mutants described above, one common feature is the formation of a first whorl of sepals, followed by a non-whorled pattern of the inner organs. This suggests that, although the whorled pattern has been established in the first whorl, it is altered in the inner 'whorls'. A true spiral phyllotaxy was never observed, nor was it described for the *fim* null alleles (Ingram *et al*., 1997), suggesting that in *Antirrhinum*, the whorled program is established and maintained by several genes in a partially redundant pathway.

From the genetic data, we can conclude that *SQUA*, *DEF* and *GLO* are involved in the establishment and maintenance of whorled phyllotaxy. This could be due to control of late *FIM* expression by the combined activity of *DEF*, *GLO* and *SQUA*. There is both genetic and molecular evidence to support this hypothesis. Flowers of *fim def* or *sty def* double mutants which exhibit low *FIM* expression, form a fourth whorl (Simon *et al*., 1994; Motte *et al*., 1998), suggesting that the formation of a fourth whorl in *def squa* or *glo squa* double mutants results from weak *FIM* activity.

SQUA is expressed early in development throughout the floral meristem and this is followed by activation of the *FIM* gene, which has a complex expression pattern, starting in the center of the flower and resolving into two stripes that define the borders between sepals and petals, and petals and stamens (Simon *et al*., 1994). By the time this final expression pattern is established, *DEF* and *GLO* expression is already visible, overlapping with that of *SQUA* in the petals and *FIM* at the borders between sepals and petals, and petals and stamens. Furthermore, in the *ple* mutant, the third whorl adopts a petal identity with ectopic expression of *SQUA* in the forming primordia. In this mutant, *FIM* is ectopically expressed between the third whorl petal primordia (Simon *et al*., 1994), again suggesting that late *FIM* expression is controlled by the combination of *DEF*, *GLO* and *SQUA*.

The C-terminus of MADS-box proteins recruits ternary factors

Our results show that *DEF*, *GLO* and *SQUA* can form ternary complexes via the C-termini. This domain is the most divergent among the different MADS-box genes, but displays conserved epitopes among members of the same gene subfamilies (Davies and Schwarz-Sommer, 1994),

which might confer the specificity of this interaction. Furthermore, the C-terminus of the MADS-box proteins seems to play a crucial role in their function. Several of the loss-of-function alleles in MADS-box genes map in the C-terminus: *cal-1* from *Arabidopsis* (Bowman *et al*., 1993; Kempin *et al*., 1995) and three alleles of *Ap1* [*ap1-4, ap1-6* and *ap1-8* (Bowman *et al*., 1993; M.Yanofsky, personal communication)]. These alleles show a range of weak to strong loss-of-function phenotypes, indicating that the C-termini of these proteins are required for their full range of functions. This is supported by experimental data in *Arabidopsis*, where ectopic expression of an Agamous cDNA lacking the C-terminus results in a dominant-negative effect with phenotypes similar to the loss-of-function alleles (Mizukami *et al*., 1996). A weak dominant-negative effect has also been reported for plants expressing chimeric cDNAs of AG and AP3 with C-terminal truncations (Krizek and Meyerowitz, 1996). Together, these data suggest that the C-termini of the MADS-box proteins might play a key role in determining their divergent biological functions, possibly by creating a network of interactions with other members of the same family or with as yet unknown factors. The C-terminus could also be the target of specific post-translational modifications that could change protein localization, interaction partners and DNA binding specificity.

Ternary complex formation as a molecular mechanism to ensure specificity of target gene recognition and activation

The genetic interaction between *DEF*, *GLO* and *SQUA* is also observed at the protein level, and we have shown that the gene products can form a ternary complex whose DNA binding affinity differs from that of the individual dimers. This increased DNA binding affinity might be meaningful only for certain promoters. It is likely that the molecular function of any complex will be promoter-specific and depend on both the structural features of the promoter itself as well as other regulatory proteins interacting with it.

Our results suggest that MADS-box protein DNA binding affinity is indeed dependent on interaction with ternary factors and that interaction of the MADS-box proteins with their heterodimer partner and ternary factors, either dimer- or promoter-specific, is largely responsible for their target recognition. This hypothesis is supported by two studies that compared plants overexpressing wild-type MADS-box proteins, and fusion proteins in which part of the MADS domain had been substituted with MADS domains from animal proteins. Surprisingly, this change in the MADS domain had only a minor effect on the overexpression phenotype compared with plants overexpressing wild-type proteins (Krizek and Meyerowitz, 1996; Riechmann and Meyerowitz, 1997). These results can be explained by taking into account the fact that the domains required for heterodimerization and ternary complex formation were not modified in the expressed proteins, thus, target recognition is hardly impaired.

