The Plant Cell, Vol. 6, 175–186, February 1994

The *TM5* MADS Box Gene Mediates Organ Differentiation in the Three Inner Whorls of Tomato Flowers

Lilac Pnueli, Dana Hareven, Limor Broday, Charles Hurwitz, and Eliezer Lifschitz [1]

Department of Biology, Technion-Israel Institute of Technology, Haifa 32000, Israel

The tomato MADS box gene no. 5 (*TM5*) is shown here to be expressed in meristematic domains fated to form the three inner whorls—petals, stamens, and gynoecia—of the tomato flower. *TM5* is also expressed during organogenesis and in the respective mature organs of these three whorls. This is unlike the major organ identity genes of the MADS box family from Antirrhinum and Arabidopsis, which function in overlapping primordial territories consisting of only two floral whorls each. The developmental relevance of the unique expression pattern of this putative homeotic gene was examined in transgenic plants. In agreement with the expression patterns, antisense RNA of the *TM5* gene conferred both early and late alterations of morphogenetic markers. Early defects consist of additional whorls or of a wrong number of organs per whorl. Late, organ-specific changes include evergreen, cauline, and unabscised petals; green, dialytic, and sterile anthers; and sterile carpels and defective styles on which glandular trichomes characteristic of sepals and petals are ectopically formed. However, a complete homeotic transformation of either organ was not observed. The early and late floral phenotypes of *TM5* antisense plants suggest that *TM5* mediates two unrelated secondary regulatory systems. One system is the early function of the floral meristem identity genes, and the other system is the function of the major floral organ identity genes.

INTRODUCTION

A relatively limited number of homeotic programs has been shown to specify the identity of floral meristems and floral organs in Antirrhinum and Arabidopsis (Coen and Meyerowitz, 1991). Genetic analysis of double and triple mutant plants has permitted the formulation of models in which combinations of three genes, each expressed in two consecutive primordial domains, are sufficient to account for and predict all known homeotic transformations of floral organs (Carpenter and Coen, 1990; Schwarz-Sommer et al., 1990; Bowman et al., 1991). Predictably, such homeotic genes were shown, upon their successful isolation, to code for proteins containing potential DNA binding domains. Unexpectedly, however, the majority of genes involved in organ identity (*DEFICIENS*, *GLOBOSA*, and *PLENA* in Antirrhinum; *AGAMOUS*, *APETALA3*, and *PISTILLATA* in Arabidopsis) and even some of the genes regulating floral meristem identity (such as *SQUAMOSA* or *APETALA1*) code for related proteins, all containing the MADS box domain (Schwarz-Sommer et al., 1990).

MADS box proteins share a very conserved, usually N-terminal, 56–amino-acid-long DNA binding domain. However, in contrast to homeodomain-containing proteins of animals, they share size, sequence, and structural homology outside the binding domain as well (Ma et al., 1991; Pnueli et al., 1991). In plant species studied thus far, proteins of the MADS box family are encoded by a dozen or more unclustered loci, most of which are expressed solely or predominantly in flowers. Because of their overall sequence and structural similarities, their involvement in many regulatory programs, and their ability to interact with other regulatory proteins (see Gruenberg et al., 1992), MADS box genes provide an excellent opportunity to study molecular mechanisms underlying variations in flower development in higher plant species. We took advantage of this opportunity in tomato.

Tomato is a day-neutral plant with a sympodial growth pattern. The juvenile stage is characterized by a gradual change in leaf shape and size. The primary vegetative shoot typically forms eight internodes before the initiation of inflorescence development. The shoot then continues its growth when a vegetative bud in the axil of the leaf just below the inflorescence develops a three-internode shoot that is again terminated by an apical inflorescence. A reiterated pattern of similar events, under the control of the *self-pruning* (*SP*) gene then follows, forming the sympodial shoot. In tomato, the apical dome of the inflorescence meristem forms the first flower; additional floral meristems arise, in a genetically controlled pattern, from lateral growing points below the first flower (Atherton and Harris, 1986).

Analysis of flower development in tomato has been hindered by the lack of both well-defined floral homeotic mutations and an accessible gene-tagging system. We have therefore attained

[1] To whom correspondence should be addressed.

an entry point via isolation of flower-specific MADS box genes. So far, seven of an estimated two dozen MADS box genes have been characterized (Pnueli et al, 1991; L. Pnueli and E. Lifschitz, unpublished data). In situ hybridization experiments reported below verify that two of the tomato MADS box genes, *TM5* and *TM6*, are expressed in the primordial domains fated to form the three inner whorls of the flower. They are then expressed in characteristic patterns in the developing and mature petals, stamens, and gynoecia. *TM5* and *TM6* ranked second in a graded series in which the recently isolated *TM16* gene (L. Pnueli and E. Lifschitz, unpublished data) was expressed in the primordia and the mature organs of all four whorls and the legitimate tomato homolog of *AGAMOUS* (see the companion paper, Pnueli et al., 1994) was expressed as expected only in stamens and gynoecia. The *AGL1* gene of Arabidopsis is already known to be expressed only in the fourth whorl (M.F. Yanofsky, personal communication), and it is expected that one-whorl MADS box genes will soon be reported in other species as well. Indeed, the *TM5* gene represents a new class of flower-specific MADS box genes (Pnueli et al., 1991). Recently, a *TM5* homolog that is expressed in the mature organs of the three inner whorls of petunia was reported (Angenent et al., 1992). We have isolated a gene from tobacco with more than 95% homology to *TM5*, and the *AGL9* gene from Arabidopsis with a virtually identical MADS box and 85% overall homology to *TM5* is also expressed in petals, stamens, and gynoecium (M.F. Yanofsky, personal communication).

Because the tomato MADS box genes were isolated solely by virtue of their partial homology with the *DEFICIENS* gene from Antirrhinum (Pnueli et al., 1991) and they are not identified with any particular gene mutation, their role in flower development remains questionable. To circumvent this inherent difficulty and investigate the developmental relevance of the aforementioned pattern of organ specificity, we have analyzed the correlation between the expression patterns of the tomato MADS box genes and the phenotypic alterations conferred by the expression of the *TM5* antisense RNA. The results obtained suggest a role for *TM5* in determining the number of floral whorls and organs as well as determining certain morphogenetic features that give organs their identity.

RESULTS

TM5 and *TM6* Are Expressed in Primordial and Developing Stages of Petals, Stamens, and Gynoecia

TM5 and *TM6* belong to a family of homeotic genes that encodes for MADS box–containing transcription factors that specify the identity of organs in flowers of Antirrhinum and Arabidopsis. *TM5* is 224 amino acids long, and *TM6* is 225 amino acids long. However, the amino acid composition of *TM5* is particularly unusual due to the high proportion of leucine residues (16%) as well as an extremely hydrophobic C-terminal domain that includes 10% methionine residues (Pnueli et al., 1991). RNA gel blot analysis of total RNA from mature floral organs suggests that both genes are expressed in mature organs of the three inner whorls—the petals, stamens, and gynoecia—but not in mature sepals. Both *TM5* and *TM6* are designated as "late" genes because their transcripts are more abundant in mature flowers than in early floral meristems (Pnueli et al., 1991).

The detailed spatial and temporal expression of the *TM5* and *TM6* genes was monitored by in situ hybridization experiments, and the results are reported below. The resultant pattern was compared with the syndrome of morphogenetic changes conferred by the *TM5* antisense RNA in each whorl of the tomato flower. Corroborating data on *TM6* are presented to demonstrate that the expression of *TM5* in the three whorls is not exceptional and that despite their partial homology, each antisense RNA confers gene-specific morphogenetic syndromes.

