



Analysis of the C-terminal region of *Arabidopsis thaliana* APETALA1 as a transcription activation domain

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Abstract

APETALA1 (API) of *Arabidopsis thaliana* is a transcription factor controlling flower development. API is a member of the MADS (MCM1, AGAMOUS, DEFICIENS, SRF) superfamily, which plays important roles in differentiation in plants and animals. MADS domains, which function most importantly in DNA binding, are found in all major eukaryotic kingdoms. In plants, MADS domain-containing proteins also possess a region of moderate sequence similarity named the K domain, which is involved in protein-protein interaction. Little is known about the function of a third, highly variable, domain designated the C domain, as it resides at the C terminus of the MADS proteins of plants. Here we report that the C-terminal domain of *Arabidopsis thaliana* API and its homologues perform a transcriptional activation function. The C-terminal region of API is composed of at least two separable transcriptional activation domains that function synergistically.

Introduction

During floral induction a transition from the vegetative stage to the reproductive stage occurs following endogenous signals such as age as well as environmental signals such as day length. Upon such an event, the shoot apical meristem (SAM) gives rise to a series of flowers. Once the transition occurs, meristem-identity genes are required to determine the fate of the newly formed floral primordia. In *Arabidopsis*, homeotic genes such as LEAFY (LFY), APETALA1 (API), APETALA2 (AP2), and CAULIFLOWER (CAL) determine floral meristem identity. Among these genes, it has been shown that LFY and API play central roles in the generation of flowers instead of shoots (Mandel *et al.*, 1992; Weigel *et al.*, 1992). *ap1* mutants form normal flower primordia, but the primordia then develop into flowers with partial shoot characteristics (Irish and Sussex, 1990). A *lfy ap1* double mutant plant develops inflorescence shoots in place of flowers, and this phenotype is more severe than in either single mutant (Huala and Sussex, 1992; Weigel *et al.*, 1992).

In addition, expression of either LFY or API under the control of a constitutive promoter is sufficient to confer floral meristem identity to cells in the shoot meristem (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995). This suggests that LFY and API have, to some extent, overlapping functions and that they appear to reinforce each other's activity. The CAL gene positively regulates both API and LFY expression, and AP2 has a similar function in which it reinforces the action of the two genes as well (Bowman *et al.*, 1993).

Several *Arabidopsis* homeotic genes that affect the identity of floral organs have been identified, including API, AP2, APETALA3 (AP3), PISTILLATA (PI), and AGAMOUS (AG). Interestingly, API participates not only in determining floral meristem identity, but also in directing organ development. API is specifically involved in determining the identities of sepals and petals. Additional phenotypes of *ap1* mutants are altered organ identities (Bowman *et al.*, 1993). In *ap1* mutants, the first whorl sepals are transformed into bract-like organs, and second whorl organs are absent

on most of plants with strong *apl* alleles. *API* RNA is at first uniformly expressed in young flower primordia and later becomes localized to sepals and petals (Mandel *et al.*, 1992). *API* expression is negatively regulated by two floral meristem-identity genes, *TERMINAL FLOWER (TFL)* and *AG*, in the inflorescence meristem and in the two inner whorls of wild-type flowers, respectively (Gustafson-Brown *et al.*, 1994).

The *API* gene has been cloned (Mandel *et al.*, 1992) as one of a group of *AG*-like genes isolated on the basis of their sequence similarity to the *AG* MADS box. *AG*-like proteins contain a MADS box (named for the conserved motif of *MCM1*, *AGAMOUS*, *DEFICIENS*, and serum response factor) (Schwarz-Sommer *et al.*, 1990) which encodes a highly conserved stretch of 56 amino acids. This MADS box is required for DNA binding (Hayes *et al.*, 1988; Norman *et al.*, 1988) and protein dimerization. The *API* protein, a typical MADS domain protein, forms a homodimer (Riechmann *et al.*, 1996a) and binds to sequences with a consensus called the CArG box (CC[A/T]₆GG) *in vitro* (Riechmann *et al.*, 1996b). Plant MADS domain proteins contain a second domain, a moderately conserved region, which is absent from those of other organisms. This domain is called the K domain for its similarity to the coiled-coil domain of keratin, and it is considered to direct protein-protein interactions (Ma *et al.*, 1991; Fan *et al.*, 1997). In addition, plant MADS domain proteins have two divergent regions called the I domain (intervening region between MADS domain and K domain) and the C domain. The I domain confers dimerization specificity (Riechmann *et al.*, 1996a). The function of the C domain is not yet known, even though it is essential for the function of *AG* (Mizukami *et al.*, 1996).

Transcription factors encoded in the homeotic genes play key roles in cell proliferation, cell differentiation, and organ development (Gehring, 1987; Kenyon, 1994). Transcriptional activators often contain two distinct domains: one is required for DNA binding to enhancer elements in mammals or the upstream activating sequence (UAS) in yeast, and the other contributes to the activation of the transcriptional machinery (Brent and Ptashne, 1985; Hope and Struhl, 1986). There are several types of transcriptional activators. Acidic activators, most frequently observed from transcriptional activators such as the *Saccharomyces cerevisiae* proteins *GCN4* and *GAL4* and the herpes simplex virus activator *VPI6*, are characterized by a relatively high content of glutamate and aspartate (Hope and Struhl, 1986; Ma and Ptashne,

1987). Glutamine-rich and proline-rich activators have been identified as well. *SPI* (Courey *et al.*, 1989) in mammals and *PosF21* (Aeschbacher *et al.*, 1991) in plants are typical glutamine-rich activators, and *CTF/NF-1* (Mermod *et al.*, 1989) in mammals and *GBF-1* (Schindler *et al.*, 1992) in plants are examples of proline-rich activators. These transcriptional activation domains are well conserved throughout the eukaryotic sphere, and they function in distantly related systems. For example, the yeast transcriptional activator *GAL4* has been shown to activate transcription in plants (Ma *et al.*, 1988), mammals (Kakidani and Ptashne, 1988; Webster *et al.*, 1988), and *Drosophila* (Fischer *et al.*, 1988). Conversely, acidic activation domains derived from some mammalian activators, such as the herpes simplex virus protein *VPI6* and the *Jun/Fos* proteins (Lech *et al.*, 1988; Struhl, 1988), efficiently stimulate transcription in yeast. On the other hand, glutamine-rich activation domain of human *SPI* does not function in yeast (Ponticelli *et al.*, 1995).