Based on the phenotypes of the double mutants and the change in DNA binding affinity found between DEF/GLO, SQUA/SQUA and the ternary complex formed by the three proteins, we propose that the target genes recognized by the complex are involved in establishing floral architecture. They are probably also recognized by the single dimers (see the proposed model, Figure 6), but the formation of a DEF/GLO/SQUA ternary complex results in a more specific regulation of downstream genes, thus guaranteeing high reliability of the floral developmental program.

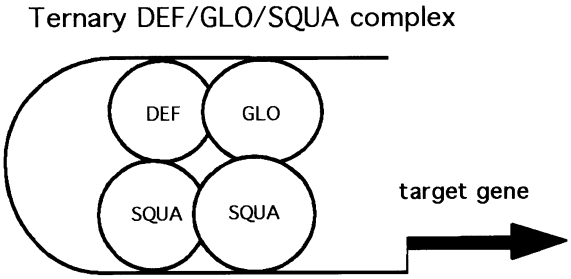

Fig. 6. A molecular model of DEF/GLO/SQUA ternary complexes based on the phenotypes and molecular data. The formation of ternary complexes between DEF/GLO and SQUA proteins produces a DNA binding complex with stronger affinity for target genes compared with the respective single hetero- or homodimers. These target genes involved in floral architecture are also partially activated by the DEF/GLO heterodimer or SQUA/SQUA homodimer, based on the fact that *def-gli*, *glo-75* or *squa-347* mutants can produce flowers with wild-type architecture. However, in the absence of DEF, GLO and SQUA, the target genes are not activated.

Materials and methods

Plant material

Plants were grown in the glasshouse at 18–25°C. *def-gli* (Schwarz-Sommer *et al*., 1992) and *glo-75* are mutants carrying transposon insertions (Tröbner *et al*., 1992). *squa-347* carries a deletion of the locus (Huijser *et al*., 1992). *def-gli*, *squa-347* and *glo-75* are all recessive mutants and considered null alleles. We crossed independently *def-gli* and *glo-75* with *squa-347*. The corresponding F_1 plants are wild type and were selfed. Double mutants were selected based on phenotypes (see Results). A total of six *def-gli squa-347* and seven *glo-75 squa-347* double mutants were initially identified from 104 F_2 plants. Thirty-two additional *def-gli squa-347* double mutants were obtained from 104 plants that resulted from selfing two *squa-347* plants heterozygous for *def-gli*. Only flowers that were 'properly' formed and did not show indeterminacy were used for phenotype characterization.

Yeast ternary factor plasmids and constructs

We used the GAL4-based yeast two-hybrid system from Clontech to analyze ternary complex formation in yeast. In order to express a third protein in the yeast strains used, an additional expression vector (pTFT1) was constructed, which has the ADH1 promoter and nuclear localization signal from pGAD424 (Clontech) as used in the two-hybrid expression plasmids (Fields and Song, 1989). The pTFT1 vector complements the *ade2* auxotrophy from *Saccharomyces cerevisiae*, and is based on pASZ12, a 2μ version of pASZ11 (Stotz and Linder, 1990). Selection can be made in media lacking adenine. Details of the vector are available upon request.

Three different constructs expressing the GLO protein were made in the pTFT1 vector by using PCR products from the GLO cDNA. The three constructs contain the N-terminal methionine of the GLO protein cloned in-frame with the nuclear localization signal. One expresses the full-size cDNA (GLO-MIKC). Two C-terminal truncations were made: the construct GLO-MIK1/2C encodes a protein from residue one to 191, lacking half of the C-terminus, and GLO-MIK encodes from amino acid 1 to 164, lacking the whole C-terminus. The open reading frame of SQUA, without the first methionine, was amplified by PCR from the cDNA and cloned as an *Eco*RI–*Xho*I fragment into *Eco*RI/*Sal*I sites in pGBT9 resulting in a GAL4 binding domain–SQUA fusion protein (SQUA–BD). The fragment was recloned as an *Eco*RI–*Pst*I fragment in pGAD424 producing a fusion to the GAL4 activation domain. Three truncations of SQUA were cloned as fusion proteins in pGAD424, resulting in the corresponding activation domain deletions: SQUA-1/2MIK1/2C contains amino acid residues 51–207, SQUA-1/2IKC contains

residues 74 to the natural stop codon and SQUA-1/2IK contains residues 74–149. pGBT-DEF, pGBT-GLO, yGLO11 (DEF-MIK-1/2C–AD) and yGLO19 (DEF-MIK–AD) SQUA-MIK1/2C were described previously (Davies *et al*., 1996). All the constructs have built-in stop codons after the last residue and were fully sequenced from both strands.