We examined the earliest possible stage of localized *TM5* and *TM6* gene expression by probing sections of wild-type and *anantha* floral meristems at the preorganogenesis stage with labeled *TM5* or *TM6* antisense RNA. Meristematic organs of the *anantha* mutant inflorescences are arrested at the preorganogenesis stage (Helm, 1951) and exhibit patterns of gene expression characteristic of floral primordia (Shahar et al., 1992; Pri-Hadash et al., 1992). Localization of *TM5* RNA in the early preorganization stage of wild-type floral buds is shown in Figure 1A, and localization of *TM6* RNA in early wild-type floral buds and in *anantha* floral meristems is depicted in Figures 1B and 1C. In both *anantha* and wild-type primordia, apical regions were only lightly marked and signals were localized to only a few cells in the center of the meristems.

At the stage at which only sepal primordia have emerged and subsequently during early organogenesis, both genes were expressed in the apical meristematic regions of the wild-type primordia that are destined to form organs of the three inner whorls but were not expressed in sepals. Expression of *TM6* at this early stage is illustrated in Figure 1D. A detailed record of *TM5* expression at successive stages is shown in Figures 1E to 1H. At least four to five layers of apical cells were labeled, indicating that cells in all apical meristematic layers (LI, LII, and LIII) express *TM5* and *TM6*. Provascular cells, which at this stage already express the dUTPase gene (Pri-Hadash et al., 1992), were not yet labeled, but were extensively marked during organogenesis (Figures 2A to 2D).

From evidence depicted in Figure 1, we infer that expression of *TM5* and *TM6* in either wild-type or *anantha* floral primordia occurs at least as early as that of floral organ identity genes, i.e., *AGAMOUS* and *APETALA3* or *DEFICIENS* and *GLOBOSA* in stages 1 to 3 of flower development in Arabidopsis and Antirrhinum (Drews et al., 1991; Jack et al., 1992; Schwarz-Sommer et al., 1992; Tröbner et al., 1992). We infer further that the low level of *TM5* and *TM6* transcripts in *anantha* floral meristems (Pnueli et al., 1991) is not the consequence of inhibition of their synthesis by the *anantha* gene mutation but rather represents the normal course of temporal gene activity at the particular stage at which *anantha* flower development is arrested.

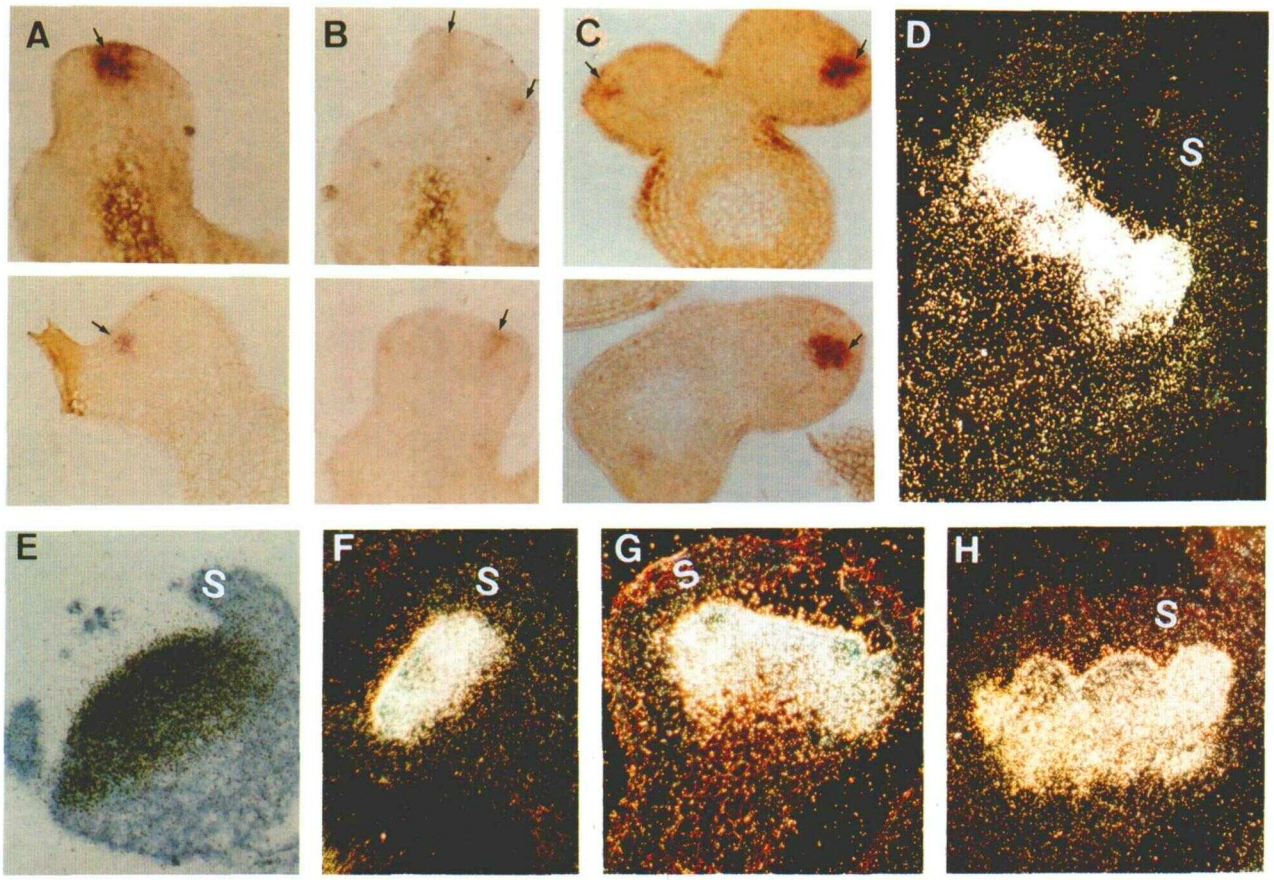

Figure 1. Spatial and Temporal Expression Patterns of the *TM5* and *TM6* Genes in Early Floral Primordia.

(A) to **(C)** Preorganogenesis stage. Digoxygenin-labeled probes; bright-field photography. Arrows point to the sites of signals.
(A) Two sections through wild-type floral meristems probed with digoxygenin-labeled *TM5* antisense RNA are shown.
(B) Localization of *TM6* transcripts in a section of wild-type floral meristems is shown.
(C) Expression of *TM6* in *anantha* floral meristems is restricted to a subset of apical cells.
(D) Localization of *TM6* transcripts in floral primordium following the appearance of sepal(s) is confined to meristematic cells fated to form the three inner whorls. Dark-field photography.
(E) to **(H)** Expression of *TM5* during sequential early organogenesis stages. Longitudinal sections; ^{35}S-labeled RNA probes.
(E) and **(F)** Localization of *TM5* transcripts in longitudinal sections of floral buds during the appearance of sepal primordia. Bright-field photography in **(E)** and dark-field photography in **(F)**.
(G) and **(H)** In advanced stages, where primordial buttresses of petals, stamens, and gynoecium emerge, all meristematic layers of the future three inner whorls are marked. Dark-field photography.

As shown in Figures 2A to 2C, the *TM5* and *TM6* genes were then expressed with no apparent temporal interruption during the formation of petals, stamens, and gynoecia. In the early specified but not fully differentiated organs, *TM5* was expressed in growing regions and, for the first time, in the developing provascular system (Figure 2A). When all floral organs acquired their final identity, *TM5* was expressed in parenchyma cells and derived differentiating tissues of all three inner organs (Figure 2B). *TM5* transcripts were specifically abundant in the vascular bundles of anthers and the receptacle. At the same stage, *TM6* was also expressed in all three whorls, but the spatial distribution of its transcripts differed in some details (compare Figure 2B with Figure 2C).