API, a homeotic gene with a MADS box, has been regarded as a transcription factor. However, it has not yet been demonstrated whether *API* itself functions as the transcriptional activator or whether it directs transcription by recruiting other transcriptional activators. Intriguingly, clusters of acidic, glutamine-rich, and proline-rich amino acid residues, often present in transcriptional activators, reside in the C-terminal region of the *Arabidopsis thaliana API* protein. We, therefore, investigated whether *API* can perform the function of a transcriptional activator in mammalian and yeast systems which have been widely used for testing transcriptional activation functions. For this purpose, different parts of *API* were fused to the yeast *GAL4* DNA-binding domain and expressed in yeast and mammalian cells with *GAL4*-binding sites upstream of the proper reporter genes (Fields and Jang, 1990). We found that the C-terminal region of *API* exhibited a transcriptional activation function. The C-region was then dissected into at least two transcriptional activation domains, one acidic residue-rich and the other glutamine-rich, that functioned synergistically. Furthermore, we demonstrated that *API* homologues of *Nicotiana sylvestris* (long-day tobacco), *Nicotiana tabacum* (day-neutral tobacco), and *Raphanus sativus* also function as transcriptional activators.

Materials and methods

Plasmid construction

Subcloning of plasmids was carried out by standard methods (Sambrook *et al.*, 1989). A series of plasmids containing different portions of the *Arabidopsis* *API* gene was constructed by inserting parts of the *API* gene downstream of the *GAL4* DNA-binding domain (amino acids 1–147) (Keegan *et al.*, 1986), which is under the control of the yeast *ADHI* promoter. Plasmid pY-AD(768–881) contains the yeast *GAL4* transcription activation domain II (amino acids 768–881) (Ma and Ptashne, 1987) fused to the *GAL4* DNA-binding domain. In order to construct pY-AD, pGAD424 (Clontech) was digested with *HindIII*, and the smaller fragment was then inserted into the *Sall* site of pGBT9 (Clontech). A phage cDNA pool was constructed from an *Arabidopsis* young flower cDNA library, and *API* cDNA fragments were obtained from it by means of polymerase chain reaction (PCR) using appropriate primers containing the start or stop codon of *API*. The *API* gene was inserted into the *SmaI* site of pBluescript II SK(–). The pY-API(1–256) plasmid was constructed by ligating the *API* fragment that was generated by digestion of pBluescript II SK-API with *BamHI* and *EcoRI* to pGBT9 linearized by the same restriction enzymes. The pY-API(88–256) plasmid was constructed by ligating pGBT9 linearized with *SmaI* (5') and *Sall* (3') to an *API* fragment generated by *AatII/Sall* digestion of pY-API(1–256). Plasmids pY-API(155–256), pY-API(1–152), and pY-API(1–191) were generated by self-ligation of pY-API(1–256) after digestion with *SacI/EcoRI*, *Sall/SacI*, and *StyI/Sall*, respectively. In order to construct pY-API(193–256), the *API* fragment was first generated from pY-API(1–256) by PCR using two primers (5'-CCGGAATTCCACAATATGCCTCCCCTC-3' and 5'-AAAAGTGCAGGTCGACGGATCGATCCCC-3'). The PCR product was digested with *EcoRI/PstI* and inserted into pGBT9, linearized with *EcoRI/PstI*.

To further analyze the transcriptional activation function of *API*, the C-terminal domain was dissected into smaller pieces using appropriate primers and the template DNA pY-API(1–256). The pY-series and pM-series plasmids were generated to investigate transcriptional activation functions in yeast and mammalian cells, respectively. Plasmid pSG424 contains the *GAL4* DNA-binding domain (amino acids 1–147) under the control of the SV40 promoter. To build the pY-series plasmids, all PCR products were digested

with *EcoRI/BamHI* and then ligated to pGBT9, digested with *EcoRI/BamHI*. The pM-series plasmids were constructed by ligating the PCR products digested with *EcoRI/BamHI* into pSG424 treated consecutively with *SacI*, Klenow, and *EcoRI*.

In order to compare the transcriptional activation function of *API* to that of its homologues *RsMADS1*, *NsMADS2*, and *NtMADS5*, equivalent deletion clones were constructed. *RsMADS1*, *NsMADS2*, and *NtMADS5* were isolated from *Raphanus sativus* (radish), *Nicotiana sylvestris* (long-day tobacco), and *Nicotiana tabacum* (day-neutral tobacco), respectively. pY- and pM-series plasmids were constructed similarly as described above. To construct the pY-series plasmids, all PCR products were digested with *EcoRI/BamHI* and then ligated into pGBT9, also digested with *EcoRI/BamHI*. The pM-series plasmids were constructed by ligating the PCR products digested with *EcoRI/BamHI* into pSG424 treated consecutively with *SacI*, Klenow, and *EcoRI*.

Yeast strain

The yeast strain HF7C [*MATa*, *ura3-52*, *his3-200*, *lys2-801*, *ade2-101*, *trp1-901*, *leu2-3,112*, *gal4-542*, *gal80-538*, *cyh^r2*, *LYS2::GAL1_{UAS}-GAL1_{TATA-HIS3}*, *URA3::GAL4_{17mers}($\times 3$)-CyCI_{TATA-laZ}] was used for the transformations. The *LYS2* gene is non-functional. The *trp1*, *leu2*, *his3*, *gal4*, and *gal80* mutations are all deletions. The *GAL1* upstream activating sequence (UAS) and the three tandem copies of the *GAL4* 17-mer consensus sequence [*GAL4_{17mers}($\times 3$)*] are responsive to either the *GAL4* transcriptional activator or a potential transcriptional activator fused to the *GAL4* DNA-binding domain. HF7C encodes *HIS3* and β -galactosidase as reporters and has *trp1*, *leu2*, *cyh^r2* as marker genes for transformant selection (Feilotter *et al.*, 1994).*

Yeast cell culture and transformation

Saccharomyces cerevisiae HF7C was grown in YPD [1% yeast extract, 2% peptone, 2% D-glucose, 1.5% agar (only in plates)] or synthetic minimal medium (SD) [0.67% yeast nitrogen base, 2% D-glucose, (10 \times) amino acids dropout solution deficient in tryptophan or histidine, 1.5% agar (only in plates)]. Yeast was transformed with appropriate plasmids by the lithium acetate method (Gietz *et al.*, 1992), and the transformants were selected on SD.