Yeast strains and growth conditions

The yeast strains HF7c (Feilotter *et al*., 1994) and Y190 (Durfee *et al*., 1993) were used to test interactions as described previously (Davies *et al*., 1996). The growth conditions for selection and transformation with pTFT1 expressing the GLO protein were standard drop-out media lacking adenine (Stotz and Linder, 1990). Protein–protein interactions were assayed on media lacking tryptophan, adenine, leucine and histidine or by LacZ (Kippert, 1995) (data not shown).

In vitro DNA binding experiments

All the constructs used in the assay have been published previously: pT7DEF, pT7GLO and pT7SQUA express full-size proteins (Davies *et al*., 1996), and pT7DEFMIK and pT7GLOMIK express the corresponding proteins without C-termini (Zachgo *et al*., 1995). Oligonucleotides used: (i) AACAGTTGGCTTGACTTTGAC; (ii) AAATGGAATGGATATAATGGC; (iii) GGACCATAAATCCATTTTCGAACTATCTTTTG; (iv) AAAAGATAGTTCGAAAATGGAATTTATGGT; (v) GGTCGCACAATCCCACAATAGAAAAATG; (vi) GCATTTTTCTATTGTGGGATTGTGCGA; (vii) TATCTTTTGCTGTTGTGTCCGAAAGTTTGTTGTCG; (viii) GGCGACAACAAACTTTCGGACACAACAGCAAAAGAT; (ix) GGCATTTTCGAAAATGGGATTGTGCGA; and (x) CCATAAATTCCACAATAGAACTATCTTTTG.

Primers (i) and (ii) were used to amplify a 125 bp fragment from the *GLO* promoter (GLOp2/3). Annealing of oligonucleotides (iii) and (iv) results in CArG box 2 (CArG2), annealing of oligonucleotides (v) and (vi) yields CArG box 3 (CArG3), and (vii) and (viii) represent the region lying between CArG2 and CArG3 of the *GLO* promoter (Tröbner *et al*., 1992). The *GLO* promoter fragment was mutagenized by PCR to obtain two DNA fragments that contained two identical CArG boxes: GLO2/2 was obtained using oligonucleotides (ii) and (ix), and GLO3/3 by using oligonucleotides (i) and (x). GLO2/2 contains twice the CArG2 motif from the *GLO* promoter, and GLO3/3 contains twice the CArG3 motif. The flanking sequences are like those in the wild-type promoter. The promoter fragments were cloned in the pADVANTAGE vector from Clontech. Radioactive labeling was carried out after digestion with *Eco*RI by 'fill in' with Klenow fragment.

The binding cocktail contained 1 ng of labeled probe, 8.5 mM HEPES pH 7.3, 8.6 μM dithiothreitol, 1.35 mM EDTA, 2.8 μg bovine serum albumin, 150 ng autoclaved herring sperm DNA, 1.5 mM spermidine, 2.5% CHAPS, 8% glycerol and 2 μl of the corresponding reticulocyte lysate in a total volume of 12 μl. When several protein combinations were mixed, each was premixed with the probe and 6 μl of each were aliquoted to other tubes to give a final volume of 12 μl. The binding reactions were incubated on ice for 30 min before loading on a 4% acrylamide:bisacrylamide (39:1) gel that was run at 150 V at room temperature. The resulting band shift was transferred to paper, dried and exposed.

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References

Bowman,J.L., Smyth,D.R. and Meyerowitz,E.M. (1989) Genes directing flower development in *Arabidopsis*. *Plant Cell*, **1**, 37–52.

Bowman,J.L., Alvarez,J., Weigel,D., Meyerowitz,E. and Smyth,D. (1993) Control of flower development in *Arabidopsis thaliana* by APETALA1 and interacting genes. *Development*, **119**, 721–743.