Most informative was the wave of *TM5* activity detected during the final stages of anther development. Initially, cells of the archesporium, which give rise to the endothecium on the outside and the sporogenous cells on the inside, were preferentially labeled (Figure 2D). Later, the primary sporogenous cells, which now occupy a more inward position, were heavily marked (Figure 2E). However, no hybridization signal was found over pollen cells. In the mature carpel, labeling of the ovules was prominent (Figure 2F), whereas in the style, *TM5* was

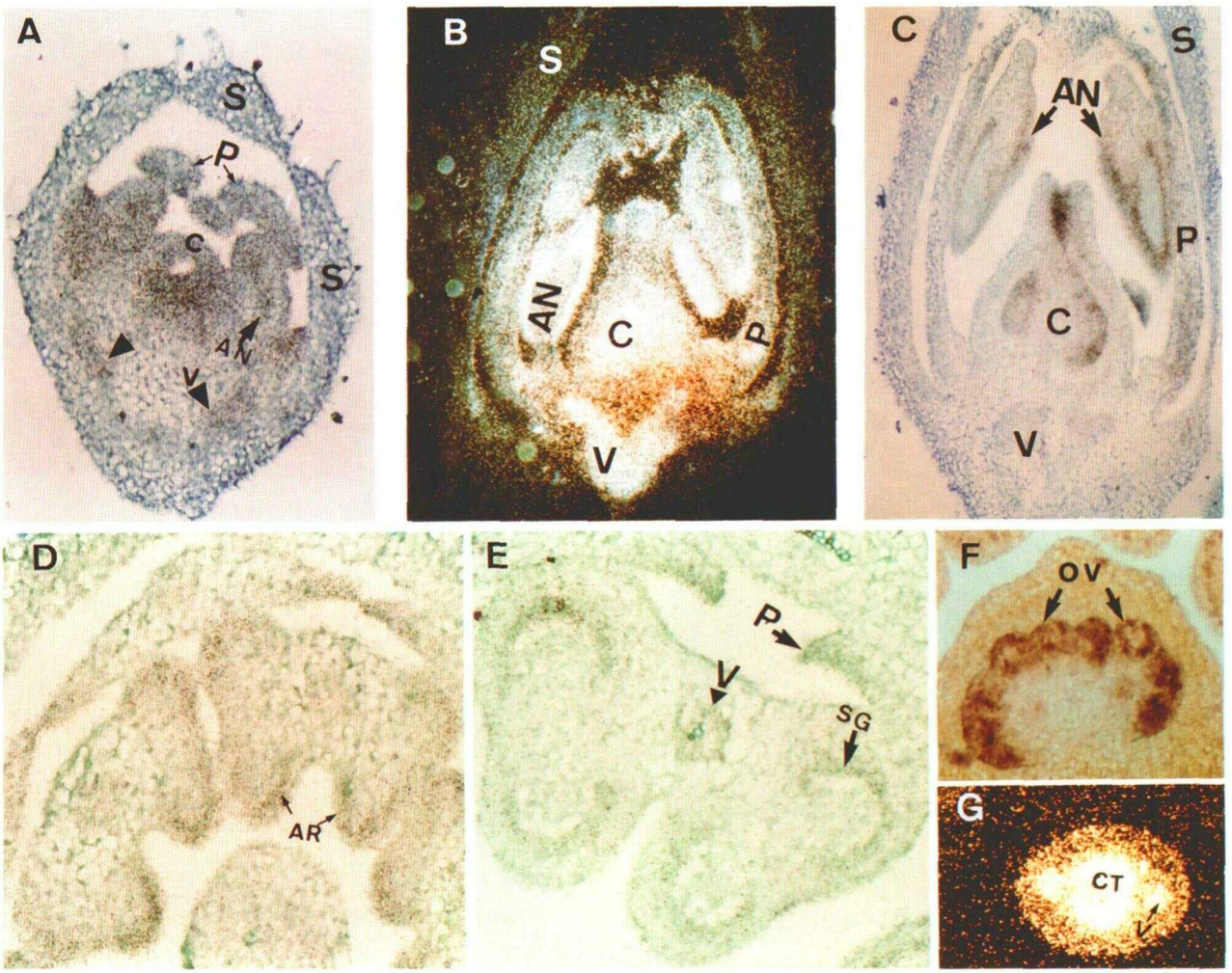

Figure 2. Expression Patterns of *TM5* and *TM6* during Development Detected by in Situ Hybridization.

(A) Localization of *TM5* RNA in a floral bud (0.8 mm long) following the emergence of all floral primordia. Primordia of petals (P), stamens (AN), and gynoecium (C) are marked. Note that cells of the provascular (V) strands (indicated by arrowheads) also express *TM5*. Sagittal section; bright-field photography.

(B) In a 1.3-mm-long floral bud, *TM5* is expressed in organs of all three inner whorls. Vascular bundles (V) are preferentially labeled. Longitudinal section, dark-field photography.

(C) *TM6* RNA is expressed in organs of the same three whorls as *TM5*, but transcripts are more localized and no overexpression is observed in the vascular system. In particular, the sites in the ovary leaves that will develop the transmitting tissue, the cells of the future placenta, and the archesporium of the anthers are marked. Same flower as in **(B)**; longitudinal section; bright-field photography.

(D) and **(E)** Hybridization of *TM5* antisense RNA with sections showing two successive stages of anther development reveals a wave of gene expression from early archesporial cells (AR) in **(D)** to sporogenous cells (SG) in **(E)**. Bright-field photography.

(F) and **(G)** In the carpel of mature gynoecium, the *TM5* RNA is expressed at high levels in the ovules **(F)**, while in the style **(G)** most transcripts are detected in the conducting tissue (CT) and the vascular bundles (V).

AN, anther; AR, archesporium; C, carpel; OV, ovules; P, petals; S, sepals; SG, sporogenous cells; V, vascular system.

expressed preferentially but not exclusively in the transmitting tissue and vascular bundles (Figure 2G). It is interesting to note that expression patterns of *TM5* and *TM6* in developing floral organs are not readily distinguishable from those of *AGAMOUS* in the mature stamens and gynoecia of Arabidopsis (Drews et al., 1991) or from those of *DEFICIENS* in petals and stamens of Antirrhinum (Schwarz-Sommer et al., 1992).

Antisense RNA of the *TM5* Gene Confers Morphogenetic Changes in the Three Inner Whorls of Transgenic Plants

The functional role of an isolated gene can be studied in transgenic plants by exploring the consequences of either ectopic expression and overexpression or repression of gene function. DNA binding domains of regulatory proteins per se are not completely specific to a given target sequence. In addition, partial sequence homology in critical interacting domains may lead to erroneous molecular interactions upon overexpression or ectopic expression of such proteins; therefore, the phenotypic consequences can be easily misinterpreted. This is particularly true when, unlike the case of the *AGAMOUS* gene (Mandel et al., 1992; Mizukami and Ma, 1992; Pnueli et al., 1994), no prior genetic evidence is available. Therefore, to analyze the developmental relevance of the MADS box genes in tomato, we resorted to gene repression by antisense RNA, which is the most reliable tool for specific modification of gene expression (Cannon et al., 1990; Mol et al., 1990; Takayama and Inouye, 1990). The *TM5* full-length cDNA clone was placed in inverted orientation under the transcriptional control of the cauliflower mosaic virus 35S (Odell et al., 1985) gene, and 30 T_1 transgenic plants were generated and analyzed.

Using the cauliflower mosaic virus 35S promoter is most advantageous in such a study because it is expressed at a high level in all tissues and cells. Therefore, in cells or tissues in which the MADS box gene is normally expressed (see Figures 1 and 2), the level of the antisense RNA should surpass that of the internal sense RNA. In tissues in which the relevant gene is not expressed, it is assumed that the presence of antisense RNA will be of no consequence.

Flowers on nine of 30 T_1 35S: antisense (*AS*)-*TM5* kanamycin-resistant plants displayed a progressive series of similar alterations of organ-specific morphogenetic markers. Twenty-one transgenic plants were phenotypically normal. The T_1 plants exhibiting the most extreme syndrome were represented by transgenic plants *TM5/AS6* and *TM5/AS111*. In wild-type flowers, the sepals initially formed a closed elongated calyx. Sepals then separated to expose pale yellow petals that became deep yellow at full opening, as shown in Figure 3A. Anthers also turned deep yellow but remained tightly joined to form an anther cone in which the gynoecium was enclosed. Note, however, that at early developmental stages, all organs were nearly as green as the sepals. In *TM5/AS6* flowers, the sepals appeared normal, but upon their separation, green rather than pale yellow petals were exposed. Petals remained green at their separation and even upon full opening; although they retained the appearance of petals, their upper third tended to become abruptly slender when compared with the gradual narrowing of wild-type flowers (Figure 3B). Unlike regular petals, the transformed petals acquired a cauline texture and never abscised or senesced even if, as frequently happens, parthenocarpic fruits were formed.