β -galactosidase assay

Relative transcriptional activation functions of the *GAL4-API* derivatives were investigated by β -galactosidase assay. Liquid β -galactosidase assay (ONPG assay) was performed as described by Yocum *et al.* (1984). In this study, the units of activity were calculated using the formula $U = 1000 \times OD_{420} / (t \times V \times OD_{600})$, where OD_{420} is the absorbance at 420 nm measured at the end of the reaction, OD_{600} is the cell density of the culture at the time of harvest, t is the duration of the β -galactosidase assay in minutes, and V is the volume of culture used for the assay (usually 0.3 ml).

DNA transfection and chloramphenicol acetyl transferase (CAT) assay

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% bovine calf serum. Transfections were carried out by the electroporation method. Briefly, DNAs (3 μ g of the pSG424 expression vector or its derivatives, 5 μ g of pG5E1b-CAT reporter plasmid, and 0.1 μ g of the luciferase expression plasmid pGL2) were transfected into COS-7 cells using the BioRad Gene Pulser apparatus at 960 μ F and 0.25 kV in 1 ml DMEM without serum. After electroporation, the cells were maintained in DMEM supplemented with 10% bovine calf serum and 5% fetal bovine serum on 100 mm dishes. At 44 to 48 h after transfection, the cells were harvested and resuspended in 200 μ l of phosphate-buffered saline (PBS) containing 5 mM EDTA. CAT activity in COS-7 cells was measured as described (Neumann *et al.*, 1987). Cell extracts were prepared by four cycles of freeze-thawing in 200 μ l of PBS containing 5 mM EDTA and centrifugation for 5 min in an Eppendorf microcentrifuge at 4 °C. CAT activity was measured in 233 μ l of a reaction mixture containing 3 μ l of [³H]-acetyl-CoA (250 μ Ci/ml; Amersham), 5 μ l of unlabeled acetyl CoA (1 mg/ml in deionized water), and 25 μ l of 10 mM chloramphenicol. The reaction mixture was gently overlaid onto 3 ml of Econofluor-2 (Packard) and then incubated at 37 °C. After 30 to 50 min, the amount of [³H]-acetyl-CoA transferred to the chloramphenicol was measured in a liquid scintillation counter (1600 CA, TRI-CARB). Plasmids pSG147-VP16 and pSG424 were used as positive and negative controls, respectively. CAT activity was normalized for transfection efficiency in each experiment measured by luciferase activity derived from pGL2.

Results*The C-terminal region of API functions as a transcriptional activation domain*

The possible transcriptional activation function of *API* was investigated in yeast and mammalian cells. Two reporter genes (β -galactosidase and *HIS3*) were used in the yeast cells to test the transcriptional activation function of *API*. Both reporter genes were controlled by a promoter that recruits *GAL4_D-API* fusion proteins to the vicinity of the reporters through *GAL4_D* domain and *GAL4*-binding site interaction. Therefore, the fusion proteins would activate transcription of the reporter genes (β -galactosidase and *HIS3*) if *API* or its derivatives contained transcriptional activation domain(s), and expression of the reporter genes were easily evaluated by measuring β -galactosidase activity or by growing the yeast cells in histidine-deficient medium. Plasmids expressing *GAL4_D*-fusion proteins containing different portions of *API* were constructed as shown in Figure 1A. Yeast cells transformed with the positive control plasmid pY-AD(768–881) containing both the *GAL4* DNA-binding domain and the *GAL4* transcriptional activation domain grew well on a histidine-deficient plate, but yeast cells with a negative control plasmid (pGBT9) containing only the *GAL4* DNA-binding domain (amino acids (aa) 1–147) did not grow at all. Yeast cells containing pY-API(1–256), pY-API(88–256), pY-API(155–256) and pY-API(193–256) that expressed the *GAL4* DNA-binding domain fused to full-length *API*, *API* aa 88–256, *API* aa 156–256 or *API* aa 193–256, respectively, grew on histidine-deficient plates (Figure 1B). In addition, all constructs containing *API* amino acids 193–256 in the C-terminal domain exhibited β -galactosidase activity derived from the other reporter gene β -galactosidase (data not shown). The data, therefore, indicate that the C-terminal region of *API* (aa 193–256) contains a transcriptional activation domain that triggers transcription of the reporter genes (*HIS3* and β -galactosidase) controlled by the *GAL4*-binding sites. On the other hand, yeast cells containing pY-API(1–152) and pY-API(1–191), which express the *GAL4* DNA-binding domain fused to *API* aa 1–152 and *API* aa 1–191, respectively, neither grew on a histidine-deficient plate (Figure 1B) nor exhibited β -galactosidase activity (data not shown). This indicates that no functional transcription activation domain resides in aa 1–191 of *API*.