Bradley,D., Carpenter,R., Sommer,H., Hartley,N. and Coen,E. (1993) Complementary floral homeotic phenotypes result from opposite orientations of a transposon at the plena locus of *Antirrhinum*. *Cell*, **72**, 85–95.

Bradley,D., Vincent,C., Carpenter,R. and Coen,E. (1996) Pathways for inflorescence and floral induction in *Antirrhinum*. *Development*, **122**, 1535–1544.

Carpenter,R., Copsey,L., Vincent,C., Doyle,S., Magrath,R. and Coen,E. (1995) Control of flower development and phyllotaxy by meristem identity genes in *Antirrhinum*. *Plant Cell*, **7**, 2001–2011.

Coen,E. and Meyerowitz,E. (1991) The war of the whorls: genetic interactions controlling flower development. *Nature*, **353**, 31–37.

Coen,E., Romero,J., Doyle,S., Elliott,R., Murphy,G. and Carpenter,R. (1990) *Floricaula*: a homeotic gene required for flower development in *Antirrhinum majus*. *Cell*, **63**, 1311–1322.

Davies,B. and Schwarz-Sommer,Z. (1994) Control of floral organ identity by homeotic MADS-box transcription factors. *Results Probl. Cell Differ.*, **20**, 235–258.

Davies,B., Egea-Cortines,M., de Andrade Silva,E., Saedler,H. and Sommer,H. (1996) Multiple interactions amongst floral homeotic MADS-box proteins. *EMBO J.*, **15**, 4330–4343.

Davies,B., Motte,P., Keck,E., Saedler,H., Sommer,H. and Schwarz-Sommer,Z. (1999) PLENA and FARINELLI: redundancy and regulatory interactions between two *Antirrhinum* MADS-box factors controlling flower development. *EMBO J.*, **18**, 4023–4034.

Durfee,T., Becherer,K., Chen,P., Yeh,S., Yang,Y., Kilburn,A., Lee,W. and Elledge,S. (1993) The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes Dev.*, **7**, 555–569.

Fan,H.Y., Hu,Y., Tudor,M. and Ma,H. (1997) Specific interactions between the K domains of AG and AGLs, members of the MADS domain family of DNA binding proteins. *Plant J.*, **12**, 999–1010.

Feilotter,H.E., Hannon,G.H., Ruddell,C.J. and Beach,D. (1994) Construction of an improved host strain for two hybrid screening. *Nucleic Acids Res.*, **22**, 1502–1503.

Fields,S. and Song,O. (1989) A novel genetic system to detect protein–protein interactions. *Nature*, **340**, 245–246.

Haughn,G.W. and Somerville,C.R. (1988) Genetic control of morphogenesis in *Arabidopsis*. *Dev. Genet.*, **9**, 73–89.

Herskowitz,I. (1989) A regulatory hierarchy for cell specialization in yeast. *Nature*, **342**, 749–757.

Huijser,P., Klein,J., Lonnig,W., Meijer,H., Saedler,H. and Sommer,H. (1992) Bracteomania, an inflorescence anomaly, is caused by the loss of function of the MADS-box gene squamosa in *Antirrhinum majus*. *EMBO J.*, **11**, 1239–1249.

Ingram,G., Doyle,S., Carpenter,R., Schultz,E., Simon,R. and Coen,E. (1997) Dual role of *fimbriata* in regulating floral homeotic genes and cell division. *EMBO J.*, **16**, 6521–6534.

Kempin,S., Savidge,B. and Yanofsky,M. (1995) Molecular basis of the cauliflower phenotype in *Arabidopsis*. *Science*, **267**, 522–525.

Kippert,F. (1995) A rapid permeabilization procedure for accurate quantitative determination of β-galactosidase activity in yeast cells. *FEMS Microbiol. Lett.*, **128**, 201–206.

Krizek,B. and Meyerowitz,E. (1996) Mapping the protein regions responsible for the functional specificities of the *Arabidopsis* MADS domain organ-identity proteins. *Proc. Natl Acad. Sci. USA*, **93**, 4063–4070.

Kunst,L., Klenz,J.E., Martinez-Zapater,J. and Haughn,G.W. (1989) AP2 gene determines the identity of perianth organs in flowers of *Arabidopsis thaliana*. *Plant Cell*, **1**, 1195–1208.