Stamens (and anthers) also remained green and, most important, did not form the typical closed anther cone but rather separated and acquired outward positions exactly like petals or sepals. Gross anatomy of the anthers was not changed, however. The anthers of tomato are bound together by lateral rows of epidermal hairs (see Chandra-Sekhar and Sawhney, 1984), and these hairs are only slightly shorter in *TM5/AS6* anthers. Diverged normal anthers are also conferred by mutations in the *dialytic* (Rick, 1947) and *hairless* (Rick and Butler, 1956) genes, in which in both cases hairs are missing throughout the plant. However, *TM5* is not related to the *dialytic* or *hairless* genes because all types of hairs in sepals, petals, leaves, or stems of *TM5/AS6* plants are normal; furthermore, *hairless* and *dialytic* are not linked to chromosome 5 in which *TM5* is localized (Pnueli et al., 1991).

Infrequently, more complex flowers evolved, suggesting an early function of *TM5* in the organization of the floral meristem. Two typical examples are shown in Figures 3C and 3D. A flower within which a second flower with an elongated pedicel replaced the whorl of stamens (reminiscent perhaps of *apetala1* of Arabidopsis) is shown in Figure 3C. A flower consisting of two or three whorls of green cauline petals, extra whorls of green stamens, and duplicated styleless ovaries is shown in Figure 3D. Flowers with the wrong number of organs per whorl were also found, but it is important to note that the organ-specific phenotypic defects observed in all of these evolved flowers are similar to those displayed by *TM5/AS6* flowers bearing a regular number of whorls.

Defects of the gynoecium were variable, but in most flowers the carpel leaves were short and were not properly fused. Most consistent and informative is the change of fate that was exhibited by epidermal cells along the styles of *TM5/AS6* plants. Normally, only elongated, multicellular types of trichomes are found on the lower one-third of the style and only occasionally on the shoulders of the ovary. As shown in Figures 4A and 4B, styles of the *TM5/AS6* plant were distinguished first by extra hairiness of the upper part of the style and the upper shoulders of the ovary and, in addition, by the ectopic formation of the short type of glandular hairs that are topped by four secretory cells (asterisks in Figures 4B and 4C). Such glandular hairs are never found on wild-type styles but only on petals, sepals, and leaves (Chandra-Sekhar and Sawhney, 1984; V. K. Sawhney, personal communication). No glandular hairs were found on the styles of the progenitor plant, on the styles of the phenotypically normal 21 T_1 transgenic *TM5/AS-X* plants, on plants transgenic for other genes, or on styles of normal segregants from T_1 plants in which one-quarter of the siblings manifested the *TM5* syndrome.

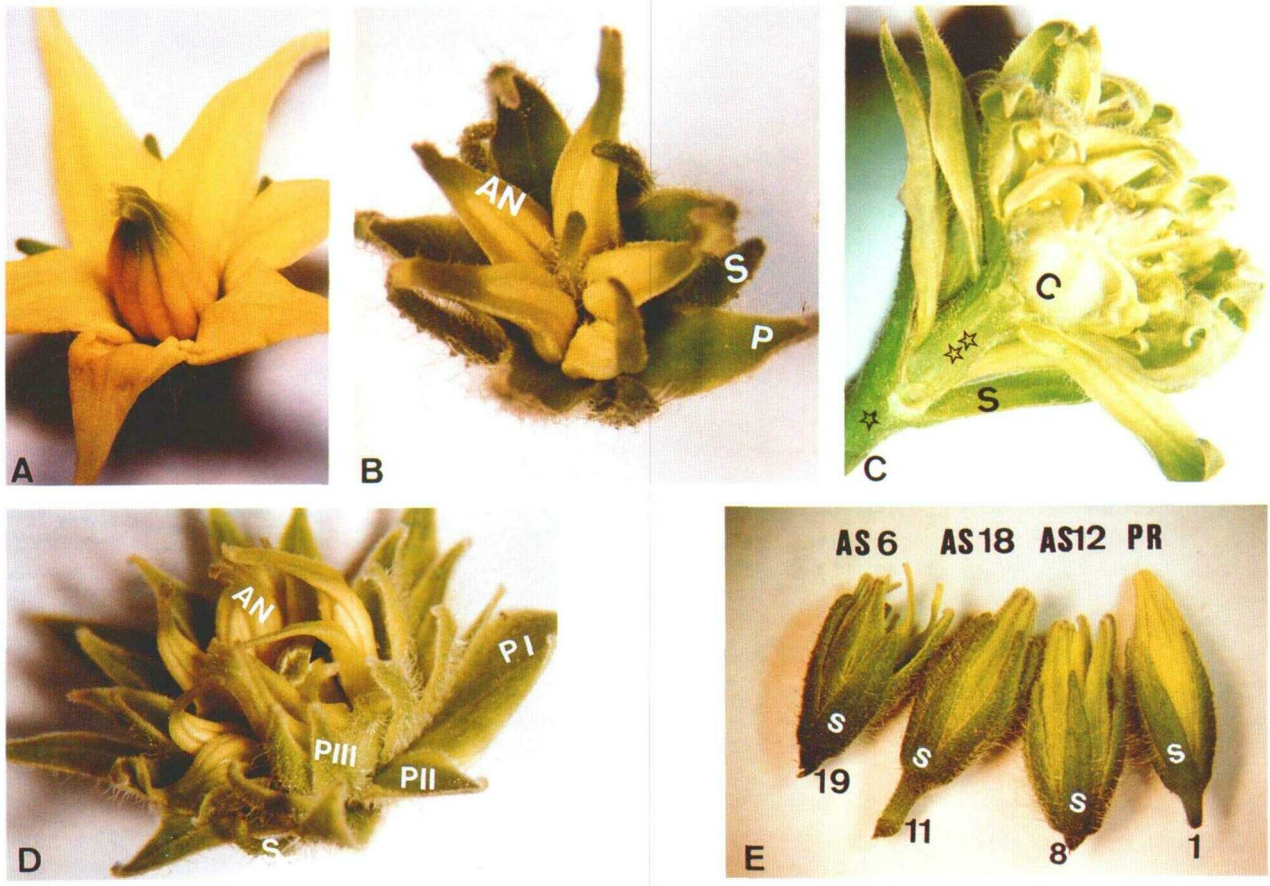

Figure 3. Phenotypes of Aberrant Flowers from Transgenic Plants Carrying *TM5* Antisense Gene.

(A) A wild-type tomato flower at anthesis.
(B) A typical *TM5/AS6* flower at anthesis.
(C) and **(D)** Complex *TM5/AS6* flowers. Note the indeterminate nature of the floral meristem in **(C)** and the multiplicity of inner whorls in **(D)**. In **(C)**, one and two stars indicate primary and secondary floral pedicels. In **(D)**, PI, PII, and PIII mark three successive whorls of petals.
(E) Left to right, flowers of transgenic *TM5/AS6, AS18, AS12,* and of the progenitor plant 2 days before anthesis. *AS12* actually represents a T_2 segregant of the primary transgenic plant *TM5/AS12* (see text). Numbers below flowers indicate the level of chlorophyll relative to wild type (PR) in petals at this particular stage.
AN, anthers; C, carpel; P, petals; S, sepals.