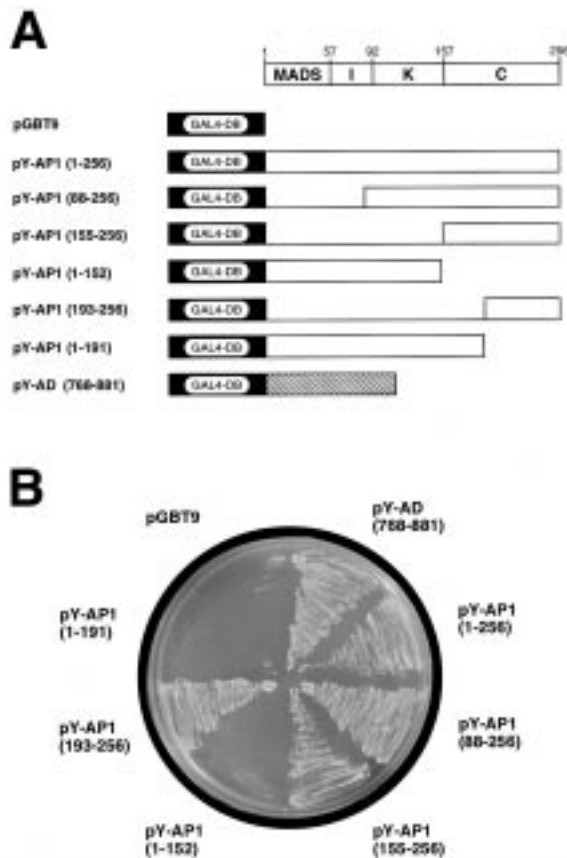


Figure 1. Determination of the transcriptional activation domain in yeast cells. **A.** Schematic diagram of proteins produced from yeast vectors (pY series). A series of *API* deletion mutants were fused in-frame downstream of the *GAL4* DNA-binding domain which was under the control of the yeast *ADHI* promoter. The numbers in parenthesis indicate the amino acid residue numbers of *API* that are present in the effector proteins. *GAL4-DB* represents the *GAL4* DNA-binding domain. Four distinct domains of *API* are shown on the top: MADS, MADS domain; I, intervening region between MADS domain and K domain; K, K domain; C, C-terminal region. The numbers above the box indicate the boundaries of each domain. **B.** Viability test of yeast cells containing plasmids shown in panel A on tryptophan- and histidine-deficient plates (SD/-Trp, -His). The transformants were selected on SD/-Trp plates until colonies grew. Then the yeast cells were streaked on SD/-Trp, -His plates to test for *HIS3* gene expression. pY-AD and pGBT9 were used as positive and negative control plasmids, respectively.

C-terminal acidic and glutamine-rich regions of API synergistically function as transcriptional activation domains

Interestingly, proline-rich, glutamine-rich, and acidic residue-rich regions, which are often present in eukaryotic transcriptional activators, are present in the C-terminal region of *API* (aa 193–256) (Figures 2A and 4). We therefore investigated the transcriptional

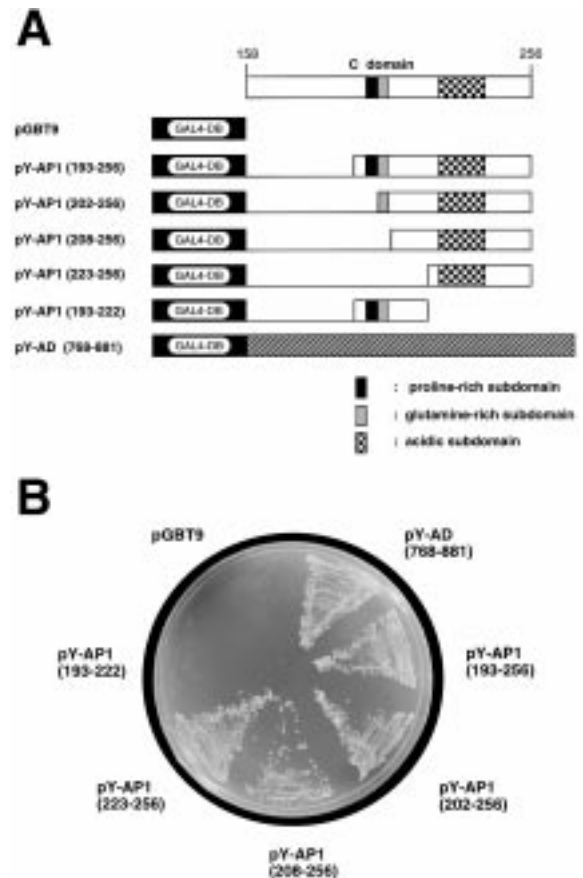


Figure 2. Determination of the minimal transcriptional activation domain of *API* in yeast cells. **A.** Schematic diagram of proteins produced from yeast vectors (pY series). Solid boxes, gray boxes, and checkered boxes represent proline-rich, glutamine-rich, and acidic domains, respectively. Other symbols depict the same as in Figure 1. The schematic diagram is not drawn to scale. **B.** Viability test of yeast cells containing plasmids shown in panel A on tryptophan- and histidine-deficient plates (SD/-Trp, -His). The methods used in this experiment are the same as those described in the legend to Figure 1B.

activation function of the C-terminal domain of *API* in two different assay systems: yeast and mammalian cells. We arbitrarily dissected the C-terminal domain of *API* into three parts: proline-rich (aa 196–201); glutamine-rich (aa 202–207); acidic (aa 228–244). A series of deletion mutants of *API* were constructed in yeast and mammalian expression vectors as shown in Figures 2A and 3A, respectively. In yeast cells, all clones except *API*(193–222) supported expression of the *HIS3* gene, which was indicated by the growth of the host yeast cells on histidine-deficient media (Figure 2B). This suggests that the transcription activation function of *API* in the yeast cells must be

C

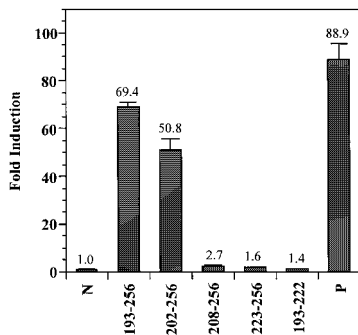


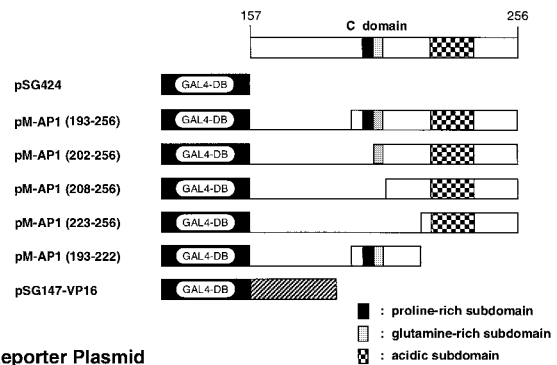
Figure 2. C. Relative transcriptional activation functions of *API* deletion mutants in yeast cells. Bars in the histogram show average β -galactosidase activities in yeast cells transformed with the plasmids shown in panel A. β -galactosidase activity in the cells transformed with the negative control vector pGBT9 was arbitrarily set at 1. The columns represent mean values, and standard deviations are indicated by error bars. N, 193–256, 202–256, 208–256, 223–256, 193–222, and P indicate cells transformed with plasmids pGBT9, pY-AP1(193–256), pY-AP1(202–256), pY-AP1(208–256), pY-AP1(223–256), pY-AP1(193–222), and pY-AD, respectively.

attributed to the acidic domain of *API* (aa 223–256). The region *API*(193–222) by itself, which contains the proline- and glutamine-rich regions, did not function as a transcriptional activation domain in the yeast cells. However, this does not rule out the possibility that the proline-rich and glutamine-rich regions might function as transcriptional activation domain in other eukaryotic cells such as plants and mammals, since human *SPI*, which is a transcriptional activator containing a glutamine-rich region, does not stimulate transcription in *Saccharomyces cerevisiae* (Ponticelli *et al.*, 1995).