Ma,H., Yanofsky,M. and Meyerowitz,E. (1991) AGL1-AGL6, an *Arabidopsis* gene family with similarity to floral homeotic and transcription factor genes. *Genes Dev.*, **5**, 484–495.

Mizukami,Y., Huang,H., Tudor,M., Hu,Y. and Ma,H. (1996) Functional domains of the floral regulator AGAMOUS: characterization of the DNA binding domain and analysis of dominant negative mutations. *Plant Cell*, **8**, 831–845.

Motte,P., Saedler,H. and Schwarz-Sommer,Z. (1998) STYLOSA and FISTULATA: regulatory components of the homeotic control of *Antirrhinum* floral organogenesis. *Development*, **125**, 71–84.

Pnueli,L., Abu-Abeid,M., Zamir,D., Nacken,W., Schwarz-Sommer,Z. and Lifschitz,E. (1991) The MADS box gene family in tomato: temporal expression during floral development, conserved secondary structures and homology with homeotic genes from *Antirrhinum* and *Arabidopsis*. *Plant J.*, **1**, 255–266.

Pollock,R. and Treisman,R. (1991) Human SRF-related proteins: DNA-binding properties and potential regulatory targets. *Genes Dev.*, **5**, 2327–2341.

Riechmann,J. and Meyerowitz,E. (1997) Determination of floral organ identity by *Arabidopsis* MADS domain homeotic proteins AP1, AP3, PI and AG is independent of their DNA-binding specificity. *Mol. Biol. Cell*, **8**, 1243–1259.

Riechmann,J., Krizek,B. and Meyerowitz,E. (1996) Dimerization specificity of *Arabidopsis* MADS domain homeotic proteins APETALA1, APETALA3, PISTILLATA and AGAMOUS. *Proc. Natl Acad. Sci. USA*, **93**, 4793–4798.

Schwarz-Sommer,Z., Huijser,P., Nacken,W., Saedler,H. and Sommer,H. (1990) Genetic control of flower development by homeotic genes in *Antirrhinum majus*. *Science*, **250**, 931–936.

Schwarz-Sommer,Z., Hue,I., Huijser,P., Flor,P., Hansen,R., Tetens,F., Lonnig,W., Saedler,H. and Sommer,H. (1992) Characterization of the *Antirrhinum* floral homeotic MADS-box gene *deficiens*: evidence for DNA binding and autoregulation of its persistent expression throughout flower development. *EMBO J.*, **11**, 251–263.

Shore,P. and Sharrocks,A. (1995) The MADS-box family of transcription factors. *Eur. J. Biochem.*, **229**, 1–13.

Simon,R., Carpenter,R., Doyle,S. and Coen,E. (1994) *Fimbriata* controls flower development by mediating between meristem and organ identity genes. *Cell*, **78**, 99–107.

Sommer,H., Beltran,J., Huijser,P., Pape,H., Lonnig,W., Saedler,H. and Schwarz-Sommer,Z. (1990) *Deficiens*, a homeotic gene involved in the control of flower morphogenesis in *Antirrhinum majus*: the protein shows homology to transcription factors. *EMBO J.*, **9**, 605–613.

Stotz,A. and Linder,P. (1990) The *ADE2* gene from *Saccharomyces cerevisiae*: sequence and new vectors. *Gene*, **95**, 91–98.

Tröbner,W., Ramirez,L., Motte,P., Hue,I., Huijser,P., Lonnig,W., Saedler,H., Sommer,H. and Schwarz-Sommer,Z. (1992) *GLOBOSA*: a homeotic gene which interacts with *DEFICIENS* in the control of *Antirrhinum* floral organogenesis. *EMBO J.*, **11**, 4693–4704.

Weigel,D. and Meyerowitz,E. (1994) The ABCs of floral homeotic genes. *Cell*, **78**, 203–209.

West,A.G., Causier,B.E., Davies,B. and Sharrocks,A.D. (1998) DNA binding and dimerization determinants of *Antirrhinum* MADS-box transcription factors. *Nucleic Acids Res.*, **26**, 5277–5287.

Zachgo,S., de Andrade Silva,E., Motte,P., Trobner,W., Saedler,H. and Schwarz-Sommer,Z. (1995) Functional analysis of the *Antirrhinum* floral homeotic *DEFICIENS* gene *in vivo* and *in vitro* by using a temperature-sensitive mutant. *Development*, **121**, 2861–2875.

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