In addition to alterations of external organ-specific markers (color, trichomes, and organ fusion), internal anatomical defects were also recorded. Flowers of *TM5/AS6* plants developed carpels with incomplete styles, in which filamentous and other unidentified structures replaced normal ovaries (Figure 4D), teratological (partially petaloid?) anthers with missing or extra pollen sacs (Figures 4E to 4G), abnormal carpels (Figure 4H), and partially duplicated or completely deformed styles, sometimes devoid of transmitting tissues (Figure 4I).

Collectively, defects in androecium and gynoecium rendered the affected plants both male sterile and female sterile. Because all efforts at pollination failed, *TM5/AS6* plants were maintained and propagated by vegetative cuttings. After four cycles of vegetative proliferation, no complete or partial phenotypic reversions were observed in flowers of *TM5/AS6* plants.

The phenotype of the *TM5/AS6* plant was by no means unique. Eight other T_1 transgenic plants mimicked an allelic series; all displayed the same range of phenotypic alterations, differing only in the severity of defects. Petals and stamens were progressively greener (Figure 3E), stamen orientation ranged from very loose cones to open outward arrangements, and the number of glandular hairs on the style increased with the severity of the syndrome. In agreement with the intensity of their green appearance, the transgenic petals retained both high levels of chlorophyll (see numbers beneath the flowers in Figure 3E) and high levels of the large and small subunits

of Rubisco, as detected by the appropriate antibodies in protein blots (data not shown).

The nine T_1 transgenic plants with recognizable defects were classified according to the intensity of the green of their petals. One plant, *TM5/AS111*, was identical to *TM5/AS6*, and another plant, *TM5/AS18*, was distinguished from *TM5/AS6* only by virtue of its light green petals. Four T_1 plants exhibited a medium coloration and the anthers disjoined only 3 to 4

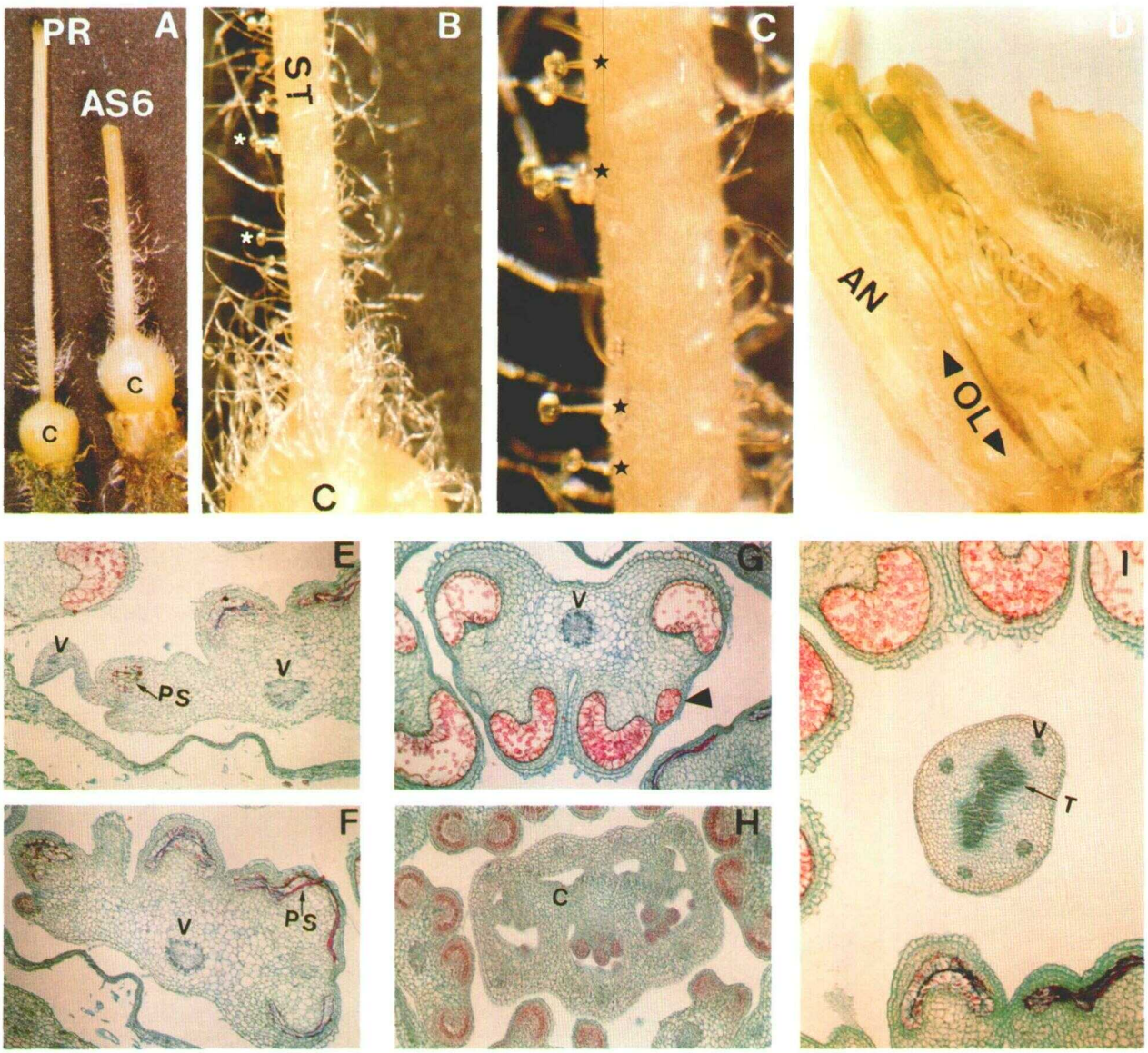

Figure 4. Organ-Specific Alterations in Flowers of *TM5/AS6* Transgenic Plants.

(A) Unlike styles and ovaries of the progenitor plant (PR), those of *TM5/AS6* and other affected T_1 plants exhibit extreme hairiness.
(B) and **(C)** Among the regular trichomes, epidermal cells of *TM5/AS6* styles also form glandular trichomes (marked by white asterisks near their top in **(B)** and by black asterisks at their bottom in **(C)**), which are not found normally on styles of wild-type flowers.
(D) Internal stigmatic filaments and ovulated outgrowths in unfused carpels of *TM5/AS6* flowers of the type shown in Figures 3C and 3D.
(E) to **(G)** Cross-sections through deformed anthers of *TM5/AS6* flowers.
(E) and **(F)** Two serial sections of the same anther.
(G) Arrow points to accessory pollen sac.
(H) and **(I)** Abnormal ovary **(H)** and style **(I)** of affected transgenic plants.
C, carpel; OL, ovary leaves; PS, degenerative pollen sac; ST, style; T, transmitting tissue; V, vascular system.

days after opening (*TM5/AS39*, *TM5/AS55*, *TM5/AS100*, and *TM5/AS110*). In two transgenic plants (*TM5/AS103* and *TM5/AS107*), the effect was modest. However, neither of these plants was male fertile or female fertile. In plants exhibiting the modest phenotypes, fewer than 5% of the flowers had multiple whorls or organs.

Two of the 21 seemingly normal and partially fertile T_1 plants, *TM5/AS44* and *TM5/AS108*, segregated at the expected Mendelian proportion T_2 progeny that displayed the typical medium class *TM5* antisense syndrome (pale green petals and stamens, dialytic anthers, and both female sterility and male sterility). One plant, *TM5/AS12*, segregated one-quarter sterile progeny with yellow–green petals (weak class). Analysis of genomic DNA indicated that only one insert was incorporated into the genome of each of the nine T_1 affected plants examined. The succession of phenotypes exhibited by nine T_1 plants and the Mendelian segregation of defective T_2 plants provided the most convincing evidence that the *TM5* antisense syndrome is due solely to the inhibition of *TM5* gene function by the antisense gene construct.