In the mammalian (COS-7) transient expression assay, pM-AP1(193–256), which contains all three domains, exhibited a 24-fold higher activity than the negative control pSG424 as seen in Figure 3B. Deletion of the proline-rich domain, *API*(202–256), only minimally affected the transcriptional activation function of *API* (compare 193–256 with 202–256 in Figure 3B). But, a dramatic decrease in the transcriptional activation function was seen when the deletion was extended into the glutamine-rich domain. The transcriptional activation function of *API* was decreased about 3.5-fold (Figure 3B, lane 208–256). These data suggest that the glutamine-rich domain plays a key role in the transcriptional activation function of *API*. When the C-terminal domain of *API* was dissected into two domains (one glutamine-rich and the other

A

Effector Plasmids



Reporter Plasmid



B

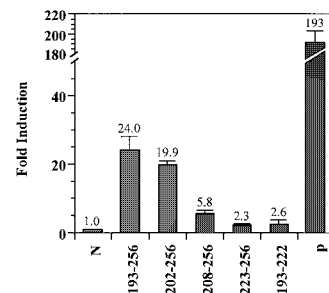


Figure 3. Determination of the minimal transcriptional activation domain of *API* in mammalian cells. A. Schematic diagram of proteins produced from mammalian expression vectors (pM series). Symbols represent the same as in Figure 1. The reporter plasmid contains 5 *GAL4*-binding sites (*GAL4* × 5), the adenovirus E1b promoter (E1b TATA), and the CAT (*CAT*) gene. B. Relative transcriptional activation functions of *API* deletion mutants in mammalian cells. Bars in the histogram show average CAT activities in mammalian cells transfected with the plasmids shown in the panel A. CAT activity in the cells transfected with the negative control vector pSG424 was arbitrarily set at 1. The columns represent mean values, and standard deviations are indicated by error bars. N, 193–256, 202–256, 208–256, 223–256, 193–222, and P indicate cells transfected with plasmids pSG424, pM-AP1(193–256), pM-AP1(202–256), pM-AP1(208–256), pM-AP1(223–256), pM-AP1(193–222), and pSG147–VP16, respectively. CAT activity was normalized with reference to the luciferase activity directed by the transfected control vector pGL2.

acidic), both domains provided weak, yet still significant transcriptional activation function (Figure 3B, 193–222 and 223–256). At least 10 independent experiments were performed to confirm this finding. The results indicate that the two parts contain minimal transcriptional activation domains. Deletion of residues 208–222 reduced the transcriptional activ-

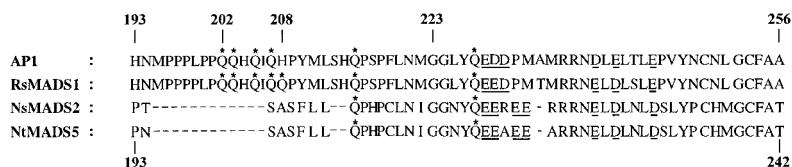


Figure 4. Comparison of amino acid sequences of the C-terminal domains of *API* and its homologues. *RsMADS1* was isolated from *Raphanus sativus* (radish). *NsMADS2* and *NtMADS5* were isolated from *Nicotiana sylvestris* (long-day tobacco) and *Nicotiana tabacum* (day-neutral tobacco), respectively. The numbers above and below the amino acids indicate amino acid numbers from the N-terminus of each gene. Asterisks and underlines indicate glutamine and acidic residues, respectively.

ity about 2-fold (compare 208–256 with 223–256 in Figure 3B). This suggests that residues 208–222 may augment the transcriptional activation function of the acidic region at the C-terminal end of *API*, possibly by assisting in the presentation of the transcriptional activator, though no known conserved sequences of transcriptional activators are recognized in this region. Combination of the two minimal transcriptional domains 193–256 (193–222 plus 223–256) resulted in about a 24-fold increase of transcriptional activation function. This is much higher than the summation of the activity of the two separate domains (193–222 and 223–256) directing 2.6- and 2.3-fold transcriptional activation functions, respectively. The data, therefore, strongly suggest that the two minimal domains function synergistically. This kind of synergy between glutamine-rich and acidic domains in mammalian and plant cells has been documented before (Klemsz and Maki, 1996; Schwechheimer *et al.*, 1998).

Synergy between the glutamine-rich and the acidic domain of *API* was also detected in yeast cells (Figure 2C). Transcriptional activation by the C-terminal domain of *API* in yeast cells was slightly weaker than that of the positive control pY-AD(768–881) which contained the transcription activation domain of *GAL4* (compare lane P with 193–256 in Figure 2C). Deletion of the proline-rich sequence (aa 193–201) reduced the transcriptional activation function of *API* by about 25% (compare lane 193–256 with 202–256 in Figure 2C). Deletion of the glutamine-rich sequence (aa 202–207) dramatically diminished transcriptional activator function of *API* about 20-fold (compare lane 202–256 with 208–256). This indicates that the glutamine-rich sequence is a key component of the transcriptional activation function of *API* in yeast cells. The results correlate well with the data obtained from mammalian cells. Transcriptional activations by 208–256, 223–256 and 193–222 in the ONPG assay were very weak, even though the β -galactosidase activities of these constructs were slightly higher than the negative control N. In order to increase the reliabil-

ity of the ONPG assay, the reactions were performed five times each. Nevertheless, the numerical values obtained in these assays may not be very accurate because of the inconsistencies of the β -galactosidase assays at low levels. On the other hand, the difference between glutamine-rich and acidic domains in yeast cells was clearly demonstrated by the histidine selection method shown in Figure 2B, where pY-*API*(223–256) containing just the acidic domain exhibited transcriptional activation functionality, while pY-*API*(193–222) containing just the glutamine-rich domain did not. We also performed western blot analysis using an antibody against the *GAL4* DNA-binding domain to investigate the expression levels of the *GAL4*-fusion proteins in mammalian and yeast cells. Similar amounts of fusion proteins were detected in both mammalian and yeast cells (data not shown). This indicates that the differences in the activities of the reporter genes (*CAT* and β -galactosidase) reflect the strength of the transcriptional activation functions of hybrid proteins *per se*.