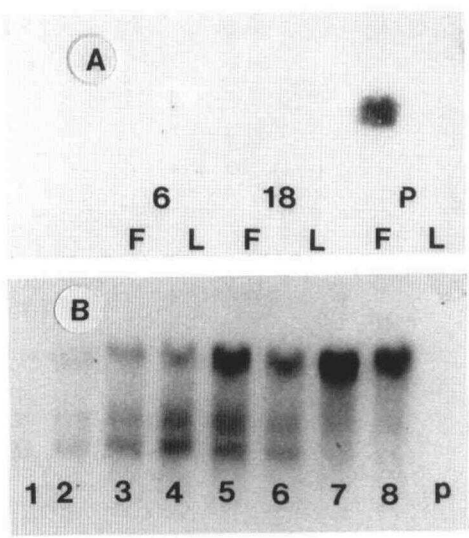

Figure 5. RNA Gel Blot Analysis of *TM5* Sense and Antisense Transcripts in Transgenic Plants.

(A) *TM5* sense transcripts are detected in flowers (F) of the progenitor plant (P) but not in flowers or leaves (L) of the transgenic plants *TM5/AS6* (6) and *TM5/AS18* (18).

(B) Expression of *TM5* antisense RNA in flowers of seven independent transgenic plants, all of which are part of the allelic series described in the text. Lane 1, *TM6* antisense flowers; lane 2, *TM5/AS12*; lane 3, *TM5*/AS110; lane 4, *TM5/AS103*; lane 5, *TM5/AS18*; lane 6, *TM5/AS6*; lane 7, *TM5/AS100*; lane 8, *TM5/AS55*; P, Progenitor.

In all cases flowers are shown 3 days before anthesis, and 5 μg total RNA was loaded per lane.

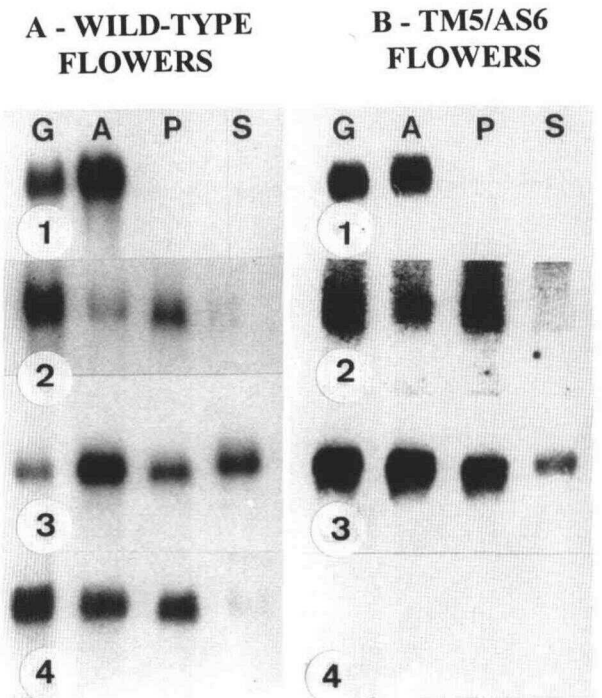

Figure 6. RNA Gel Blot Analysis of Three MADS Box Genes in Floral Organs of Transgene *TM5/AS6*.

(A) and **(B)** Comparison of expression of four MADS box genes in the four floral organs of progenitor **(A)** and *TM5/AS6* **(B)** plants reveals that *TM5* sense RNA was abolished in all three organs of the transgenic plants (B4), in which it is otherwise normally expressed (A4). Transcripts of the tomato MADS box genes *TAG1* (Pnueli et al., 1994), *TM6* (Pnueli et al., 1991), and *TM16* (L. Pnueli and E. Lifschitz, unpublished data) were not affected by the presence of *TM5* antisense RNA. G, gynoecium; A, androecium; P, petals; S, sepals; A1 and B1, *TAG1* probe; A2 and B2, *TM6* probe; A3 and B3, *TM16* probe; A4 and B4, *TM5* probe.

TM5 Antisense RNA Abolishes *TM5* Sense RNA, But Levels of *TM6*, *TM16*, and *AGAMOUS* Transcripts Remain Unaffected

The levels of *TM5* sense RNA transcripts in leaves and flowers of the progenitor plant and of transgenes *TM5/AS6* and *TM5/AS18* are shown in Figure 5A. No *TM5* sense RNA was detected in flowers of these transgenic plants or in flowers of other tested transformants (110, 111, 100, 101, and 103; data not shown). The expression of *TM5* antisense RNA in several T_1 plants is recorded in Figure 5B, in which only weak correlation was found between the level of antisense RNA and the severity of phenotypic alterations.

We also examined the effect of *TM5* antisense RNA on the expression of other MADS box genes in each of the four whorls of mature *TM5/AS6* flowers. The results are shown in Figure 6, in which A1-4 designate progenitor RNA and B1-4 designate

TM5/AS6 RNA. The tomato *AGAMOUS* homolog (Figures 6A1 and 6B1) was still expressed in whorls 3 and 4 of the transgenic plants, *TM6* (Figures 6A2 and 6B2) was expressed in its regular second, third, and fourth whorls, and *TM16* (Figures 6A3 and 6B3) was expressed in all four whorls as in wild-type flowers. The *TM5* transcripts were completely abolished in all floral organs of the *TM5/AS6* flowers (Figures 6A4 and 6B4). Therefore, antisense *TM5 RNA* does not interfere with the expression of three other MADS box genes, *TM5* sense RNA is not required for the expression of the three other genes in mature organs, and the expression of these genes is insufficient to overcome the disturbances inflicted by the disappearance of *TM5* sense transcripts.

DISCUSSION

In many systems, the developmental role of genes coding for regulatory proteins depends primarily on several post-transcriptional and post-translational regulatory mechanisms (e.g., Lawrence, 1991). The multiplicity of MADS box genes, the high levels of homology throughout all of their encoded protein domains, and their ability to interact with other regulatory proteins (Gruenberg et al., 1992) dictate further caution in the assignment of function solely on the basis of gene expression patterns or sequence similarities. This is particularly the case when genetically anonymous members of the gene family are studied, as in the tomato (Pnueli et al., 1991) or petunia (Angenent et al., 1992). Indeed, in petunia, although it has been claimed that a MADS box gene, *FBP1*, is expressed in two whorls and its protein product is found in only one of them (Angenent et al., 1992), this claim has been disputed by further functional studies (Angenent et al., 1993). Conceivably, the assignment of a developmental role to a given MADS box gene should be inferred from functional tests only, and such criteria should also be applied to the assignments of functional homologies between genes from different plant families.

Morphogenetic Alterations Conferred by *TM5* Antisense RNA Are Correlated with the Temporal and Spatial Expression Patterns of the Wild-Type Gene

We present evidence that early and late morphogenetic changes, both homeotic and disruptive, occur in agreement with the observed temporal and spatial expression patterns of the *TM5* gene. In situ hybridization experiments showed that *TM5* (and *TM6*) is expressed in the central apical zone of floral meristems at the preorganogenesis and early organogenesis stages. In accordance with this finding, antisense RNA of the *TM5* gene confers early alterations that are reflected in changes in the number of whorls, number of organs per whorls, or even in the determinacy of the floral meristem.

TM5 transcripts are subsequently found at high levels in meristematic territories fated to form petals, stamens, and gynoecia. The gene is then expressed continuously throughout differentiation in a characteristic spatial pattern in tissues of petals, stamens, and gynoecia. In agreement with this expression pattern, antisense RNA of the *TM5* gene confers morphogenetic alterations in all three whorls.

There is no obvious effect of *TM5* antisense expression in vegetative parts of the plants, or in the sepals, in which *TM5* is not normally expressed. In nine independent T_1 plants transformed with the antisense construct of the *TM5* gene and in progeny of an additional three T_1 plants, the second whorl consists of petals that are green throughout the life span of the flowers. Their cauline texture, failure to abscise, and delayed senescence are also characteristic of sepals. Nevertheless, the parameters of shape and size, which are dictated by the rate and direction of cell divisions, remain similar to those of petals. In the third whorl, the anthers diverge like sepals or petals and remain green like sepals; although sporogenic tissues and pollen sacs are deformed, the overall anatomy of the stamens is maintained. Prominent among the variable defects of the gynoecia is the ectopic formation of glandular hairs, which are normally found only on sepals and petals. It is certainly an earmark for a homeotic change of fate of the epidermal cells of the pistils. Incomplete fusion of carpels and the failure to form normal styles are also observed. Although several morphogenetic markers are altered in each of the three organs, the combined defects are not sufficient to declare a change in organ identity. Because even floral organs of the severely modified flowers (Figures 3C and 3D) exhibit the typical range of morphogenetic alterations and *TM5* sense RNA is completely abolished in affected transgenic plants, the severity of the same phenotypes, but no other contradictory homeotic changes, seems likely to represent complete inactivation of *TM5*.

The syndrome conferred by the antisense RNA of the *TM5* gene seems to be gene specific because an identical range of phenotypic alterations is observed in 12 independent transgenic plants. No overlapping phenotypes were obtained in plants transformed by the *TM6* antisense gene (data not shown), which is expressed in the very same three whorls and in fact confers carpelloid stamens rather than green dialytic ones, or by two other tomato MADS box genes, such as *TM4* and *TM8*, in a similar number of transformants (L. Pnueli and E. Lifschitz, unpublished results). Similarly, the tomato *TM5* antisense RNA failed to elicit any phenotypic alterations in 18 transformants in tobacco in which a *TM5* homolog with 95% overall amino acid sequence identity exists (E. Lifschitz, unpublished results). The fidelity of antisense specificity among members of the MADS box gene family is most dramatically illustrated by the gene-specific phenotypes displayed in plants bearing the antisense orientations of the entire tomato AGAMOUS gene (Pnueli et al., 1994).

The *TM5* antisense syndrome is clearly distinguished from that of the *green petal* mutation of petunia in which second whorl organs are sepaloid but third and fourth whorl organs are not affected (Angenent et al., 1992). More important, the *TM5* syndrome is also distinguished from homeotic alterations

caused by group B genes (i.e., *DEFICIENS*, *GLOBOSA*, *PISTILLATA*, and *APETALA3*), which confer carpelloid identity on the third whorl (Coen and Meyerowitz, 1991) concomitantly with sepaloid identity on the second whorl.

It is unlikely that the failure of the transgenic plants to form carpelloid stamens along with sepaloid petals is due to the loss or extreme modification in Solanacea of the basic features of the combinatorial floral organ identity gene system. The tomato *AGAMOUS* homolog, which provides the interactive group C function, is expressed in tomato with the "correct" two-whorl pattern, and its sense RNA induces the phenotypes expected from its known function in Arabidopsis and Antirrhinum (see the accompanying paper, Pnueli et al., 1994). It also functions in transgenic plants of the closely related tobacco species in the manner that is expected from Arabidopsis (Mandel et al., 1992). Another possible scenario is that variations in the efficiency of the inhibition mechanism, coupled with altered temporal and spatial expression of the antisense orientation *TM5* gene by the spliced 35S promoter, may lead to erratic or mosaic inhibition. Carpelloidy of stamens would not materialize according to such a view because the developmental control in tomato is relatively robust and is not altered by minor or mosaic changes in gene expression caused by the *TM5* antisense RNA. However, carpelloid stamens are characteristic of flowers of the *TM6* antisense transgenes (data not shown), and such a homeotic transformation is not associated with greening of petals or stamens or with dialytic arrangement of stamens. *TM6* was shown here to be expressed in practically the same developmental domains as *TM5*.

The mode of expression, the pattern of defects incurred by the antisense RNA, and the unchanged expression pattern of the tomato *AGAMOUS* gene in the transgenic plants suggest that *TM5* is not a functional homolog of group B or C genes or involved directly in the transcriptional regulation of these genes. Thus, we suggest that it is involved in other regulatory functions.

Hypothetical Developmental Hierarchies Uncovered by *TM5*

MADS box genes display a graded stepwise expression pattern. *TM16* is expressed in four whorls, *TM5* and *TM6* in three whorls, *AGAMOUS* and others in two whorls, and *AGL1* of Arabidopsis (M.F. Yanofsky, personal communication) in only one whorl. One wonders whether this expression regime reflects a hidden rationale of the floral program. Unless the expression of MADS box genes like *TM5*, *TM6*, and *TM16* is regulated by a particular combination of organ identity genes in one whorl and by another blend in the next, the existence of signals from yet another regulatory system (see below) must be involved.

Irrespective of how *TM5* gene activity is modulated, the following three speculative schemes are considered to interpret the role of its protein product:

(1) The TM5 protein is recruited at several different stages by different transcription complexes to regulate gene expression during flower development.

(2) In the second, more regimented scheme, the TM5 protein participates at the early preorganogenesis stage in a program that establishes the correct size of the floral meristem and determines the number of whorls. Such a program operates downstream of and is probably induced by the floral meristem identity genes (such as tomato homologs of *LEAFY* or *FLORICAULA*, and of *APETALA1* and *SQUAMOSA* in Arabidopsis and Antirrhinum, respectively). Later on and following the activation of the floral organ identity system, the TM5 protein is available in the primordia of the three inner whorls for participation in transcription complexes assembled by the "combinatorial program" to regulate just a fraction of the genes that provide flower organs with their final identity. Its malfunction is reflected, therefore, by disturbances in two distinguishable developmental systems and at two different stages. The implication is that the major organ identity genes organize primarily a downstream regulatory system in which the product of TM5 is but one of the players. The final cytodifferentiation genes are activated by this mediator system and not directly by the organ identity genes. It is possible that the products of the major organ identity genes like *AGAMOUS* or *DEFICIENS* also participate independently of their primary role in this secondary mediatory system.

(3) The third scheme takes into account that although the above "piecemeal" interpretation of the morphogenetic alterations in each of the three organs is favored, it may prove to be inadequate. It is possible to argue that the tendency toward sepaloidy, as reflected by the numerous modifications inflicted by inactivation of *TM5* in all organs, is the most important phenomenon; consequently, the primary role of the wild-type *TM5* gene could be to repress "sepaloid" genes in the three inner whorls. Suppose that the syndrome displayed by the *TM5/AS6* plant does not represent the extreme consequences of null activity. Given the tendency for heterodimerization among MADS box genes (Tröbner et al., 1992), a possible scenario is that a homodimer of yet another MADS box gene partially salvages the sepaloid functions of a heterodimer in which *TM5* is one of the partners. If, indeed, a complete abolishment of the *TM5* function will result in a proven sepaloidy of all three inner whorls, we will have to accommodate, within current models, an additional major regulatory program that mediates the shift from sepaloidy to three distinguished inner whorls.

METHODS

Plant Material

Tomato material for transformation was derived from the K9 line. K9 was bred for superior regeneration rates and derived from the F_3

population of a *Lycopersicon peruvianum* × *L. esculentum* hybrid (see Koornneef et al., 1986). We have backcrossed this line as a maternal parent to the cultivar Tiny Tim (short internodes, determinate growth habit, cherry fruits; LA154, kindly provided by C.M. Rick, University of California, Davis), and progeny of the second backcross were tested for their regeneration ability. The chosen segregant, designated TK9/8, is constantly propagated by cuttings in sterile cultures and is the sole source of material for transformation. It is homozygous for *self-pruning(sp)* and *dwarf (d)* (Rick and Butler, 1956) and bears cherry fruits.

Transformation

The leaf disc procedure according to the methods of Horsch et al. (1985) and Koornneef et al. (1986) was applied to both leaves and internodes. Explants were incubated 1 day before and 2 days after infection on petunia feeder cells and subsequently transferred to 2Z medium (Koornneef et al., 1986). Explants were then transferred to the same medium except that zeatin was reduced to 0.2 mg/L for shoot formation. Shoots were rooted on MSO medium (McCormick, 1991) supplemented with 0.02 mg/L indole-3-butyric acid. All media contained 200 mg/L carbenicillin and 100 mg/L kanamycin. T_2 segregants were tested for kanamycin resistance by growing shoot explants on rooting medium supplemented with kanamycin (200 mg/mL). The *TM5* and *TM6* full-length cDNA clones (EcoRI inserts, Pnueli et al., 1991) were fused in opposite orientation to the 35S promoter in the pBI121 vector (Clontech), and this plasmid was mobilized to Agrobacterium LBA4404 using triparental mating (Ditta et al., 1980).