API homologues isolated from different species function as transcriptional activators

Several *API* homologues have been isolated from different species such as radish and tobacco. *RsMADS1* was isolated from *Raphanus sativus* (radish), and *NsMADS2* and *NtMADS5* were isolated from *Nicotiana sylvestris* (long-day tobacco) and *Nicotiana tabacum* (day-neutral tobacco), respectively. The C-terminal sequence of the *API* homologue in *R. sativus*, which belongs to the mustard family along with *Arabidopsis thaliana*, shares very high homology with the *A. thaliana API* (compare *API* and *RsMADS1* in Figure 4). On the other hand, the *API* homologues in *NsMADS2* and *NtMADS5* lack the proline- and glutamine-rich sequence at amino acids 195–206 as compared with *API* of *A. thaliana* (Figure 4). The sequence from 215 in *API* of *A. thaliana* and its equivalent sequences in the other species are rather

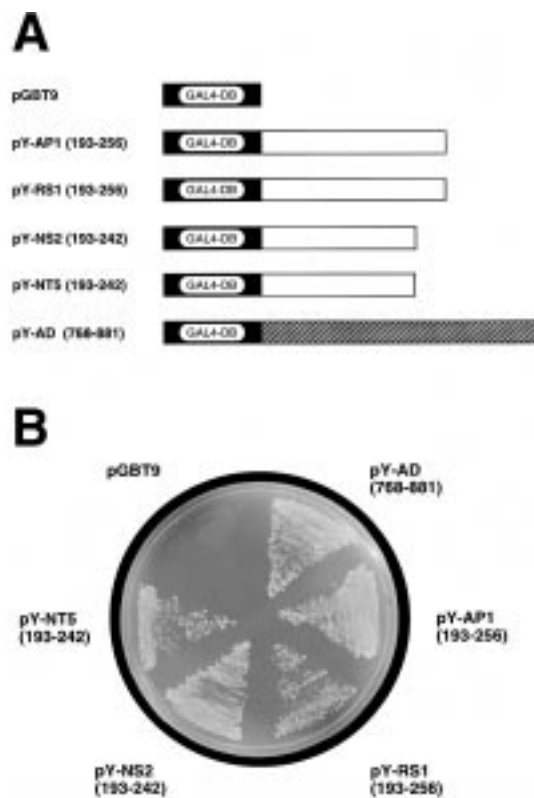


Figure 5. Transcriptional activation functions of the C-terminal domains of *API* and of their homologues in yeast cells. **A.** Schematic diagram of proteins produced from yeast vectors (pY series). The C-terminal domain of *API* (amino acids 193–256) and its equivalent in *API* homologues (shown in Figure 4) were inserted into pGBT9 to investigate the transcriptional activation function of the *API* homologues. **B.** Viability test of yeast cells containing plasmids shown in panel A on a tryptophan- and histidine-deficient plate (SD/-Trp, -His). The methods used in this experiment are the same as those described in the legend to Figure 1B.

well conserved among the different species (Figure 4). The transcriptional activation functions of the C-terminal domains of *RsMADS1*, *NsMADS2*, and *NtMADS5* were investigated in yeast and mammalian cells. All *API* homologues tested showed the transcriptional activation function in yeast cells as indicated by the growth of plasmid-containing cells in histidine-deficient medium (Figure 5B). The *API* homologues also directed transcription of the reporter gene in mammalian cells (Figure 6B). The transcriptional activation function of *API* and of *RsMADS1* was 2–3-fold higher than those of *NsMADS2* and *NtMADS5*. The relative activities of these proteins were comparable to those of *API*(202–256) and *API*(208–256) in which the glutamine-rich domain is present and absent, respectively. This result also indicates that

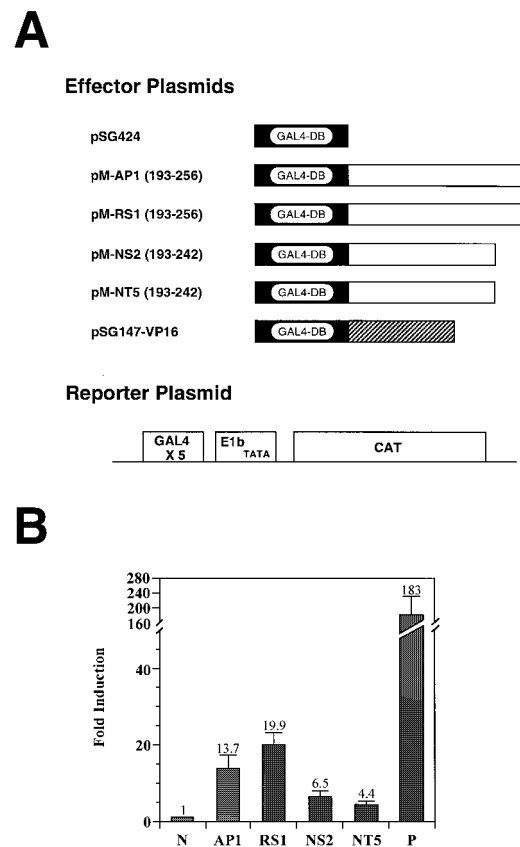


Figure 6. Transcriptional activation function of *API* homologues in mammalian cells. **A.** Schematic diagram of proteins produced from the mammalian expression vector (pM series). The C-terminal domain of *API* (amino acids 193–256) and its equivalents in *API* homologues (shown in Figure 4) were inserted into pSG424 to investigate the transcriptional activation function of the *API* homologues in mammalian cells. **B.** Relative transcriptional activation function of *API* homologues in mammalian cells. The methods used in this experiment were the same as those described in the legend to Figure 3B.

the glutamine-rich sequence plays an important role in the transcriptional activation function of *API*.