Nucleic Acids and in Situ Hybridization Procedures

RNA was extracted according to published protocols (Samach et al., 1991). Other procedures were performed as described by Maniatis et al. (1982) or Ausubel (1988). In situ hybridization with ^{35}S-labeled sense or antisense RNA was conducted according to the method of Cox and Goldberg (1988). Tissues were fixed in FAA (3.7% formaldehyde: 50% ethanol and 5% acetic acid) embedded in paraplast plus. Sections (8-μm thick) were placed on slides coated with vectabond (Vector Laboratories) dewaxed with xylene, dehydrated through ethanol series, and hybridized overnight with 8×10^5 cpm of fragmented probe. Exposure time was 12 days. Digoxygenin-labeled RNA probes were prepared according to the manufacturer's recommendation (Boehringer Mannheim) and staining done was according to the protocol provided by E. Coen (John Innes Institute, Norwich, U.K.).

ACKNOWLEDGMENTS

We thank Dr. Tamar Gutfinger for her help with the in situ hybridization experiments and Dr. Ry Meeks-Wagner for helpful comments on the manuscript. This work was supported by grants from the U.S.–Israel Binational Science Foundation (No. 91-00138), the German–Israeli Biotechnology Projects (No. BMFT/MOSTGR1170), and the U.S.–Israel Binational-Agricultural Research and Development Fund (BARD).

Received September 30, 1993; accepted December 2, 1993.

REFERENCES

Angenent, G.C., Busscher, M., Franken, J., Mol, J.N.M., and van Tunen, A.J. (1992). Differential expression of two MADS box genes in wild-type and mutant petunia flowers. Plant Cell **4,** 983–993.

Angenent, G.C., Franken, J., Busscher, M., Colombo, L., and Tunen, A.J. (1993). Petal and stamen formation in petunia is regulated by the homeotic gene *fbp1.* Plant J. **4,** 101–112.

Atherton, J.G., and Harris, G.P. (1986). Flowering. In The Tomato Crop, J.G. Atherton and J. Rudich, eds (New York: Chapman and Hall), pp. 167–200.

Ausubel, F.M., Brent, R., Kingston, R.E., Moor, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., eds (1988). Current Protocols in Molecular Biology. (New York: Wiley).

Bowman, J.L., Smyth, D.R., and Meyerowitz, E.M. (1991). Genetic interactions among floral homeotic genes of *Arabidopsis.* Development **112,** 1–20.

Cannon, M., Platz, J., O'Leary, M., Sookdeo, C., and Cannon, K. (1990). Organ-specific modulation of gene expression in transgenic plants using antisense RNA. Plant Mol. Biol. **15,** 39–47.

Carpenter, R., and Coen, E.S. (1990). Floral homeotic mutations produced by transposon-mutagenesis in *Antirrhinum majus.* Genes Dev. 4, 1483–1493.

Chandra-Sekhar, V.N., and Sawhney, V.K. (1984). A scanning electron microscope study of the development and surface features of floral organs of tomato (*Lycopersicon esculentum*). Can. J. Bot. **62,** 2403–2413.

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

Cox, K.H., and Goldberg, R.B. (1988). Analysis of plant gene expression. In Plant Molecular Biology: A Practical Approach, C.H. Shaw, ed (Oxford: IRL Press), pp. 1–35.

Ditta, G., Stanfield, S., Corbin, D., and Helinski, D.R. (1980). Broad host range DNA cloning system for gram negative bacteria: Construction of a gene bank of *Rhizobium meliloti.* Proc. Natl. Acad. Sci. USA **77,** 7347–7351.

Drews, G.N., Bowman, J.C., and Meyerowitz, E.M. (1991). Negative regulation of the Arabidopsis homeotic gene *AGAMOUS* by the *APETALA-2* product. Cell **65,** 991–1002.

Gruenberg, D.A., Natesan, S., Alexandre, C., and Gilman, M.Z. (1992). Human and *Drosophila* homeodomain proteins that enhance the DNA binding activity of serum response factor. Science **257,** 1089–1095.

Helm, J. (1951). Vergleichende Betrachtungen über die Entwicklung der infloreszenz bei Lycopersicon esculentum Mill und bei einer Röntgenmutante. Zuchter **21,** 89–95.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G., and Fraley, R.T. (1985). A simple and general method for transferring genes into plants. Science **227,** 1229–1231.

Jack, T., Brockman, L.L., and Meyerowitz, E.M. (1992). The homeotic gene *APETALA3* of Arabidopsis thaliana encodes a MADS box and is expressed in petals and stamens. Cell **68,** 683–697.

Koornneef, M., Hanhart, C., Jongsma, M., Toma, I., Weide, R., Zabel, P., and Hille, J. (1986). Breeding of a tomato genotype readily accessible to genetic manipulation. Plant Sci. *45,* 201–208.

Lawrence, P. (1991). The Making of the Fly. (Oxford: Blackwell Scientific).

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, A.M., Bowman, J.L., Kempin, S.A., Ma, H., Meyerowitz, E.M., and Yanofsky, M.F. (1992). Manipulation of flower structure in transgenic tobacco. Cell **71**, 133–143.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

McCormick, S. (1991). Transformation of tomato with *Agrobacterium tumefaciens*. In Plant Tissue Culture Manual B6 (Dordrecht: Kluwer Academic Publishers), pp. 1–9.

Mizukami, Y., and Ma, H. (1992). Ectopic expression of the floral homeotic gene *AGAMOUS* in transgenic Arabidopsis plant alters floral organ identity. Cell *71*, 119–131.

Mol, J., van der Krol, A., van Tunen, A., van Blokland, R., de Lange, P., and Stuitje, A. (1990). Regulation of plant gene expression by antisense RNA. FEBS Lett. *268*, 427–430.

Odell, J.T., Nagy, F., and Chua, N.-H. (1985). Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature **33**, 810–812.

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.

Pnueli, L., Hareven, D., Rounsley, S.D., Yanofsky, M.F., and Lifschitz, E. (1994). Isolation of the tomato *AGAMOUS* gene *TAG1* and analysis of its homeotic role in transgenic plants. Plant Cell **6**, 163–173.

Pri-Hadash, A., Hareven, D., and Lifschitz, E. (1992). A meristem-related gene from tomato encodes a dUTPase: Analysis of expression in vegetative and floral meristems. Plant Cell **4**, 149–159.

Rick, C.M. (1947). Partial suppression of hair development indirectly affecting fruitfulness and the proportion of cross-pollination in a tomato mutant. Am. Natur. **81**, 185–202.

Rick, C.M., and Butler, L. (1956). Cytogenetics of the tomato. Adv. Genet. **8**, 267–382.

Samach, A., Hareven, D., Gutfinger, T., Ken Dror, S., and Lifschitz, E. (1991). Biosynthetic threonine deaminase gene of tomato: Isolation, structure and upregulation in floral genes. Proc. Natl. Acad. Sci. USA **88**, 2678–2682.

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.J., Hansen, R., Tetens, F., Lönnig, 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.

Shahar, T., Hennig, N. Gutfinger, T., Hareven, D., and Lifschitz, E. (1992). The tomato 66.3-kD polyphenoloxidase gene: Molecular identification and developmental expression. Plant Cell **4**, 135–147.

Takayama, K.M., and Inouye, M. (1990). Antisense RNA. CRC Crit. Rev. Biochem. Mol. Biol. **25**, 155–184.

Tröbner, W., Ramirez, L., Motte, P., Hue, I., Huijser, P., Lönnig, W.-E., 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.