Discussion

API, a homeotic gene with a MADS box, has long been believed to be a transcription factor. However, it had not yet been demonstrated that *API* by itself could function as a transcriptional activator. Here we report that the C-terminal region of *API* contains transcriptional activation domains that work in yeast and mammalian cells through its glutamine-rich and acidic segments. Several lines of evidence suggest that the transcriptional activation domains in the C-terminal

region of *API* also function in plant cells, but a similar activity of *API* in plant cells has yet to be confirmed. First, many transcriptional activators are composed of a DNA-binding domain and transcriptional activation domains (Brent and Ptashne, 1985; Hope and Struhl, 1986). *API* has been regarded a transcription factor, because the *API* gene product is similar in sequence to MADS domain proteins, some of which have been demonstrated to be transcription factors (e.g. *SRF* and *MCM1*) and because the MADS domain of *API* binds to CArG-box sequences *in vitro* (Riechmann *et al.*, 1996b). Therefore, it would not be surprising to find that *API* by itself could function as a transcriptional activator. Second, the transcriptional activator sequences are well conserved among mammalian, plant, and yeast cells (Hope and Struhl, 1986; Ma and Ptashne, 1987; Courey *et al.*, 1989; Mermod *et al.*, 1989; Aeschbacher *et al.*, 1991; Schindler *et al.*, 1992). A transcriptional activator of one group can function in a different group (Fischer *et al.*, 1988; Kakidani and Ptashne, 1988; Lech *et al.*, 1988; Ma *et al.*, 1988; Struhl, 1988; Webster *et al.*, 1988). Third, *API* contains a glutamine-rich and an acidic domain which can serve as minimal transcriptional activation domains (see above). These two domains are often present and work synergistically in transcriptional activators of plants (Schwechheimer *et al.*, 1998).

Plant MADS domain proteins are composed of four distinct domains (MADS, I, K, C). MADS domain is well conserved among the MADS domain proteins. The K domain is primarily conserved at the level of secondary structure retaining the ability to form amphipathic α -helices. These domains play important roles in DNA-protein interactions and/or protein-protein interactions. The C domain is highly variable, and its function is not yet well understood. The C-terminal domain is likely to be involved in specific functions of different groups of MADS domain proteins. Furthermore, the importance of the C-terminal domain in MADS domain proteins is well documented in *AG* by ectopic expression of *AG* and its mutants (Mizukami *et al.*, 1996). Here we report that *API* homologues function as transcriptional activators through their C-terminal domains. The MADS domain proteins lacking transcriptional activation domains may still function as transcription factors through protein-protein interaction with MADS domain proteins containing transcriptional activators such as *API* and its homologues.

The transcriptional activation domain of *API* is composed of at least two separable domains that can function in mammalian cells. One is rich in glutamine residues, and the other is rich in acidic residues. In combination the two domains show synergism (Figure 3B). Such a synergistic activity of glutamine-rich and acidic domains in plant cells has been recently demonstrated by Schwechheimer *et al.* (1998). Intriguingly, the glutamine-rich region by itself does not function as a transcriptional activation domain in yeast cells (pY-API(193–222) in Figure 2B). This result coincides with the observation that the glutamine-rich activation domain of human *SPI* does also not stimulate transcription in *Saccharomyces cerevisiae* (Ponticelli *et al.*, 1995). This result is possibly due to the lack of some TBP-associated factors (TAFs) targeted to the metazoans' non-acidic activators, such as dTAFII110 and hTAFII130, in yeast cells (Reese *et al.*, 1994).

API is known as an early-acting gene in the genetic pathway of floral induction (Mandel *et al.*, 1992). Activation of *API* is triggered in the primary response to floral inductive signals, both environmental and endogenous signals. *API* is expressed in newly formed floral primordia, and it plays a central role in the generation of flowers instead of shoots. *API* functions partially redundantly with *LFY* to specify floral meristem fate. Genetic analyses suggest that *API* may trigger expression of key genes required in a following stage, such as *AGL6* (Savidge *et al.*, 1995). However, direct transcriptional activation of *AGL6* by binding of *API* to the *AGL6* promoter remains to be elucidated. In addition, *API* specifies the identity of sepals and petals during later stages of flower development. How does *API* activate different sets of genes at different developmental stages? One feasible explanation could be that *API* activates the transcription of different sets of genes by interacting with different transcription factors. Recently, *AG* (a member of the MADS proteins) was shown to interact with *AGLs* (other members of MADS proteins) to form heterodimers through the K domains (Fan *et al.*, 1997). Putative homodimeric or heterodimeric *API* molecules might function as transcriptional activators in conjunction with other MADS proteins with different DNA-binding specificities. A similar mode of transcriptional activation has been observed for jun/jun and jun/fos complexes (Hirai *et al.*, 1990). Protein-protein interactions among the MADS proteins are under investigation.

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References

- Aeschbacher, R.A., Schrott, M., Potrykus, I. and Saul, M.W. 1991. Isolation and molecular characterization of *PosF21*, an *Arabidopsis thaliana* gene which shows characteristics of a b-Zip class transcription factor. *Plant J.* 1: 303–316.
- Bowman, J.L., Alvarez, J., Weigel, L., Meyerowitz, E.M. and Smyth, D.R. 1993. Control of flower development in *Arabidopsis thaliana* by *APETALA1* and interacting genes. *Development* 119: 721–743.
- Brent, R. and Ptashne, M. 1985. A eukaryotic transcriptional activator bearing the DNA specificity of a prokaryotic repressor. *Cell* 43: 729–736.
- Courey, A.J., Holtzman, D.A., Jackson, S.P. and Tjian, R. 1989. Synergistic activation by the glutamine-rich domains of human transcription factor *SPI*. *Cell* 59: 827–836.
- 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.
- Fields, S. and Jang, S.K. 1990. Presence of a potent transcription activating sequence in the p53 protein. *Science* 249: 1046–1049.
- Feilolter, H.E., Hannon, G.J., Ruddell, C.J. and Beach, D. 1994. Construction of an improved host strain for two hybrid screening. *Nucl. Acids Res.* 22: 1502–1503.
- Fischer, J.A., Giniger, E., Maniatis, T. and Ptashne, M. 1988. *GAL4* activates transcription in *Drosophila*. *Nature* 332: 853–856.
- Gehring, W.J. 1987. Homeo boxes in the study of development. *Science* 236: 1245–1252.
- Gietz, D., St Jean, A., Woods, R.A. and Schiestl, R.H. 1992. Improved method for high efficiency transformation of intact yeast cells. *Nucl. Acids Res.* 20: 1425.
- Gustafson-Brown, C., Savidge, B. and Yanofsky, M.F. 1994. Regulation of the *Arabidopsis* floral homeotic gene *APETALA1*. *Cell* 76: 131–143.
- Hayes, T.E., Sengupta, P. and Cochran, B.H. 1988. The human c-fos serum response factor and the yeast factors *GRM/PRTF* have related DNA-binding specificities. *Genes Dev.* 2: 1713–1722.
- Hirai, S., Bourachot, B. and Yaniv, M. 1990. Both Jun and Fos contribute to transcription activation by the heterodimer. *Oncogene* 5: 39–46.
- Hope, I.A. and Struhl, K. 1986. Functional dissection of a eukaryotic transcriptional activator protein, *GCN4* of yeast. *Cell* 46: 885–894.
- Huala, E. and Sussex, I.M. 1992. LEAFY interacts with floral homeotic genes to regulate *Arabidopsis* floral development. *Plant Cell* 4: 901–913.
- Irish, V.F. and Sussex, I.M. 1990. Function of the *APETALA-1* gene during *Arabidopsis* floral development. *Plant Cell* 2: 741–753.
- Kakidani, H. and Ptashne, M. 1988. *GAL4* activates gene expression in mammalian cells. *Cell* 52: 161–167.
- Keegan, L., Gill, G. and Ptashne, M. 1986. Separation of DNA binding from the transcription-activating function of a eukaryotic regulatory protein. *Science* 231: 699–704.
- Kenyon, C. 1994. If birds can fly, why can't we? Homeotic genes and evolution. *Cell* 78: 175–180.
- Klemsz, M.J. and Maki, R.A. 1996. Activation of transcription by *PUI1* requires both acidic and glutamine domains. *Mol. Cell Biol.* 16: 390–397.
- Lech, K., Anderson, K. and Brent, R. 1988. DNA-bound Fos proteins activate transcription in yeast. *Cell* 52: 179–184.
- Ma, J. and Ptashne, M. 1987. A new class of yeast transcriptional activators. *Cell* 51: 113–119.
- Ma, J., Przibilla, E., Hu, J., Bogorad, L. and Ptashne, M. 1988. Yeast activators stimulate plant gene expression. *Nature* 334: 631–633.
- Ma, H., Yanofsky, M.F. and Meyerowitz, E.M. 1991. *AGL1-AGL6*, an *Arabidopsis* gene family with similarity to floral homeotic and transcription factor genes. *Genes Dev.* 5: 484–495.
- Mandel, M.A. and Yanofsky, M.F. 1995. A gene triggering flower formation in *Arabidopsis*. *Nature* 377: 522–524.
- Mandel, M.A., Gustafson-Brown, C., Savidge, B. and Yanofsky, M.F. 1992. Molecular characterization of the *Arabidopsis* floral homeotic gene *APETALA1*. *Nature* 360: 273–277.
- Mermoud, N., O'Neill, E.A., Kelly, T.J. and Tjian, R. 1989. The proline-rich transcriptional activator of *CTF/NF-1* is distinct from the replication and DNA binding domain. *Cell* 58: 741–753.
- 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.
- Neumann, J.R., Morency, C.A. and Russian, K.O. 1987. A novel rapid assay for chloramphenicol acetyl transferase gene expression. *BioTechniques* 5: 444–447.
- Norman, C., Runswick, M., Pollock, R. and Treisman, R. 1988. Isolation and properties of cDNA clones encoding *SRF*, a transcription factor that binds to the c-fos serum response element. *Cell* 55: 989–1003.
- Ponticelli, A.S., Pardee, T.S. and Struhl, K. 1995. The glutamine-rich activation domains of human *SPI* do not stimulate transcription in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 15: 983–988.
- Reese, J.C., Apone, L., Walker, S.S., Griffin, L.A. and Green, M.R. 1994. Yeast TAFIIs in a multisubunit complex required for activated transcription. *Nature* 371: 523–527.
- Riechmann, J.L., Krizek, B.A. and Meyerowitz, E.M. 1996a. Dimerization specificity of *Arabidopsis* MADS domain homeotic proteins *APETALA1*, *APETALA3*, *PISTILLATA*, and *AGAMOUS*. *Proc. Natl. Acad. Sci. USA* 93: 4793–4798.
- Riechmann, J.L., Wang, M. and Meyerowitz, E.M. 1996b. DNA-binding properties of *Arabidopsis* MADS domain homeotic proteins *APETALA1*, *APETALA3*, *PISTILLATA*, and *AGAMOUS*. *Nucl. Acids Res.* 24: 3134–3141.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Savidge, B., Rounsley, S.D. and Yanofsky, M.F. 1995. Temporal relationship between the transcription of two *Arabidopsis* MADS box genes and the floral organ identity genes. *Plant Cell* 7: 721–733.
- Schindler, U., Terzaghi, W., Beckmann, H., Kadesch, T. and Cashmore, A.R. 1992. DNA binding site preferences and transcriptional activation properties of the *Arabidopsis* transcription factor *GBF1*. *EMBO J.* 11: 1275–1289.
- 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.
- Schwechheimer, C., Smith, C. and Bevan, M.W. 1998. The activities of acidic and glutamine transcriptional activation domains in

- plant cells: design of modular transcription factors for high-level expression. *Plant Mol. Biol.* 36: 195–204.
- Struhl, K. 1988. The *JUN* oncoprotein, a vertebrate transcription factor, activates transcription in yeast. *Nature* 332: 649–650.
- Webster, N., Jin, J.R., Green, S., Hollis, M. and Chambon, P. 1988. The yeast *UASG* is a transcriptional enhancer in human HeLa cells in the presence of the *GAL4 trans*-activator. *Cell* 52: 169–178.
- Weigel, D. and Nilsson, O. 1995. A developmental switch sufficient for flower initiation in diverse plants. *Nature* 377: 495–500.
- Weigel, D., Alvarez, J., Smyth, D.R., Yanofsky, M.F. and Meyerowitz, E.M. 1992. *LEAFY* controls floral meristem identity in *Arabidopsis*. *Cell* 69: 843–859.
- Yocum, R.R., Hanley, S., West Jr, R and Ptashne, M. 1984. Use of *lacZ* fusions to delimit regulatory elements of the inducible divergent *GALI-GAL10* promoter in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 4: 1985–1998.